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Effect of complement C5a in tumor progression and the local tumor microenvironment.

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EFFECT OF COMPLEMENT C5A IN TUMOR PROGRESSION AND LOCAL
TUMOR MICROENVIRONMENT

By

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B. A. Earlham College 2003
M. S. University of Louisville 2008

A Dissertation
Submitted to the Faculty
of the Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements for the Degree of

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Department of Microbiology and Immunology
University of Louisville
Louisville, KY

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A Dissertation Approved on

June 16, 2011

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Jun Yan

Dissertation Director

Douglas Taylor

DEDICATION

This work is dedicated to my family and friends,
for their continued encouragement and support.

ACNOWLEDGMENTS

I would like to express my thanks and appreciation to my mentor Dr. Jun Yan, MD PhD for his encouragement, support, and patience throughout my study in his lab. His training and guidance helped me become a better scientist, while his compassion and understanding gave me strength during challenging times. To all my past and present labmates for their training, time, and discussion; without the example of their work and skills, I would not have gained a strong foundation in research and appreciation for collaboration. I can not thank my family enough for the support and patience they gave me and helping care for my family during busy times. Most importantly, to my husband, Robert, for holding on during long, tough times apart and getting me through difficult days to ensure I accomplished my goal, and to my daughter, Josabel, for being tough when I had to go to the lab and we were apart more than she wanted to be. Without all these people above, I would not have been able to complete this journey.

ABSTRACT

EFFECTS OF COMPLEMENT C5A IN TUMOR PROGRESSION AND THE LOCAL TUMOR MICROENVIRONMENT

Lacey Jo Gunn

June 16, 2011

Monoclonal antibodies directed toward tumor associated antigens are FDA approved anticancer reagents used commonly in the clinic. Administered antibodies initiate tumor cell death through several mechanisms. Improving immune mediated mechanisms, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) may improve treatment success. However, in many tumors, complement activation and propagation is limited by tumor cell overexpression of complement regulatory proteins (CRPs). Thus, introduction of complement components into the tumor, specifically anaphylatoxin C5a, may provide a key strategy to improve existing, approved anti-tumor treatment. Currently the role of C5a in the tumor remains unclear.

The receptor for C5a, C5aR, is expressed predominantly by neutrophils and cells of the innate immune system. The human ovarian adenocarcinoma cell line, SKOV-3, was demonstrated to overexpress CRP CD55 which inhibits the release of C5a. As a result, a SCID xenograft model was established to determine the effect of C5a in the tumor microenvironment. The model allowed study of the effect of C5a on innate

immune cells exclusively, as well as the introduction of C5a in the presence of CD55. *In vivo and in vitro* studies were performed to clarify the controversial role of C5a in the tumor and the effect on innate immune cell phenotype and cytotoxic function.

The release of local C5a from tumor cells led to enhanced infiltration of macrophages and NK cells, and neutrophils as related to tumor size. C5a also increased production of proinflammatory and tumor cytostatic mediator TGF- β by infiltrating macrophages. Furthermore, C5a downregulated the generation of iNOS and arginase by infiltrating cells, tumor cell VEGF and total tumor TNF- α production. *In vitro* studies provided support of C5a significantly improving cytotoxicity of NK cells against tumor cells, and significantly reducing immunosuppression by infiltrating neutrophils. Importantly, C5a in the tumor caused no major systemic alterations or adverse side effects.

The result of local C5a release into the tumor microenvironment had a dramatic effect on tumor growth through the recruitment and activation of innate immune cells. C5a altered phenotypes of infiltrating immune cells toward anti-tumorigenic and improved tumor cell cytotoxicity. Based on the findings of C5a in the SKOV-3 xenograft model in the context of published work involving C5a in the tumor, concentration of local C5a is proposed to be critical in determining its role in tumor progression.

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CHAPTER 1

THE COMPLEMENT SYSTEM: A CRITICAL ARM OF THE IMMUNE RESPONSE

Complement activation:

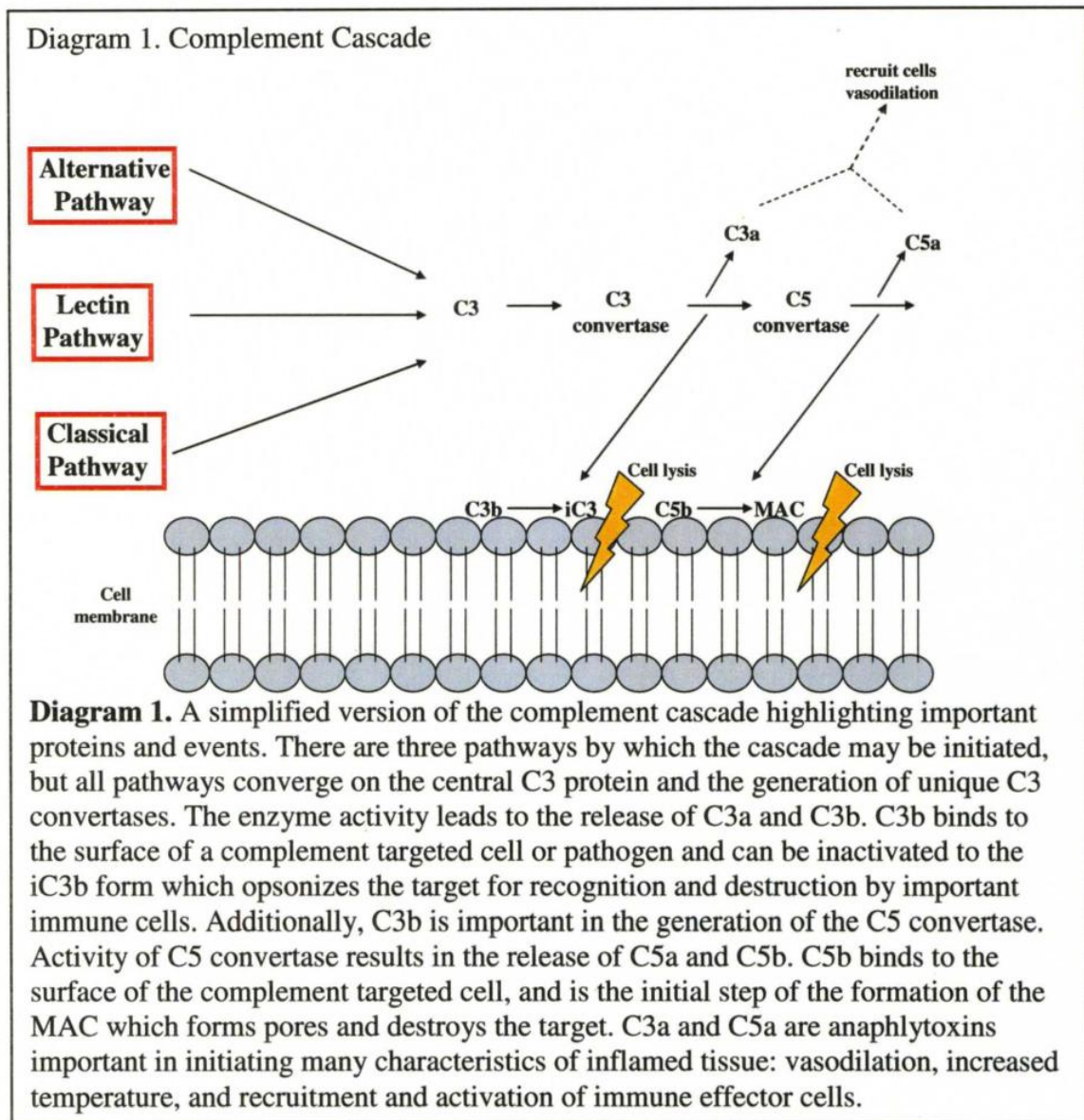
Complement is an important component of the immune system, and activation of the complement cascade involves a variety of proteins, including opsonins, convertases, pore-forming proteins, anaphylatoxins, and regulatory proteins. The events producing and releasing these proteins results in the propagation and control of a multi-faceted system by which to contribute and perpetuate immune reactions during an effective immune response [1]. Activation of complement can occur via the classical pathway following the aggregation of antigen-antibody complexes, lectin pathway in the case of infection with microbes expressing surface mannose molecules, and alternative pathway which occurs spontaneously or on the surface of foreign substances or molecules [2]. Although initiation of the cascade can vary, all three pathways converge in the formation of the C3 protein.

The generation and subsequent cleavage of C3 leads to the three effector mechanisms of the complement system: binding and formation of the membrane attack complex (MAC) which can directly lyse targeted cells, deposition of iC3b on cells or microbes decorating them as targets for destruction by cells of the innate immune system, and the release of anaphylatoxins which chemoattract and activate immune cells to the

desired location [1]. Anaphylatoxins are responsible for converting danger signals from the fluid phase of the host into an orchestrated cellular response [3]. Anaphylatoxin C5a, a potent inflammatory mediator is released into the local environment and is responsible for several functions. C5a chemoattracts cells of the innate immune system to the site of infection or damage [3, 4], activates immune cells upon interaction with the C5a receptor (C5aR) resulting in oxidative burst in neutrophils, phagocytosis augmentation, and enzymatic granule release, as well as vasodilation of local blood vessels to enhance immune cell entry and exchange of cells and factors from the circulation into the local infected or injured tissue [3, 5]. C5a is quickly degraded by carboxypeptidases available abundantly in tissues and serum by cleaving the carboxy-terminal arginine residue to C5a *desArg* [1]. The degradation product maintains some chemotactic and proinflammatory properties of C5a but the potency is greatly reduced [3].

As a result of the extensive and potent effects of complement activation, the complement cascade is highly regulated at several levels by complement regulatory proteins (CRPs) which can effectively dampen the intense proinflammatory response initiated by complement activation and the release of C5a. CRPs are expressed on the membranes of normal cells and can also exist in soluble form [6, 7] to limit the extent of complement activation and reduce normal/healthy cell damage. CD35, CD46, CD55, and CD59 are all known members of the regulators of complement activation family [7]. CD35 (complement receptor 1 CR1) is expressed on haematopoietic cells, monocytes, and granulocytes; it regulates the activity of C3 convertase. CD46 (membrane cofactor protein) also regulates C3 convertase activity and is expressed broadly with the exception of erythrocytes. CD59 and CD55 are membrane proteins expressed normally on nearly all

cells of the body. CD59 (Protectin) prevents the formation of the MAC, limiting cell lysis of complement opsonized cells, also a function of CD46 and CD55. CD55 (decay-accelerating factor) is responsible for the accelerated dissolution of C3 convertase, and as a result, the C5 convertase. Rather than proteolytically, in the case for other regulatory proteins, CD55 accelerates the dissociation of the components of the convertases eliminating release of anaphylatoxins [7].



CHAPTER 2

THE COMPLEX RELATIONSHIP BETWEEN ARISING TUMORS AND THE IMMUNE SYSTEM

Inflammation and Cancer:

Immunosurveillance:

Immunosurveillance refers to the prevention of cancer development as a result of immune cell recognition and elimination of transforming cells. Various cells and components of the innate immune system possess the capacity to recognize and eliminate cancerous cells [8] (Diagram 2), and have been implicated in protecting the host against tumor development in many research models, as well as human cases [3, 8-11]. Three main results of immune cell activity have been demonstrated as being critical to preventing the development of cancer in the host: protection against viral infection, resolution of inflammation in order to dissolve an inflammatory microenvironment ideal for tumor development, and destruction of tumor cells based on tumor cell expression of innate immune cell activating receptors [11]. The innate immune system, including the complement system, has the capacity to recognize alterations produced by transformed cells and destroy these cells [3, 11]. However, as the defined mechanism of “immunoediting” illustrates, only a few tumor cells under the scrutiny and attack of immune cells need adapt the features necessary to evade destruction and take a strong hold in the host [11, 12]. In the case of this event, these transformed cells are able to

escape detection and begin growing while using the inflammatory mediators produced locally to fuel progression [9, 12]. As with any group of growing cells, growth factors, inhibition of apoptosis, and supply of blood are required. Cells of the immune system are recruited and stimulated by tumor products to aid in the acquisition of these vital survival and growth requirements [9, 12, 13] (Diagram 2, adapted from).

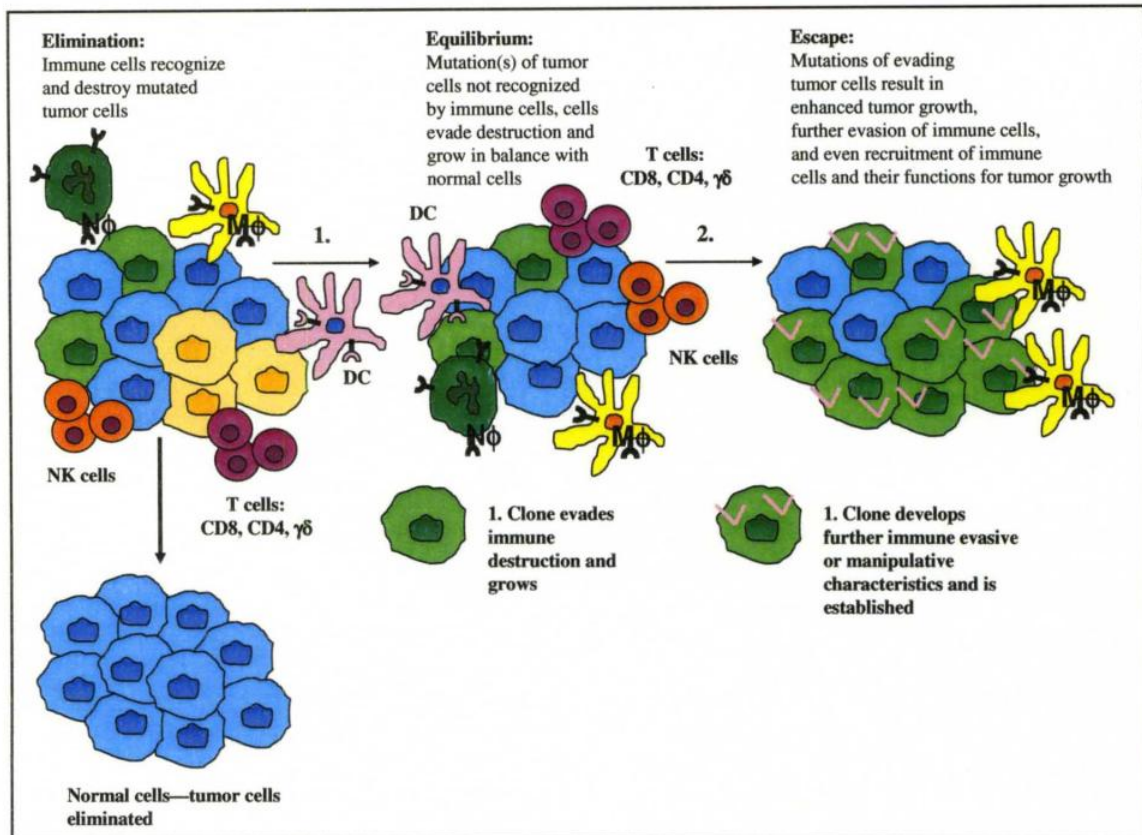


Diagram 2: Tumor destruction or growth in the presence of host immune cells. During the elimination stage, mutating or transforming host cells are recognized and destroyed by multiple cells of the immune system. This results in restoration of normal cell growth in the tissue free of cancerous cells. Alternatively, mutating cells may develop a mutation which results in evasion of immune cell recognition and subsequent destruction, and these transformed cells grow in equilibrium with normal host cells in the presence of immune cells. However, an additional mutation leading to enhanced growth or recruitment of immune cells in order to elicit their properties for tumor growth rather than destruction occurs during escape, and cancer develops in the host.

Immune cell manipulation by tumor:

Cancer often develops in adults following undetected/asymptomatic inflammatory disease [14]. When the vast majority of cancer patients present clinically they have an established tumor that successfully evaded and/or enlisted immune cells responding to the tumor to avoid destruction and promote its growth. Tumor and associated stromal cells respond to proinflammatory factors produced locally by infiltrating immune cells, which leads to tumor production of mediators, such as cytokines, inhibitory molecules, or dysregulation of receptors, to effectively alter the immune response in the tumor microenvironment to benefit tumor growth [9, 13, 15]. The generation of an environment that fosters a continuous, tumor promoting response by infiltrating immune cells and tumor cells, is thought to be perpetuated by the environmental conditions established mimicking chronic inflammation [13]. The properties of inflammation efficiently promote and protect the tumor including ischemia, edema, immune cell infiltration, and new blood vessel formation for delivery of additional nutrients [8, 12, 14]. Immune cells in the tumor are required for the construction of new blood vessels and tumor growth enhancement [14].

Immunotherapy:

Debate over the role of immune cells in cancer destruction and prevention lasted decades, but it is well established that the immune system plays an important role in the prevention of cancer development [11]. In the more recent decades of cancer research, manipulating and enhancing immune cell migration and anti-tumor activities has been a main focus of immunologists. Many methods of immunotherapy in cancer have targeted developing and enhancing the tumor specific T cell response through the administration

of activated dendritic cell vaccines, or administration of an individual's T cells following culture and expansion with tumor associated antigens (TAAs) [16]. Whereas these methods seem promising, they are complicated, expensive, and limited by requirements of the individual patient's as a cell source [16]. These restrictions to successful treatment have encouraged shifting the focus to other immunological targets for enhancing an anti-tumor response [11]. Innate immune cells are attractive targets of the immune system as a result of large circulating numbers, and the lack of prerequisite priming event(s) to be effectively cytotoxic [10]. In the case of macrophages, subsets are known to play an important role in tumor growth and progression [13, 17], targeting and reversing this pro-tumorigenic phenotype may prove beneficial in providing additional modes of immunotherapy [18, 19].

Complement and Cancer:

The complement system can discern alterations of endogenous molecules that are related to tumors and become activated [3]. In several tumor models, complement proteins have been indicated in tumor regression [20-22]. Great potential has been shown for components of the complement cascade being targeted or incorporated into cancer patients' treatment regimes with anti-tumor monoclonal antibodies to improve the most common and nearly only anticancer reagents approved by the FDA [4, 23]. Rituximab is a monoclonal antibody against CD20, and is used in the frontline of treatment of patients with non-Hodgkin's B cell lymphoma (NHL). The mechanism of rituximab action against tumor cells is suspected to include complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [24, 25]. Therefore, complement

activation and propagation of the complement cascade is critical to the therapeutic efficacy of rituximab treatment and other monoclonal anti-tumor antibody therapies [24, 26-28]. The opsonization of cells following rituximab or other antibody binding and complement activation is critical to leading to MAC formation on and innate immune cell destruction of targeted cells [24, 28, 29]. In mouse xenograft and syngeneic tumor models, cobra venom factor treatment to deplete complement and C1q knockout mice treated with rituximab demonstrated a significant reduction of the anti-tumor properties [26-28]. It is apparent from the above studies that regardless of whether or not the tumor is targeted for lysis via the MAC formation on the membrane, opsonization of the tumor cells by C3b or iC3b deposition, or release of C5a to recruit and activate innate immune cells leading to subsequent destruction of the tumor, activation of complement proves to be an important contributor to mechanisms by which tumor cells can be eliminated.

Antibody therapy with anti-EGFRs (epidermal growth factor receptor) effectively kills tumor target cells predominantly through ADCC [23]. Further study of anti-EGFR pairings has shed light on the important contribution CDC provides when also elicited by therapy. The potency of the complement killing system has been shown using a combination of Cetuximab and Matuzumab, directed toward unique epitopes of EGFR [23]. This combination approach was capable of activating complement as demonstrated by C1q and C4b deposition detected on tumor cells and enhanced killing of human squamous and glioblastoma cancer cells via CDC. Effective CDC even occurred in dual anti-EGFR therapy despite high expression of CRPs by these tumor cells [23].

As previously eluded, there are many complement cascade activating and regulatory proteins with the potential to effect the development of cancer. Several

complement proteins have been identified as playing a role in cancer elimination. C1q was found to be responsible for activating the tumor suppressor gene, WOX1, *in vitro* in human prostate cancer cells, DU145, enhancing cancer cell apoptosis [21]. Additionally an anti-tumor agent, the small peptide, tachyplesin was determined to interact with C1q and thereby activate the classical pathway of complement resulting in the cleavage of C3 and C4 and assembly of the MAC on tumor cells [22]. When TSU tumor cells, another prostate cancer line, were treated with tachyplesin in the presence or absence of serum, death in TSU cells was significantly greater in the presence of serum [22]. TSU cells were targeted for destruction by MAC formation as evident by the fact that cultures were positive for C4b, C3b, and C5b-9 by ELISA [22].

The above evidence illustrates the importance of complement activation in the anti-tumor response. Complement as an immune target is favorable for two important reasons: the components of the complement system are soluble, as opposed to cells and can more easily access the tumor and secondly, complement proteins can be made highly available as activation is generated locally and key proteins are made by cells in the environment [30]. Tumor cell expression of membrane and soluble CRPs inhibiting complement has been observed in the setting of antibody therapy renders the developing cancer greater resistance to CDC [6, 29]. The expression of CRPs has been demonstrated to be upregulated by many types of human tumor cells also indicating activation of complement within the tumor has potential anti-tumor effects, as the tumor develops complement evasive mechanisms [6, 20, 31].

In a study of non-small cell lung carcinoma patients, despite Her2/neu overexpression by the tumor cells, administration of anti-Her2/neu antibody provided

only 15% therapeutic efficacy as a result of decreased CDC due to tumor cell CD55 and CD59 expression [30]. In studies involving treatment of NHL patients, expression of CRPs was related to disease severity and response to rituximab therapy; therapy non-responsive patients expressed higher levels of CD46 and CD59, and patients with bulky disease expressed high levels of CD55 and CD59 [24, 32]. Immunohistological studies on 5 types of human carcinomas indicated that expression of CRPs is widely enlisted by tumors to inhibit complement functions [6]. Additionally tumor stromal cells stained heavily for CD55 and CD59 CRPs, without positive cells occurring in the adjacent normal tissues, suggesting the tumors release a soluble form of these regulatory proteins [6]. These clinical examples illustrate complement activation is critical in the tumor microenvironment for tumor cell destruction and optimal treatment efficacy. Adaptation of tumor cells to limit complement within the microenvironment indicates inhibiting CRPs or directing complement components to the tumor may have anti-tumor potential.

C5a in Cancer:

Translational research efforts support a positive role for C5a in treatment protocols necessary to the anti-tumor response. β -glucan adjuvant therapy significantly improves monoclonal anti-tumor antibody therapy against tumor growth. β -glucan, a polysaccharide isolated from yeast cell wall, has been demonstrated to prime complement receptor 3 (CR3) on neutrophils for enhanced tumor killing. Complement activation during this therapy is critical for efficacy, as it leads to the release of C5a, which is responsible for recruiting β -glucan primed neutrophils [33]. Expression of CD55 by the human ovarian cancer cell line, SKOV-3, *in vivo* renders the tumor capable of

eliminating therapeutic efficacy of β -glucan/ HER2/neu antibody combination treatment by inhibiting C5a release [20]. Additionally, *in vitro* studies involving human breast cancer cells demonstrated improvement in tumor cell killing by neutrophils following treatment of cells with anti-HER2/neu monoclonal antibody fused to either C5a or C5a *desArg* [4, 34]. The fusion antibodies were also able to recruit more neutrophils *in vitro* and were effective in tumor cell killing at doses at which the standard HER2/neu antibody shows no effect on tumor cells [4].

Previously, two groups reported on the effect of complement component C5a in the tumor microenvironment revealing conflicting roles. In 2005, Kim et al. reported C5a produced by distinct clones of EMT6 (murine mammary sarcoma) cells transfected with mouse C5a expressing plasmid had a significant effect on tumor growth *in vivo*. EMT6 C5a clones producing projected low levels of C5a, capable of inducing minor migration of J774 macrophage cells *in vitro*, resulted in the most substantial reduction in tumor growth and regression as compared to control EMT6 cells or EMT6 C5a clones that induced abundant J774 cells to migrate [35]. The group reported C5a expressing tumors showed significant increase in the number of apoptotic cells at various time points post tumor development, and cell cycle progression was inhibited at all phases. Additionally, C5a tumor bearing mice experiencing complete tumor regression resisted tumor growth upon re-challenge [35]. However, no clear immunological or alternative mechanism mediated by C5a which attributes to this limited tumor growth was defined.

Contrary to these findings, in 2008, the Lambris group demonstrated a pro-tumorigenic role of C5a generated via the classical pathway within the tumor microenvironment. In this TC-1 murine cervical cancer model, C5a was shown to recruit

significantly more myeloid-derived suppressor cells (MDSCs) of the granulocytic subset into the tumor [36]. C5a also enhanced the production of immunosuppressive factors, ROS and RNS (reactive oxygen or nitrogen species), by the monocytic subset of MDSCs resulting in the effective inhibition of the anti-tumor specific CD8 T cell response and increased tumor burden [36]. C5aR deficient mouse tumors showed significantly more CD8 T cells throughout the tumor, and depletion of these cells eliminated the tumor growth disparity between the WT and C5aR lacking mice [36].

C5a release and activation of C5aR expressing cells produces many of the important events of inflammation used by tumors: contraction of smooth muscle and vasodilation enhancing blood flow to the location resulting in edema and the migration of neutrophils and macrophages into the site [3, 5, 37]. Additional valuable conditions of inflammation on tumor growth include ischemia and new blood vessel formation for delivery of additional nutrients and cells which C5a does not directly contribute [8, 12, 14]. However, the effects of C5a have apparent pro-tumorigenic effects and have been shown to be responsible for tumor progression in a single model [36]. On the other hand, as a potent immune modulator in acute inflammation, as well as demonstrating a role, yet undefined, in tumor regression [35], C5a may prove an effective and necessary component of the complement system in tumor destruction.

Innate immune cells in cancer:

Innate immune cells including neutrophils, macrophages, natural killer (NK) cells and dendritic cells are well known for their role in mounting the initial immune attack after recognition of pathogens during infection, as well as repairing and restoring host

tissue following the resolution of the infection, but they are capable of more. Innate immune cells also contribute in the immune surveillance of a host to inhibit the survival of cancerous cells during tumor development [8, 12]. As evident by studies demonstrating an increased occurrence and burden of spontaneous and carcinogen-induced tumors developing in mice depleted of NK cells and monocytes/macrophages, or deficient in perforin, TNF-related apoptosis induced ligand (TRAIL), or GM-CSF [8, 10, 17, 38]. Not only are innate immune cells critical to the elimination of cancer cells early in tumor development, but also in controlling and limiting the duration of the inflammatory response, as chronic inflammation has been linked to cancer development [9, 12].

Studies by Hicks et al. with spontaneous regression/complete resistance (SR/CR) mice clearly showed critical importance of an anti-tumor innate immune cell response. The group demonstrated a strain of mice resistant to tumor growth or disease development. The existence of innate immune cell rosettes forming around cancer cells were responsible for eliminating tumor cells, and this anti-tumor response was transferable to and long lasting in non-resistant, wild-type mice following the adoptive transfer of innate immune cell subsets from SR/CR mice [39]. It has also been shown innate immune cells are important in the efficacy of combination anti-tumor therapy consisting of β -glucan and a monoclonal antibody. The effect of this treatment is mediated by neutrophils demonstrated by depletion experiments eliminating neutrophils effectively eliminates all therapeutic efficacy [33, 40]. Another study using rituximab also revealed the importance of innate immune cell involvement in antibody mediated therapy and tumor eradication, proposing that rituximab leads to the activation of

complement resulting in subsequent release of innate immune cell recruiting chemokines (CCL3 and CCL4) [27]. Depletion studies by this group demonstrated each subset of innate immune cells; neutrophils, macrophages, or NK cells; showed each played a critical role in tumor eradication in rituximab treatment.

Antibodies bound to tumor surface antigens trigger the activation of immunoglobulin G fragment C receptors (Fc γ receptors) expressed predominantly by innate immune cells initiating a retrograde signaling cascade resulting in cytokine release, oxidative burst, increased phagocytosis, and more potent ADCC by the receiving cell [41-43]. Drozitumab, a human monoclonal antibody to death receptor 5 (DR5), and rituximab have been shown to require signaling through Fc γ receptors in order to be effective in anti-tumor therapy [41]. Binding of the Fc γ receptors on tumor infiltrating innate immune cells from Rag2 knockout mice to drozitumab decorated tumor cells lead to engagement with drozitumab and DR5, resulting in tumor cell apoptosis [41]. Fc γ receptors on innate immune cells are found to be targeted in anti-tumor therapy resulting in cytotoxic activity by innate immune cells elicited against the tumor.

CHAPTER 3

OVARIAN CARCINOMA AND A RELEVANT MODEL SYSTEM FOR TARGETING C5A AND INNATE IMMUNE CELLS FOR IMPROVEMENT OF IMMUNOTHERAPY AGAINST TUMORS

Ovarian carcinoma:

Epithelial ovarian cancer is diagnosed at a rate of approximately 205,000 cases per year world wide. More than 13,000 women die with ovarian cancer annually in the United States, making it the most lethal gynecologic cancer nationally (American Cancer Society). Due to the overall lack in diagnostic markers, most ovarian cancer patients are not diagnosed until metastatic stage, with a 5 year overall survival rate of 30-40% [44]. Standard treatment of care following diagnosis of ovarian carcinoma consists of surgical debulking followed by chemotherapy [44]. In ovarian carcinoma as in other types, significant research has shown an important immune component including tumor infiltrating populations of highly immune suppressive cells (including MDSCs and regulatory T cells) that inhibit anti-tumor immune cell activity, as well as produce factors that promote the tumor [14, 45]. Strong immunosuppressive and tumor promoting environments established in ovarian cancer patients lend to the complications in treating tumors by a single modality without addressing the tumor immune component.

Necessity of this research:

Activation of the complement cascade affects the local environment, cell recruitment, cell activation and has profound effects on host outcome. Complement proteins are capable of initiating and sustaining an immune response. C5a, in particular, is an incredibly potent mediator of immune cells through its chemotactic and activation functions. Targeting a key component of the immune system, such as C5a, in order to destroy tumor is critical for successful therapy combating tumor progression mechanisms of limiting immune cell infiltration [10, 20] and stimulation [10, 46], as well as inhibition of CDC in treatment protocols [7, 24]. Study of the role of C5a in the tumor microenvironment may demonstrate a method by which to circumvent the limitations CRPs have on the complement cascade since these have been observed to be upregulated on many human tumors [6, 24, 25, 29]. Subsequently, the limitations tumors have on immune cell recruitment and activation locally may be reversed through C5a activity.

C5a can have far-reaching immunological effects by influencing each immune cell type; however, due to the conflicting published accounts and the breadth of C5a activity, we feel it is important to address the effect of C5a in a limited system.

Immunocompromised SCID model use allowed for dissecting the resultant immune response C5a triggers in the tumor and host by the innate immune cells, exclusively. Additionally, the tumor cell line of choice, SKOV-3 human ovarian adenocarcinoma cells are highly aggressive [47] and express abundant surface CD55 [20]. A reduction in growth of this tumor by the addition of C5a would prove encouraging. The direct incorporation of C5a into the tumor microenvironment also circumvents the need for antibodies directed toward CD55. Given that this antigen is expressed on nearly all

normal cells of the body, elimination of blockade treatment will ensure no damage to healthy tissue.

Harnessing the potent chemotactic property of C5a may enhance innate immune cell migration into the tumor. Once cells infiltrate, activation via C5a may foster an immune stimulating microenvironment, generating proinflammatory innate immune cells that will produce anti-tumor factors and act as superior effector cells affording adaptive immune cell benefits in entry and anti-tumor responses. These are all potential results of introducing C5a in the tumor due to its potent immune response initiating characteristics. Previously performed studies involving C5a in the tumor microenvironment *in vivo* reveal the role of this potent immune mediator is as of yet inconclusive.

It is hypothesized the potent innate immune cell chemoattractant, C5a, expressed from the tumor microenvironment can alter immune cell infiltration into the tumor by promoting recruitment of innate immune cells, changing the infiltrating cell milieu and potentially the phenotype and activity of these cells to destroy tumor cells.

CHAPTER 4

MATERIALS AND METHODS

SCID mice and implantable tumor cell:

All mice used in the *in vivo* tumor growth studies were treated in agreement with the established laws and guidelines of the institution. Protocols for tumor studies in mice were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Tumor growth studies were performed on mice 6-10 weeks of age. Severe Combined Immunodeficient (ICR SCID) mice were purchased from Taconic Farms (Germantown, NY, USA) or Harlan Laboratories (Fox Chase Cancer Center, Philadelphia, PA, USA). SKOV-3 tumor cells are a human ovarian adenocarcinoma cell line that express HER-2/neu antigen acquired from the American Tissue Type Collection (ATCC, Manassas, VA, USA). SKOV-3 cells were injected subcutaneously (s.c.) at a dose of $7-10 \times 10^6$ cells per SCID mouse with BD matrigel matrix basement membrane (BD Biosciences, Bedford, MA, USA).

***In vitro* tissue culture:**

All cell lines used were cultured at 37°C in a humidified incubator with 5% CO₂. Each cell line was cultured in specific growth medium. Media supplements used for cell culture were obtained from the following vendors: fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA); 200 mM L-glutamine (L-gln), antibiotic-

antimycotic solution (10,000 IU/ml penicillin, 10,000 ug/ml streptomycin, 25 ug/ml amphotericin B), Geneticin, G418 Sulfate all from Cellgro Mediatech, Inc. (Manassas VA, USA). SKOV-3 cells and isolated white blood cells when used for cytotoxicity studies were cultured in McCoy's 5A (Caisson Laboratories; North Logan, UT, USA or MIDSCI; St. Louis, MO, USA) supplemented with 10% FBS, 1% L-gln, and 1% antibiotic-antimycotic solution. Transfected SKOV-3 cell lines were cultured in the same media with the addition of 600 ug/ml geneticin. J774 murine macrophage cells were grown in DMEM with 4.5 g/L glucose supplemented with 10% FBS, 1% L-gln, and 1% antibiotic-antimycotic solution. RPMI with L-gln (Cellgro Mediatech, Inc.) was supplemented with 5% FBS, DNase I, collagenase from *Clostridium histolyticum*, and hyaluronidase from sheep testes (Sigma, St. Louis, MO, USA) to produce the 10X tumor lysis buffer for single cell tumor suspension preparation. Yac-1 luciferase expressing cells were generously provided by Dr. Zhang at University of Louisville, Brown Cancer Center. These cells were cultured in RPMI supplemented with 10% FBS, 1% L-gln, and 1% antibiotic-antimycotic solution.

Generation of C5a expressing tumor cell line:

SKOV-3 WT cells were transfected with murine C5a secreting and empty vector DNA plasmids generously provided by Dr. Fangting Liang at the Veterinary School of Medicine at Louisiana State University. Cells were seeded at 9×10^5 cells per well in a 6-well plate the night before the transfection in McCoy's 5A media supplemented with 10% FBS and 1% L-glutamine. The following day, wells 70-80% confluent were transfected using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, 18324-012)

protocol. Each well was washed twice with 1X PBS prior to the addition of the Lipofectamine/DNA plasmid complexes. After overnight incubation, the transfection complexes were removed and fresh media was added to the wells. After 2-3 days of cell growth, selection media containing 600 ug/mL geneticin was added over transfected cells. Once all cells in the control/Lipofectamine only wells had died, supernatants of confluent transfected cell wells were screened by C5a ELISA (BD Pharmingen, Sparks MD), and a limiting dilution assay was performed in a 96-well format with the cells from positive wells. Following the growth of single colonies from the limiting dilution wells, supernatants of single clones were screened by C5a ELISA. Positive clones were expanded in T25 flasks, supernatants were screened once cells reached confluency and cells were injected s.c. into the mammary fat pads of ICR SCID mice.

After tumors had developed to 5-6 mm in diameter, tumors were excised and half of the tumor was pulverized in growth medium. Large tumor pieces were removed and the cell suspension was washed and suspended in selection media and seeded into a 6-well plate. Culture media was changed as necessary, and supernatants over confluent wells were screened for C5a by ELISA for expression maintenance. The other half of the tumor was frozen, sectioned, and immunohistochemistry staining with mouse anti-C5a antibody (BD Pharmingen) was performed. Following *in vivo* passage, the SKOV-3 C5a tumor cell clone positive by both ELISA and IHC staining, was expanded and frozen down for all experiments in this study.

C5a ELISA:

Nunc-Immuno BreakApart and LockWell MaxiSorp wells (VWR International, LLC, Batavia, IL) were coated with 2 μ g/ml purified rat anti-mouse C5a monoclonal antibody (BD Pharmingen, 558027) diluted in ELISA coating buffer with pH of 9.35 and incubated overnight at 4°C. The following day, the coated plate was washed and blocked with 300 μ l per well of assay diluent for 1-2 hours at room temperature. Cell culture supernatants over transfected cells were screened undiluted, and the standard curve with mouse C5a (BD Pharmingen, 622597) was prepared in assay diluent at concentrations ranging from 55.5 pg to 14.2 ng/ml. Samples and standards were incubated for 2 hours at room temperature on a shaker at 200 rpm. After washing the plate, the biotinylated rat anti-mouse C5a antibody (BD Pharmingen, 558028) was applied at a concentration of 2 μ g/ml in assay diluent and the plate was incubated with rocking at room temperature for 1 hour. Streptavidin-HRP conjugate (BioSource, Invitrogen, 43-4323) was added at 0.65 μ g/ml in assay diluent, and the plate was incubated for 30 minutes at room temperature on a shaker. Wells were thoroughly washed and TMB Conductivity Substrate (BioFX Laboratories, Inc., Owings Mills, MD, TMBC-1000-01) that had been allowed to reach room temperature was added to each well. The plate was left uncovered and incubated in the dark at room temperature for 10-15 minutes. Following the colorimetric reaction, equal volume stop solution was applied to each well and the absorbance value at 450 nm was read immediately on a BioRad Model 680 Microplate Reader using the Microplate Manager 5.2.1 software.

Immunohistochemistry and immunofluorescence:

Excised tumor samples were divided in half using a scalpel blade, and tumors were placed with the center of the tumor down into standard size Cryomolds (Tissue Tek, Sakura Finetek USA, Inc., Torrance CA, 4583) over a thin layer of O.C.T. Compound (Tissue Tek) freezing media. Tissues were completely covered in O.C.T. and snap frozen in 2-methylbutane over dry ice. Frozen tumor blocks were kept at -80°C and sections were cut on a Leica CM 1900 cryostat (Leica), 5mm sections were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburg, PA, 12-550-15) and stored at -20°C after drying. Tumor tissue was fixed to the slides in ice cold acetone at room temperature for 20 minutes. Following fixing, tumor tissue was rehydrated in 1X PBS for 5 minutes. A hydrophobic boundary was applied surrounding the tissue on the slide, and tissue samples were treated with 3% hydrogen peroxide for 30 minutes at room temperature in a humidity chamber. Slides were rinsed in 1X PBS 3 times for 3 minutes each. Blocking buffer (3% BSA in 1X PBS) was applied to the tissue and samples were blocked at room temperature for 2 hours.

After slides were blocked, buffer was removed by shaking the slide and drying excess liquid with a kim wipe. Biotin and streptavidin blocking steps were performed with Vector Avidin/Biotin Blocking Kit (Vector Laboratories, Inc. Burlingame, CA SP-2001) using instructions provided with the kit. After rinsing the slides one time in PBS following each blocking step, biotinylated rat anti-mouse C5a antibody (BD Pharmingen 558028) was added to appropriate tissue sections at 2µg/mL diluted in blocking buffer. Slides were incubated in the humidity chamber overnight at 4°C. After incubation, slides were washed in 1X PBS 3 times for 3 minutes each. Streptavidin-HRP from the Vector

Peroxidase Substrate Binding Kit AEC (Vector Laboratories, SK-4200) was prepared according to the kit instructions, applied to each section on the slides, incubated in the dark for 8-10 minutes for visualization of the areas that bound the biotinylated antibody. Upon visualization of the colorimetric reaction occurring, slides were rinsed in tap water, and counterstain with hematoxylin was performed.

Tumor tissue slides were prepared with only slight differences for immunofluorescence staining. Once tumors were sectioned and applied to the slide, the slides were kept overnight at room temperature and allowed to dry in the fume hood. Following drying, the slides were fixed in ice cold acetone for 12 minutes. The blocking step was performed at room temperature for 1-2 hours using a blocking buffer containing 1% BSA, 5% rat serum, and 1:400 dilution anti-CD16/32 Fc γ receptor antibody. Staining with directly labeled fluorescent antibodies, HMa2 Alexa Fluor 647 (sharing epitope binding with DX5) (Biolegend, San Diego, CA, 103511) and F4/80 APC (Biolegend, 123116) , was performed at 1:50 dilution for 45 minutes at 37°C in a humidity chamber with appropriate isotype controls conducted in parallel. The appropriate isotype controls were performed simultaneously on similar tissue sections with Armenian hamster IgG isotype Alexa Fluor 647 (Biolegend 400924) and Rat IgG2a isotype APC (Biolegend, 400511). Slides were washed and then a DAPI (Invitrogen, D3571) stain was added to each section for 30 sec-1 min.

***In vitro* cell growth kinetics:**

Wild-type and transfected SKOV-3 cell (SKOV-3 WT, SKOV-3 C5a and control vector (CV)) growth was measured *in vitro*, in real-time, using the ACEA system which

measures cell attachment to the culture plate surface containing a conductor allowing for detection of the impedance of electrical current upon adherence (Acea Biosciences, Inc.; San Diego, CA, USA). Each cell line was plated in quadruplicate, in the Acea 96 well plate format, at 5×10^3 cells per well. Cells were incubated at 37°C in a humidified 5% CO₂ incubator for 40 hours. Cell indices read out based on the number of cells attached to the bottom of the plate well was used to calculate the growth of each cell line over the course of time. The Acea assay system reports cell index, a value calculated by measuring impedance of electrical current on the well bottom which occurs when cells in culture adhere to the surface.

***In vitro* cytotoxicity:**

During cytotoxicity experiments utilizing the Acea system, SKOV-3 WT cells were plated and allowed to adhere and grow for about 24 hours, until reaching a cell index of 1-1.6. Several innate immune cell populations were used in various cytotoxicity experiments all purified from SCID mice. Naïve mice spleens were used in preparation of innate immune leukocytes. Following single cell suspension and red blood lysis treatment, splenocytes were added to a T75 flask in medium for 45 minutes. Non-adherent cells were collected for cytotoxicity studies, and the white blood cell samples contained mostly NK cells and neutrophils, excluding adherent macrophages. These innate immune cells were added at an effector to tumor cell ratio of 20:1.

Gr-1+CD11b+ cells sorted from SKOV-3 C5a and SKOV-3 CV tumors following *in vivo* growth were added to wells containing the growing SKOV-3 WT cells in the presence or absence of SCID mouse white blood cells isolated from the spleen as

described previously. The Gr-1+CD11b+ sorted tumor infiltrating cells were added at a ratio of 2:1. Tumor and effector cells were co-incubated overnight, and SKOV-3 WT cell cytotoxicity was calculated at 28 hours. NK cells were purified and pooled from naïve SCID spleens using the EasySep magnetic beads kit for positive selection of DX5+ cells (StemCell Technologies, British Columbia, Canada, 18755). Purity of NK cells used for cytotoxicity assays was analyzed by flow cytometry and was found to be around 80%, with red blood cells again being the predominant contaminant. Recombinant mouse C5a protein (R&D systems, Minneapolis, MN, 2150-C5/CF) was diluted in medium and added to the wells to determine levels of C5a that may enhance macrophage and/or NK cell cytotoxicity of tumor cells.

In the Yac-1 cytotoxicity assay, Yac-1 cells transfected to express luciferase were utilized to identify if the addition of C5a could enhance NK cell specific killing of tumor cells. Yac-1 Luciferase (Luc) cells were plated in a 96-well plate at 2×10^4 per well. Positive control wells included only Yac-1 cells and negative control wells had added Triton X at 1%. Experimental wells included purified NK cells prepared as mentioned above at a ratio to the Yac-1 Luc cells of 1:1 with or without the addition of recombinant mouse C5a in decreasing concentrations and IL-2 cytokine (R&D systems, 1150-ML/CF) was added to each well at 100ng/mL. Tumor cells and NK cells were co-cultured for 6 and 12 hours, following which cells were lysed and combined with Luciferase enzyme assay reagent (Promega, Madison, WI, E1500) and luminescence released by live Yac-1 Luc in each well was collected as a reading of relative light units. Each condition was performed with 3-6 replicates.

***In vitro* chemotaxis assay:**

SKOV-3 CV and C5a cells were each cultured in a T25 flask to 90% confluency and fresh media was applied to the cells. Flasks were returned to the 37°C incubator with humidity and 5% CO₂ for 16-18 hours. Supernatant was collected from each flask and debris and cells were pelleted at 1500 rpm for 5 minutes. The supernatant, approximately 5 ml, was then transferred to the sample reservoir of a Centriprep Ultracel YM-3 (Millipore, Billerica, MA, 4303) unit for concentrating the C5a produced by the cells. The YM-3 was spun at 3000 x g in a Sorvall RC-3B refrigerated centrifuge for 2 hours. After centrifugation, the filtrate was collected in a 15 ml conical and the retentate, approximately 1 ml, containing the C5a was collected and stored at 4°C until the chemotaxis assay the following day. J774 murine macrophage cell line (ATCC, Manassas, VA) was used as responder cells in the chemotaxis assay. Concentrated supernatants were added to the bottom well of a HTS Transwell 96 well plate with 5.0µm Polycarbonate Membranes (Corning, Corning, NY), and J774 cells were added to the upper chamber of the transwell plate at 1x10⁵ cells per well. The plate was incubated at 37°C for 3 hours before the J774 murine macrophage cells that were chemoattracted to the bottom well were collected and counted. Migrated cells were collected from the wells and counted by trypan exclusion. Each concentrated media condition was plated in triplicate for the chemotaxis assay.

Flow cytometry and cell sorting:

Single cell suspensions of each tissue or blood sample were prepared before antibody staining was performed. Briefly, peripheral blood was acquired from mice

through retro-orbital bleeding and collected in heparin, ACK lysis was performed twice for 5 then 3 minutes, cells were washed and resuspended in 100 μ l of 1X PBS and placed on ice. Spleen samples were processed into a single cell suspension using the end of a syringe plunger in a filter, washed, and pelleted. Cells were re-filtered, ACK lysis was performed one time for 30 seconds, cells were then counted and put on ice. Excised tumor masses were minced with a scalpel blade and transferred to a 15ml conical tube in a total of 10mls of 1X tumor lysis buffer in supplemented McCoys 5A media. Tumor samples were digested in lysis buffer for 45 minutes with continuous rotation at 37°C. Following digestion, larger tumor pieces were allowed to settle, the supernatants were transferred to a new tube, and cells pelleted by centrifugation. Tumor cell pellets were resuspended, filtered, and pelleted, then cells were counted and placed on ice.

Staining protocols for surface markers on the prepared single cell suspensions were performed on ice beginning with incubation with anti-CD16/32 Fc γ receptor antibody for 15 minutes. Gr-1-PE Cy5.5 and CD11b-APC were used to identify neutrophils, F4/80-PE and CD11b-APC were used to identify macrophages, and DX5-APC and CD11b-PECy5.5 were used to identify NK cells. Antibody combinations were added to appropriate tubes of cells following the blocking step; tubes remained on ice for staining for 20 minutes, followed by washes with 1X PBS. All antibodies were purchased from Biolegend with the exception of the blocking antibody which was prepared from hybridoma culture in the lab. Stained samples were collected on FACS Calibur following analysis of collected events using FloJo software.

Quantitative RT-PCR:

Small portions of excised tumors were removed with a scalpel blade and weighed. The tumor pieces were placed in tubes containing 1 mL of Trizol reagent (Invitrogen) and kept at -80°C for less than two weeks before RNA extraction. Samples were thawed on ice, then each sample was placed in a small culture dish and processed individually by pressing flat and breaking up the tumor with the back end of a 3 mL syringe plunger until many small pieces were generated. Then all Trizol and tumor pieces were pipetted back into the tube and samples were vortexed briefly. While processing tumor tissue for extraction, samples were kept on ice. Following sample preparation, samples were placed in a rack at room temperature for 5 minutes. Then 200 µL of chloroform was added to each tube followed by manual, vigorous shaking of the samples for 15 seconds, and another 2 minute incubation at room temperature. Samples were then spun at 4°C for 15 minutes at 12,000 x g and the top aqueous layer was carefully removed and pipetted into a clean tube. Isopropanol was added at a volume of 500 µL in order to precipitate the RNA, tubes were capped and inverted 3-4 times to mix well and incubated at room temperature for 10 minutes. The same spin as above was performed on samples with the exception of lasting 10 minutes. The isopropanol supernatant was removed and pellets of RNA were washed with 75% ethanol and vortexed followed by a final spin at 4°C for 5 minutes at 7,500 x g. Supernatants were removed and pellets were dried for 10 minutes on the bench top before resuspended in small volume of RNase free water. RNA samples were quantitated immediately utilizing the quantifying program. Reverse transcription (BioRad) reactions were performed using 1 µg of each RNA sample and the cDNA generated was used for all subsequent qRT-PCR gene expression reactions.

Cytospin and stain:

Cells remaining following sort for tumor infiltrating Gr-1+CD11b+ and CD11b+ cells for cytotoxicity assay were applied at 100 μ L with a pipette to the Shandon pre-made cuvettes (Shandon Inc, Pittsburg, PA) with slide inserted. Only a small number of cells were used for visualization. Cells were spun in Shandon Cytospin 3 centrifuge at 100 rpm for 2 minutes. After spinning, the slide was allowed to air dry, cells were fixed to the slide with methanol, and then allowed to completely air dry again before staining. Protocol Hema 3 solution I and II (Fisher Diagnostics, Middletown, VA) were used for cell visualization. Slides were submerged in solution I for 30 seconds followed by water then solution II for 1 minute. Slides were rinsed in water, dried and a cover slip was applied. Cells analyzed by microscopy under 20X and 40X magnification. Images were captured using the Nikon Eclipse E400.

Graphing and statistical analysis:

Prism 4.0 (GraphPad Software: San Diego, CA, USA) was utilized in creating graphs and analyzing data collected from *in vitro* and *in vivo* studies. Following C5a ELISA the standard curve was graphed, linear regression test performed, and sample concentrations were extrapolated from the standard curve. Analysis of tumor growth significance and significance between the percentages of infiltrating cells following flow utilized the Student's t test. The survival curves were generated using the Kaplan-Meier method and the log rank test was used to analyze significance between the curves representing two groups. The correlation between cell infiltrates and tumor weight was analyzed using Pearson's correlation. For the Yac-1 Luc cytotoxicity assays, specific

cytotoxicity was calculated using the read out values by the following equation: relative light units of experimental well/average relative light units of positive control wells=cytotoxicity, (1-cytotoxicity) x 100=% specific lysis.

CHAPTER 5

C5A EXPRESSION FROM SKOV-3 CELLS

Expression of functionally active mouse complement component C5a from SKOV-3 human tumor cell line

The human ovarian adenocarcinoma cell line, SKOV-3, has been shown to express the CRP CD55 which demonstrated a significant role in reducing therapeutic efficacy of β -glucan/anti-HER-2/neu combination treatment [20]. CD55 accelerated inhibition of C5a release resulted in diminished tumor infiltration of β -glucan primed neutrophils [20]. SKOV-3 cells were selected for the transfection of mouse C5a to determine if C5a expression and the chemotactic effects of C5a from the tumor could enhance the recruitment of leukocytes to the tumor microenvironment, eliminating the negative effects of CD55.

The double stranded DNA construct used encoded for mouse C5a (secreted) and geneticin resistance for selection of positively transfected clones. Prior to transfection of SKOV-3 wild-type (WT) cells, a kill assay was performed in which the geneticin G418 concentration appropriate for killing all living SKOV-3 WT cells in no less than a weeks time was determined: 600 ug/mL. In addition to survival in selective media by transfected cells, ELISA was used for detection of C5a released into the supernatants, and SKOV-3 WT cells were confirmed not to express C5a (Table 1). Identification of C5a positive cells from the originally transfected cells that grew out of selection was followed by a

limiting dilution assay to isolate single clones that expressed substantial levels of C5a by ELISA (Table 2, 3). Selected clones were expanded for injection and screened by ELISA prior to *in vivo* passage in SCID mice.

Table 1.

absorbance	ng/mL C5a	sample ID
0.1425	-0.288	blank
0.137	-0.2982	SKOV-3 WT
0.1345	-0.3029	LLC
0.1735	0.02734	7
0.184	0.05469	6
0.251	0.10938	5
0.339	0.21875	4
0.633	0.4375	3
1.043	0.875	2
1.563	1.75	1

Table 1: C5a ELISA of SKOV-3 WT cells and standard control samples 1-7. LLC (Lewis Lung Carcinoma) cell supernatant screened as tumor sample negative control.

Table 2.

absorbance	ng/mL C5a	sample ID
0.148	0.085644	Blank
0.92	1.150966	1B6
0.276	0.2482458	1D11
0.947	1.188813	1C10
0.281	0.2552545	2B6
0.399	0.4206598	3D8
0.252	0.2146041	5B6
2.805	3.793246	2B5
0.137	0.053404	1G8
0.261	0.2272197	4C7
0.237	0.193578	1D10
0.232	0.1865693	1F2

absorbance	ng/mL C5a	sample ID
0.12	0.0600549	Blank
0.167	0.132585	2B4
0.498	0.6433822	1B2
0.174	0.1433873	1F5
0.136	0.084746	1E 7
0.188	0.164992	1B7
0.157	0.117153	2D2
0.337	0.394928	1C8

Table 2: C5a ELISA for SKOV-3 C5a transfected clones. Clones from the limiting dilution assay 96 well plates were screened once wells reached confluency. Cells from positive wells were collected, expanded, and re-screened in preparation for subcutaneous injection, or *in vivo* passage. *In vivo* growth of the chosen clone was critical to the establishment of a SKOV-3 C5a expressing cell line.

Table 3.

absorbance	ng/mL C5a	sample ID
0.182	-0.0563	blank
0.367	0.14101	1B6
0.136	-0.1053	1C10
1.15	0.97595	2B5
0.115	-0.1277	1B8

Table 3: C5a ELISA screen of SKOV-3 C5a supernatants from cells used for *in vivo* passage: Re-screen of supernatant of expanded cells injected s.c. in individual SCID mice for *in vivo* passage (1×10^7 or greater cells). Screening results demonstrate variations in C5a concentrations contained in cell supernatants during culture. 1C10 previously had high concentrations of C5a, and following *in vivo* passage the 1C10 clone was selected as a result of high concentration of C5a produced in culture supernatants and positive staining by IHC. Lack of detection of C5a in the supernatant of the 1C10 SKOV-3 C5a clone demonstrates the expression of C5a may be transient, or kinetics of C5a secretion may vary following transfection. This creates some difficulty in quantifying the C5a production by cells.

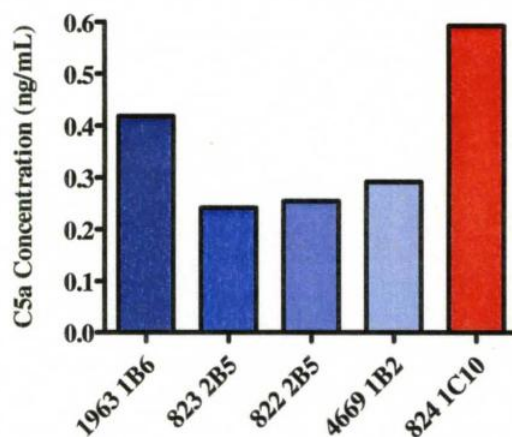


Figure 1: SKOV-3 C5a transfected clones maintain C5a secretion following *in vivo* passage. Screening of culture supernatant for C5a by ELISA revealed C5a production could be detected from clones following growth *in vivo*. 824 1C10 was the SKOV-3 C5a transfected clone selected for all subsequent *in vivo* and *in vitro* experiments due to the highest expression levels of C5a following *in vivo* passage. (In addition, it was the only tumor sample following *in vivo* passage in which C5a staining was observed abundantly in IHC experiments.) SKOV-3 C5a is 824 1C10 clone passaged *in vivo*.

Table 4.			Table 4.		
absorbance	ng/mL C5a	sample ID	absorbance	ng/mL C5a	sample ID
0.178	0.10101	821 10/15	0.081	0.0453	823 10/24-28
0.204	0.11619	823 10/24	0.071	0.03967	824 10/24
0.108	0.06067	821 10/24	0.178	0.10101	822 10/24-28
0.12	0.06751	822 10/15	0.176	0.09984	4669 10/28
0.105	0.05896	824 10/15	0.848	0.53492	824 10/24-28
0.081	0.0453	823 10/15	0.159	0.08997	1963 10/28
0.118	0.06637	2B5 10/15	0.203	0.11561	2B5
0.113	0.06352	821 10/24-28	0.581	0.35085	4669
0.738	0.45715	2B5 10/24-28	0.162	0.0917	822
0.268	0.15416	1963 10/24	0.273	0.15715	1963
0.13	0.07324	2B5 10/24	0.086	0.04899	Blank

0.125	0.01756	blank	0.141	0.08638	822 d4
0.114	-0.0298	824 24hr	0.106	-0.0642	822 d9
0.118	-0.0126	824 51hr	0.128	0.03046	823 24hr
0.115	-0.0255	824 72hr	0.139	0.07777	823 72hr
0.142	0.09068	824 inj d6	0.177	0.24122	823 51hr
0.302	0.77889	2B5 27hr	0.161	0.1724	823 d6
0.308	0.8047	2B5 48hr	0.152	0.13369	4669 24hr
0.131	0.04336	2B5 d5	0.154	0.14229	4669 48hr
0.395	1.17891	2B5 d7	0.195	0.31865	4669 75hr
0.132	0.04767	821 24hr	0.157	0.1552	1963 24hr
0.134	0.05627	821 48hr	0.218	0.41758	1963 51hr
0.124	0.01326	821 75hr	0.155	0.1466	1963 16hr
0.092	-0.1244	821 d6	0.125	0.01756	4669 16hr
0.128	0.03046	821 d4	0.142	0.09068	821 16hr
0.12	-0.004	821 d9	0.104	-0.0728	822 16hr
0.18	0.25413	822 24hr	0.105	-0.0685	823 16hr
0.169	0.20681	822 48hr	0.126	0.02186	824 16hr

Table 4: Time course for optimization of the detection of C5a by ELISA of cell lines following *in vivo* passage. Positive clones that had been successfully passaged *in vivo* were grown *in vitro* and supernatant from these cells was collected at various time points following plating. Clones revealed different time points for peak C5a expression *in vitro*.

Table 5.

absorbance	ng/mL c5a	sample ID
0.261	1.140633	SKOV-3 C5a 1
0.165	0.323909	SKOV-3 CV
0.217	0.766301	SKOV-3 C5a 2

Table 5: C5a ELISA of SKOV-3 C5a (824 1C10) and CV. Both cell lines had been passaged *in vivo* and were screened for production of C5a by ELISA prior to using the cells in experiments. C5a production maintenance throughout culturing and experiments was routinely screened. Sample SKOV-3 C5a 1: 2 days culture in T25 flask. Sample SKOV-3 C5a 2: 4 days culture in T25 flask. Variation in C5a concentration was observed in samples acquired over the same cells at different times.

Table 6.

absorbance	ng/mL C5a	sample ID
0.296	0.63	1C10
0.318	0.7	1C8
0.244	0.46	1D11
0.199	0.32	1B2
0.328	0.73	2B6
0.516	1.33	2B5
0.327	0.73	824 1C10
0.482	1.22	824 1C10

Table 6: C5a production screen of pre-*in vivo* passage clones frozen down. Re-screen after cells were frozen revealed maintenance of C5a expression and the SKOV-3 C5a *in vivo* passage clone (824 1C10) was screened prior to use in an experiment.

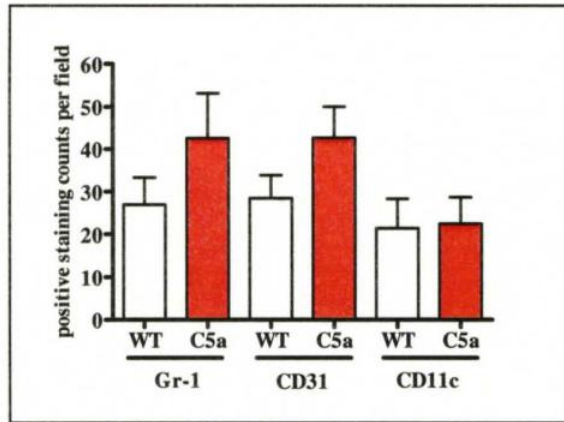
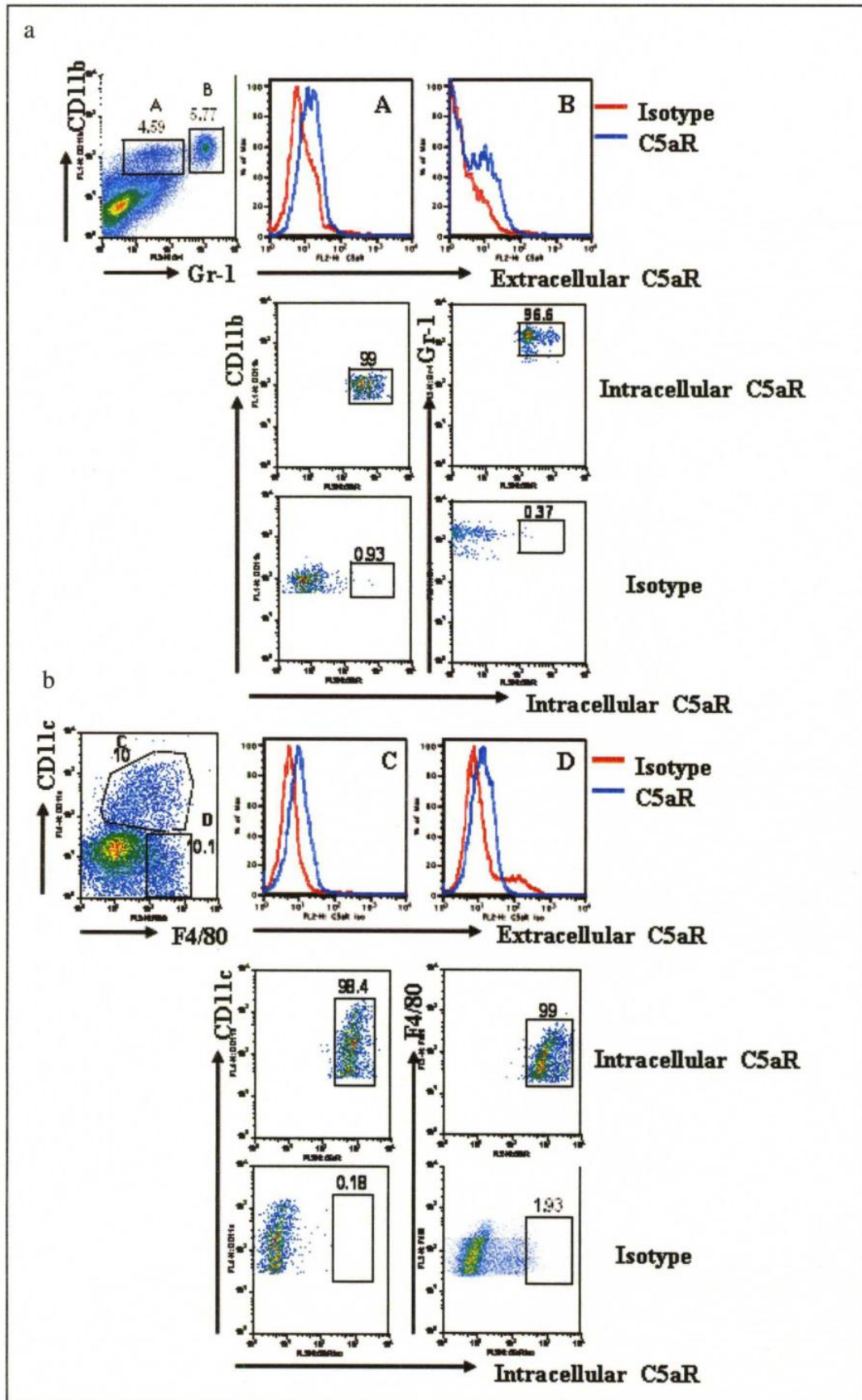


Figure 2: Summary of results for IHC staining of SKOV-3 WT and SKOV-3 C5a. Following excision of tumors from individual mice, half of the tumor was frozen for sectioning and staining. Tissues were stained with biotinylated anti-mouse Gr-1 antibody, anti-mouse CD31, and anti-mouse CD11c to analyze if any differences occurred in the presence of these cell surface markers between tumor types as a result of the expression of C5a. It was determined by this method that no significant differences between the cell lines for the infiltration of neutrophils (Gr-1) or dendritic cells (CD11c), or the density of blood vessels (CD31) was apparent.

Tumors for *in vivo* passage mice were monitored and allowed to grow until tumors reached 5-6 mm diameter. Following excision and culture of *in vivo* passaged SKOV-3 C5a positive clones, maintenance of C5a expression was confirmed by performing ELISA (Fig. 1) on culture supernatants. Of note, it was observed at some time points that C5a levels were low to undetectable in previously positive clones before and after *in vivo* passage (Table 2, 3, 4, 6). However, subsequent ELISA would again confirm positive expression. Kinetics of C5a expression based on cell status, phase of growth, or condition of growth media is critical in the screening process. Following time course experiments, it was difficult to pinpoint a time post plating at which SKOV-3 C5a cells could be screened and C5a levels would consistently be detected, further clarifying a transient C5a expression and complicating C5a quantification. 2B5 clone from before *in vivo* passage was utilized as a positive control for these experiments (Table 4). C5a expressing transfected cells expressed lower ranges of ng/mL to pg/mL concentrations (1.2 ng/mL-250 pg/mL) (Fig. 4a). Initial screening by IHC comparing SKOV-3 C5a and SKOV-3 WT tumors for infiltration of Gr-1+ and CD11c+ cells, as well as the presence of CD31+ cells revealed no significant difference (Fig. 2).

The cells of primary interest in C5aR response were the cells of the innate immune system. Splenocytes from SCID animals were prepared and stained for flow cytometric analysis of the general expression of C5aR both extracellularly and intracellularly (Fig. 3a-c). All cells screened, NK cells; monocytes; neutrophils; macrophages; and dendritic cells; showed minimal to moderate levels of the receptor expressed on the surface, and nearly all of each subset screened expressed the C5aR intracellularly (Fig. 3a-c). In addition, to ensure the C5a expression from SKOV-3 tumor

cells would not directly affect the growth of the tumor cells, but rather, alterations in growth would be a result of the receptor ligand interaction on host cells, SKOV-3 WT cells were also stained for C5aR surface expression (Fig 3d).



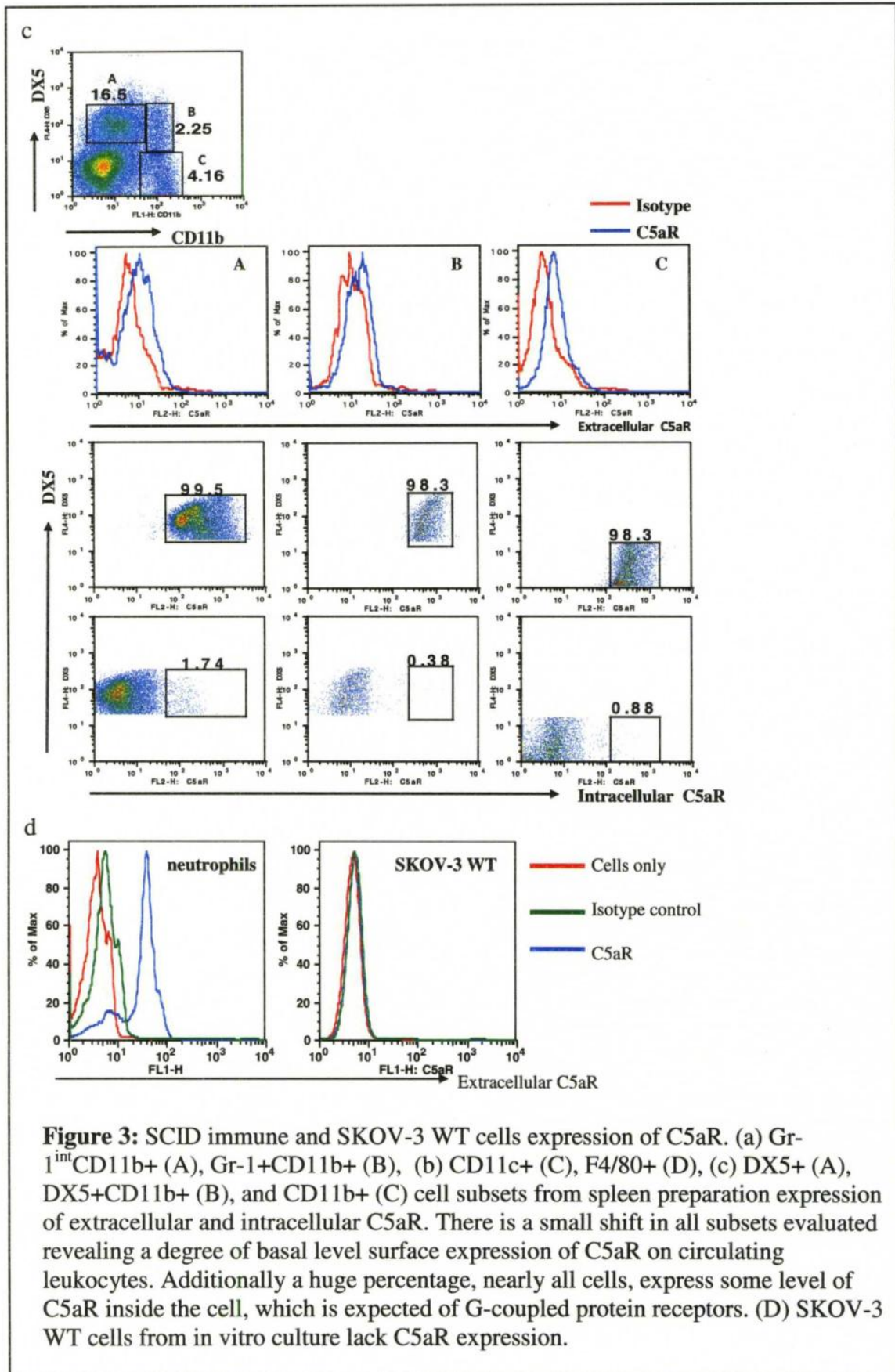


Figure 3: SCID immune and SKOV-3 WT cells expression of C5aR. (a) Gr-1^{int}CD11b⁺ (A), Gr-1⁺CD11b⁺ (B), (b) CD11c⁺ (C), F4/80⁺ (D), (c) DX5⁺ (A), DX5⁺CD11b⁺ (B), and CD11b⁺ (C) cell subsets from spleen preparation expression of extracellular and intracellular C5aR. There is a small shift in all subsets evaluated revealing a degree of basal level surface expression of C5aR on circulating leukocytes. Additionally a huge percentage, nearly all cells, express some level of C5aR inside the cell, which is expected of G-coupled protein receptors. (D) SKOV-3 WT cells from in vitro culture lack C5aR expression.

SKOV-3 WT tumor cells were also transfected with control vector (CV) plasmid, lacking C5a expression (Table 5). These cells were prepared in a slightly different way due to lack of C5a expression. The transfection and initial growth selection process was identical, but the numerous rounds of screening by ELISA was omitted, as well as the limiting dilution assay. Once transfected cells grew out of selection following the transfection, several clones were passaged *in vivo* and determined to lack C5a expression by ELISA (Table 5, Fig. 4a,d).

In order to determine the transfection or introduction of C5a expression did not introduce any growth disparities into the CV or C5a cell lines, *in vitro* growth kinetics of all relevant cell lines was monitored in real time using the Acea assay system. Both C5a and CV transfected clones displayed an initial *in vitro* growth enhancement over SKOV-3 WT, which was no longer apparent after 12 hours of culture (Fig. 4c); all three cell lines were determined to grow equally well. *In vitro* growth studies did not look greater than 40 hours following plating of cells due to restraints in well size; however it may be important to determine long term *in vitro* growth similarities. SKOV-3 WT cells seem to be growing well at 40 hours and at later time points may begin to grow significantly better than the transfected cell lines. *In vivo* data presented below suggests otherwise, since SKOV-3 WT and CV cells grow similarly *in vivo* up to 38 days (Fig. 5a).

Additionally, to determine the C5a expressed by C5a expressing transfected SKOV-3 cells following *in vivo* passage was functionally active, an *in vitro* chemotaxis assay was performed using transfected cell culture supernatants that had been approximately 6 times concentrated by centrifugation. Chemotactic functionality of C5a

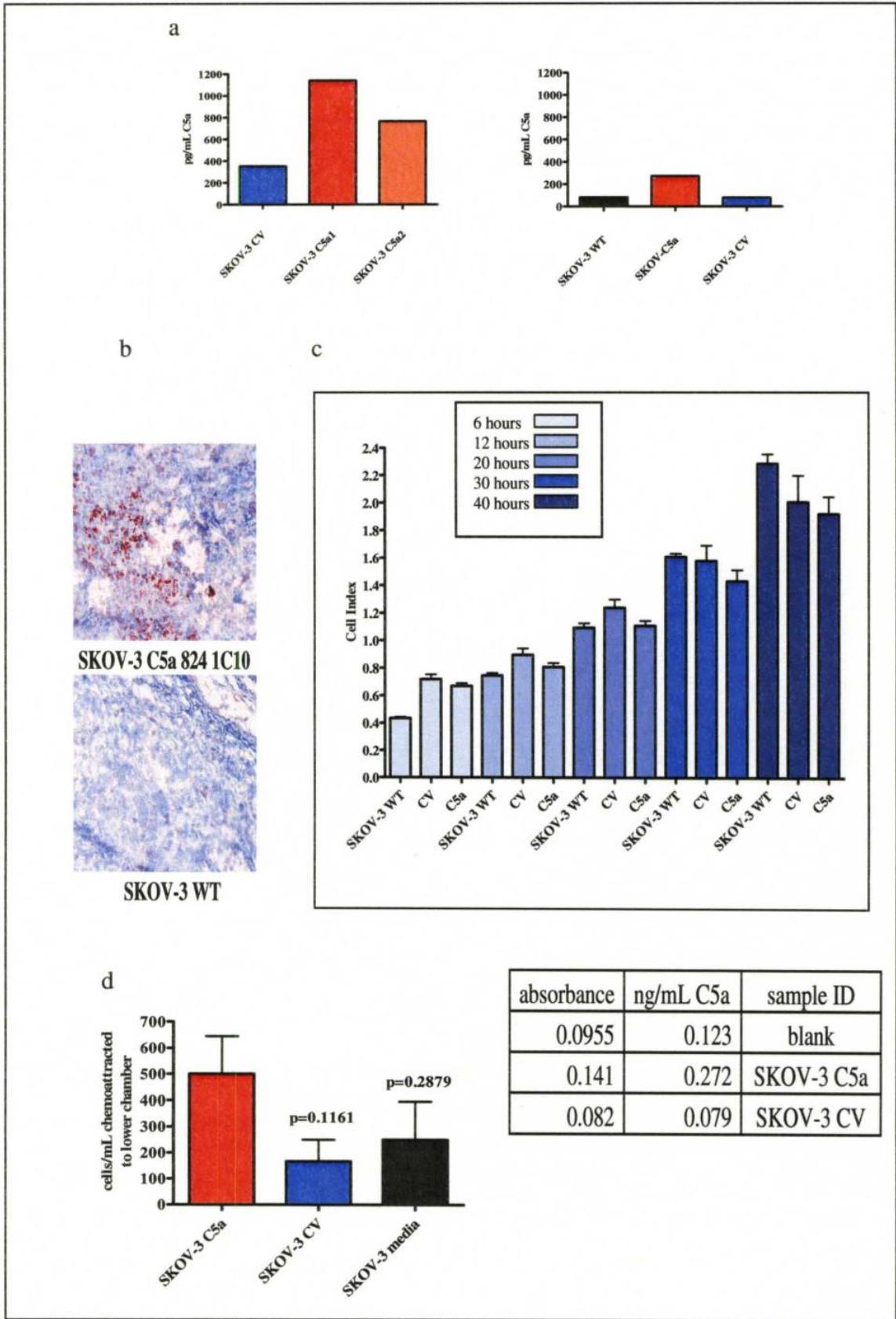


Figure 4: SKOV-3 C5a, CV, and WT C5a expression and *in vitro* characterization of SKOV-3 cell lines. (a) ELISA assays were performed before experiments using SKOV-3 C5a (824 1C10) cells or supernatants *in vivo* or *in vitro*. Screening, by ELISA, of supernatants from SKOV-3 C5a, CV, and WT cells throughout the time of study revealed that levels of C5a expressed by SKOV-3 C5a transfected cell line varied during culture. The bar graph on the left shows C5a ELISA acquired 4-10-2009, and the bar graph on the right from 4-27-2010. The SKOV-3 C5a clone selected for experimental study demonstrated different levels of C5a depending on size of culture vessel, cell confluency, time in culture, amount of time supernatant had been applied. (b) Following tumor excision from SCID mice injected with isolated SKOV-3 clones expressing C5a or the CV, one half of the tumor was frozen for sectioning and analysis of C5a expression by IHC, with rat anti-mouse C5a monoclonal antibody. (c) Following transfection before monitored for growth differences *in vivo*, SKOV-3 C5a and CV cells grown and screened for C5a expression following *in vivo* passage were plated to determine the existence of *in vitro* growth disparities from SKOV-3 WT cells due to the transfection or the introduction of C5a expression. (d) C5a functional activity was assessed through chemotactic assays. Overnight culture supernatants, similar to ELISA samples, were collected from confluent T25 flasks and concentrated by centrifugation. The migration of J774 murine macrophage cell line from the transwell to the supernatant (from the samples in the 4/27/2010 ELISA (a)) in the bottom well was quantified by microscope evaluation of trypan blue negative cells collected from the lower chamber following a three hour incubation.

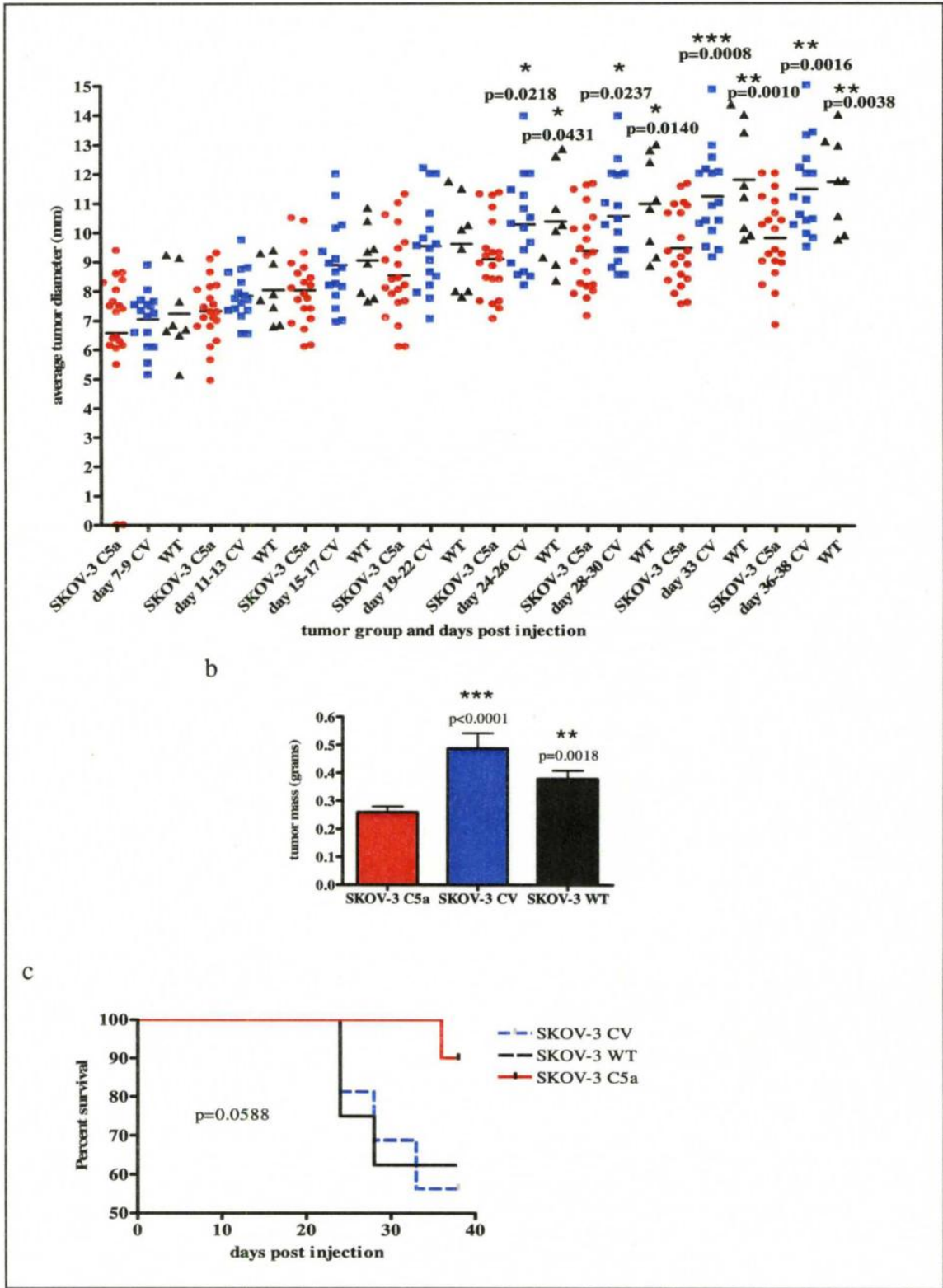
in the transfected SKOV-3 supernatant was determined by the enhanced migration, although not significantly, of J774 murine macrophage cell line to the SKOV-3 C5a transfected cell supernatant (Fig. 4d). The SKOV-3 C5a cell line was successfully generated following transfection, limiting dilution assay for acquisition of a single clone generated line, and multiple rounds of ELISA C5a screening with IHC confirmation of the 824 1C10 SKOV-3 C5a clone as the cell line used for study. It must also be considered that even though the cell culture supernatants were serum free, that some other agent may have been enhanced in the culture supernatant to affect the chemotaxis of the J774 cells. For example, exosomes released by tumor cells may have an influence on migrating cells, and the composition of these exosomes following transfection may be altered [48].

SKOV-3 C5a expressing tumor cells have significantly reduced growth *in vivo*

Using the ICR SCID-immunocompromised/SKOV-3 tumor model was beneficial on two fronts. It permitted for focus on the effect of C5a on innate immune cells' infiltration and functional activity in the tumor, exclusively. Cells of the innate immune system express abundant C5aR so they were hypothesized to be the main targets. Figure 3 demonstrates that indeed the innate immune cells (Fig. 3a-c) circulating through SCID mice express both surface and intracellular C5aR while the tumor cells did not (Fig. 3d). Additionally, this model allowed study of an aggressive human tumor type, SKOV-3 ovarian adenocarcinoma which overexpresses the CRP CD55 [20]. Overexpression of CD55 ultimately results in accelerated elimination of the C5 convertase, completely terminating the release of C5a endogenously [7].

SCID mice were implanted with $7-10 \times 10^6$ of SKOV-3 C5a, SKOV-3 CV, or SKOV-3 WT cells s.c. Solid tumor growth at the site of injection was monitored and measured every 2-4 days following development. All tumor cell lines demonstrated similar initial *in vivo* growth; however, beginning around day 24 following tumor cell injection, SKOV-3 C5a tumors revealed significant reduction in tumor progression (Fig. 5a). Tumor development in SCID mice bearing SKOV-3 C5a tumors slowed, and these mice could have survived longer than control tumor-bearing mice which reached the 12mm diameter endpoint measurement tumor size (Fig 5c) to determine enhanced survival. However, as required by animal care restrictions, mice bearing tumors with a diameter of 15mm must be euthanized, and SKOV-3 C5a mice were euthanized for the purpose of simultaneous study with SKOV-3 CV and WT mice with endpoint tumors. Upon excision at an endpoint time between 31 and 38 days for three separate

experiments, C5a expressing tumors weighed significantly less than both CV and WT tumors (Fig. 5b). At sacrifice, the outward appearance of SKOV-3 CV mice tumors were necrotic, which was not the case for the smaller SKOV-3 C5a tumors that showed limited or no necrosis (Fig. 5d).



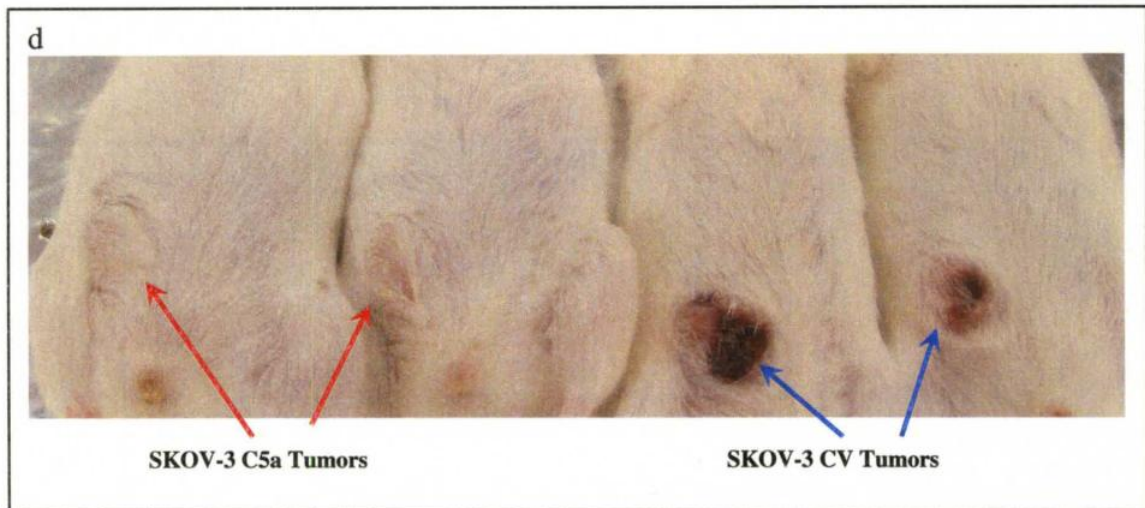


Figure 5: SKOV-3 C5a *in vivo* tumor growth is significantly reduced. (a) SCID mice were injected s.c. with SKOV-3 C5a (n=20), SKOV-3 CV (n=16), or SKOV-3 WT (n=8) and growth was monitored by measuring and averaging two perpendicular tumor diameters. Around day 24 post tumor cell injection, SKOV-3 C5a tumors grew significantly less well. (b) The significance in tumor growth could also be visualized upon excision when tumors were weighed. (c) Only 2 mice bearing C5a expressing SKOV-3 tumors reached 12 mm before the endpoint as compared with CV (7) and WT (5) SKOV-3 tumor bearing mice. (d) Image taken following sacrifice of tumor bearing mice reveals gross differences in appearance between SKOV-3 C5a (red arrows) and SKOV-3 CV (blue arrows). The necrotic phenotype was also observed in mice bearing SKOV-3 WT tumors (not shown).

CHAPTER 6

EFFECT OF C5A RELEASE FROM SKOV-3 CELLS IMPACTS THE TUMOR MICROENVIRONMENT GREATLY: INFILTRATING POPULATIONS AND RELEASE OF IMMUNE AND GROWTH MEDIATORS

Innate immune cell subsets enhanced infiltration of SKOV-3 C5a expressing tumors

Cells of the innate immune system have been shown to be important to mount an anti-tumor response [33, 39, 49], as well as play an important role in sustaining the immunosuppressive environment and angiogenic switch promoting tumor growth and metastasis [15, 50]. Expression of C5a from the tumor environment may harness the anti-tumor response of these cells because it potently stimulates these cells expressing C5aR for a proinflammatory response. However, this potency of C5a as a proinflammatory peptide and its pronounced role in death following sepsis [3, 34, 51] requires caution when utilizing it *in vivo* as a chemotactic and stimulatory agent. It was important to note any dramatic systemic effects as a result of the introduction of C5a even from the limited environment of the tumor of these animals. Tumors were examined for the role of C5a in enhancing the migration of innate immune cells into the tumor tissue.

NK cells were identified by the expression of DX5 and/or CD11b, macrophages by the expression of F4/80 and/or CD11b, and neutrophils by the expression of high levels of Gr-1 and CD11b. As an additional marker with DX5, CD11b expression by NK cells has been associated with activation and maturation [52]. Minimal significant systemic effects were observed in the percentage of circulating innate leukocyte subsets

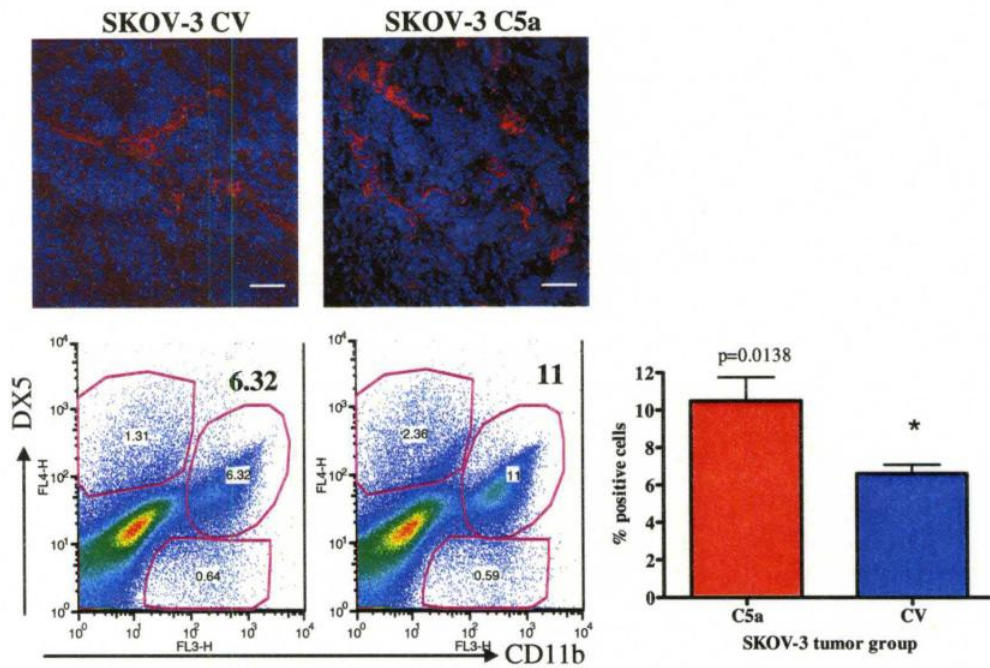
in the peripheral blood and spleens aside from an increase in NK cells in both sample types of C5a tumor-bearing mice (data not shown). Additionally, naïve mouse spleen samples contained the highest percentage of neutrophils (Gr-1 high CD11b high), significantly more than C5a and control tumor bearing mouse spleens (data not shown). Despite the reduction of neutrophils in C5a tumor bearing mice spleens, significantly more occurred than in the spleens of CV tumor bearing mice indicating the reduction in neutrophils was less dramatic with C5a expression.

However, several changes in the percentage of infiltrating innate leukocytes were observed between SKOV-3 C5a and CV tumors. C5a tumors showed both an increased percentage in infiltrating DX5+CD11b+ NK cells (Fig. 6a) and F4/80+, F4/80+CD11b+ subsets of macrophages (Fig. 6b). Although by flow cytometric analysis there was no difference in the percentages of Gr-1+CD11b+ cells infiltrating the tumor, when correlated with tumor weight, more Gr-1+CD11b+ neutrophils were found to infiltrate smaller C5a expressing tumors (Fig. 6c). C5a appears to be enhancing the infiltration of both NK cells and macrophages, two distinct subsets of innate immune cells that have been shown to be important in tumor immunity [10, 39, 49, 53, 54].

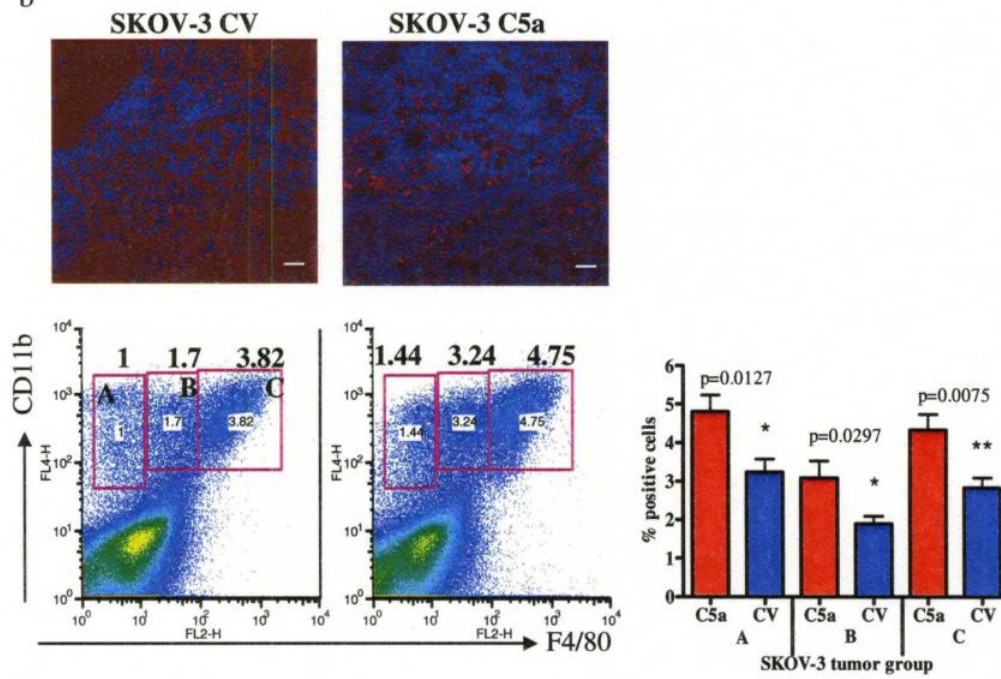
Neutrophils express the most abundant C5aR of all innate immune cells and should therefore be the most sensitive in responding to C5a release in the tumor [5]. However, there is not evidence that C5a is recruiting more neutrophils to the tumor. The short-lived cells may be infiltrating the tumor, activated to kill tumor cells, and dying, so increased percentages of Gr-1+CD11b+ cells are not detected at this time point, and the shorter half-life of these cells results in little accumulation. The heightened sensitivity of neutrophils to C5a may be the reason a difference in infiltration of these cells is only seen

when correlated with tumor size which may result directly from the amount of C5a released *in vivo*. It is important to note these tumor infiltrating neutrophils were demonstrated to express C5aR extracellularly (Fig. 6c), despite the presumed continuous release of C5a into the environment. A previous tumor C5a model demonstrated infiltrating Gr-1+CD11b+ cells only expressed intracellular C5aR [36]. SKOV-3 C5a tumors have shown enhanced infiltration in two subsets of migrating innate leukocytes, additionally C5a release may enhance the cytotoxic effector function and/or anti-tumor phenotype of these cells upon arrival.

a



b



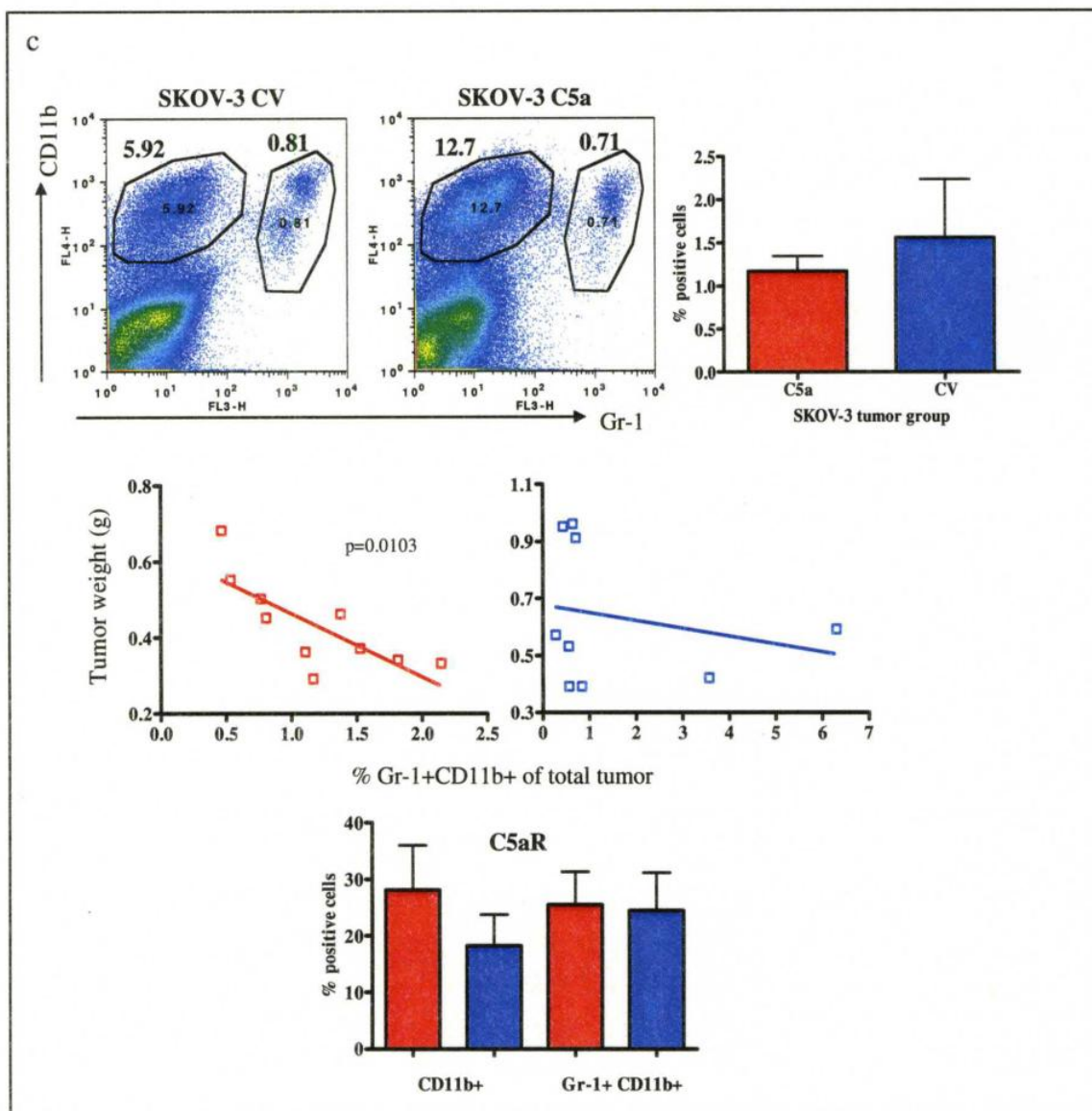


Figure 6: Enhanced infiltration of innate immune cell subsets in SKOV-3 C5a tumors. When control tumor bearing mice tumor size reached endpoint measurements at 31-38 days post injection, all mice were sacrificed and tumor samples were collected and processed for staining with innate immune cell surface antigen recognizing antibodies. Differences in percentages of innate immune cells infiltrating the tumor occurred in both the (a) DX5+CD11b+ NK cell and (b) F480intCD11b+ and F4/80+CD11b+ macrophage subsets. No difference was seen between SKOV-3 C5a and CV tumor (c) Gr-1+CD11b+ infiltrate percentages; however, when considering both the percentage of neutrophil infiltrates and the weight/size of the excised tumor, C5a tumors showed a correlation between smaller tumor size and greater percentages of Gr-1+CD11b+ cells infiltrating. The SKOV-3 C5a Gr-1+CD11b+ and CD11b+ infiltrating subsets expressed surface C5aR to similar levels, despite the C5a released *in vivo*.

Tumor microenvironment analysis reveals a significant decrease in the production of pro-tumorigenic factors

A small portion of tumor sample was cut away following tumor weight measurement. This portion was processed for quantitative real time-polymerase chain reaction (qRT-PCR) analysis of pro- and anti-tumorigenic gene levels to identify alterations in the total tumor microenvironment as a result of C5a expression. Cytokine levels produced by only innate immune cells presumably have a much different profile than if adaptive cells are present as would be the case in an immunocompetent host; however, of importance is the effect C5a has on the cells that highly express the appropriately receptor, constitutively.

When the total tumor samples were analyzed by qRT-PCR, many cytokine genes evaluated did not show a difference in expression at the mRNA level: inducible nitric oxide synthase (iNOS), transforming growth factor-beta (TGF- β), interleukin (IL) IL-6, IL-10, IL-12, interferon-gamma (IFN- γ), (Fig. 7b) granzyme B, or perforin (Fig. 7c). Due to the enhanced infiltration of NK cells into SKOV-3 C5a tumors molecules important in their cytotoxic function were of interest, granzyme B and perforin mRNA levels were examined but no significant difference in the levels of expression of these two genes was noted. However, there were several genes with striking differences. Two genes, vascular endothelial growth factor (VEGF) and arginase, which have been shown to promote tumor growth and inhibit the local immune response, were shown to be present in significantly lower levels in C5a expressing tumors (Fig. 7a). In addition, tumor necrosis factor-alpha (TNF- α) expression levels of mRNA were also significantly lower in SKOV-3 C5a tumor compared to SKOV-3 CV and WT (Fig. 7a) (Table 7). TNF- α was originally discovered based on its tumor killing function, but since its discovery, recent

tumor studies including human and murine, have identified this cytokine as being a key mediator in promoting tumor growth [55, 56].

In subsequent experiments, upon tumor excision, cells were prepared and sorted for dissecting which portion of tumor cells were responsible for the expression of certain genes of interest (Fig 8). First, tumors were divided into tumor cells and infiltrating leukocytes using the CD11b surface marker to separate these populations. Of interest was defining the source of VEGF to identify whether the role of C5a in its reduction was potentially directly on the immune population or indirectly leading to reduced tumor VEGF. Following qRT-PCR of both populations, it was clear the tumor cells were the source of VEGF and SKOV-3 CV and WT tumor cells revealed elevated levels of VEGF mRNA expression (Fig. 8a).

In the previous tumor samples, arginase was observed to be expressed at significantly lower levels in SKOV-3 C5a tumors (Fig. 7a); however, in the separated samples, arginase mRNA levels were not significantly different between either cell group, but it was determined the source of arginase in the tumor was derived predominantly from infiltrating leukocytes (Fig. 8b). Additional mediators, TGF- β and iNOS, were re-evaluated in the separated samples to further examine the presence of any differences in purified leukocyte samples. No difference was observed in levels of TGF- β in CD11b+ cells, but iNOS was shown in significantly greater levels in the CD11b+ cells sorted from SKOV-3 CV tumors (Fig. 8c). Both VEGF and iNOS have been linked to promoting tumor cell growth and poor survival in human studies [57, 58]. The release of C5a within the tumor microenvironment results in the reduction of iNOS in the infiltrating leukocytes and VEGF in the tumor cells.

Tumor associated macrophages have gained a great deal of attention in recent years, highlighted as bearing extreme importance on shaping the tumor microenvironment depending on macrophage phenotype which is related to the set of factors produced by the cells [13]. Macrophages in the tumor are most often characterized as pro-tumorigenic, expressing mediators to support tumor growth and suppress the immune system from targeting tumor cells for destruction. Because C5a preferentially recruited a greater percentage of macrophages to the tumor, determination of C5a alteration of tumor associated macrophage phenotype was important. An additional sorting experiment was required to isolate F4/80+ infiltrates from tumor cells and other infiltrating leukocytes. The results of these qRT-PCR experiments revealed no difference between the SKOV-3 C5a or CV tumor infiltrating macrophages in the case of VEGF, iNOS, TNF- α or IL-12 (Fig 9). F4/80+ cells infiltrating SKOV-3 C5a tumors made significantly less arginase, confirming lower arginase levels determined previously by whole tumor qRT-PCR experiments. Also surprisingly revealed was SKOV-3 C5a F4/80+ cells made significantly more TGF- β . This immune mediator is presently almost exclusively identified as pro-tumorigenic and immunosuppressive [59-62]. TGF- β suppresses cytotoxic T cells and enhances immune regulatory T cells and macrophages to promote tumor growth [59], even tumor cells themselves acquire the means to produce TGF- β . However, TGF- β is also critical to cytostatic cell pathways and inhibiting developing tumors from growing in developing stages [60-63]. The results for the above qRT-PCR experiments have been summarized in Table 7.

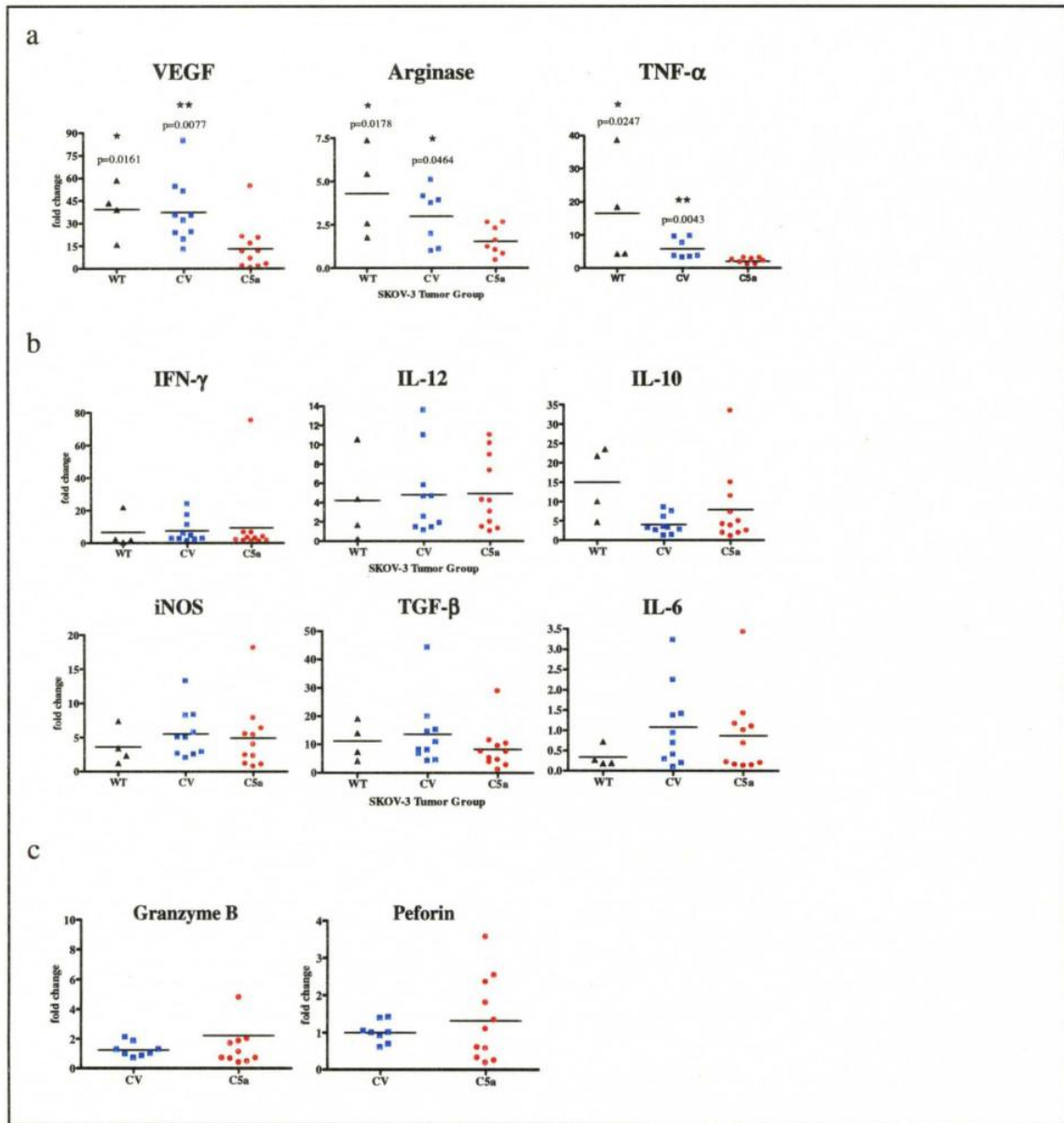


Figure 7: Immune factors expressed in the tumor microenvironment *in vivo*. A small portion of the excised tumors at end point were processed in Trizol for RNA extraction and following reverse transcription to cDNA were screened for the presence of known immune mediators important in tumor development qRT-PCR. (a) Levels of VEGF, arginase, and TNF- α mRNA were significantly different between SKOV-3 C5a and the SKOV-3 CV and WT control tumors, with each gene showing downregulation as a result of C5a in the tumor. (b) Other important modulators of immune and tumor cells were evaluated in tumor samples, but these showed no significant difference including IFN- γ , IL-12, IL-10, iNOS, TGF- β , IL-6. (c) Two important effector molecules in NK cell cytotoxicity, granzyme B and perforin, were also evaluated by qRT-PCR after determining NK cell enhanced infiltration of SKOV-3 C5a tumors, however, no difference was seen in the expression levels of these molecules between tumor groups.

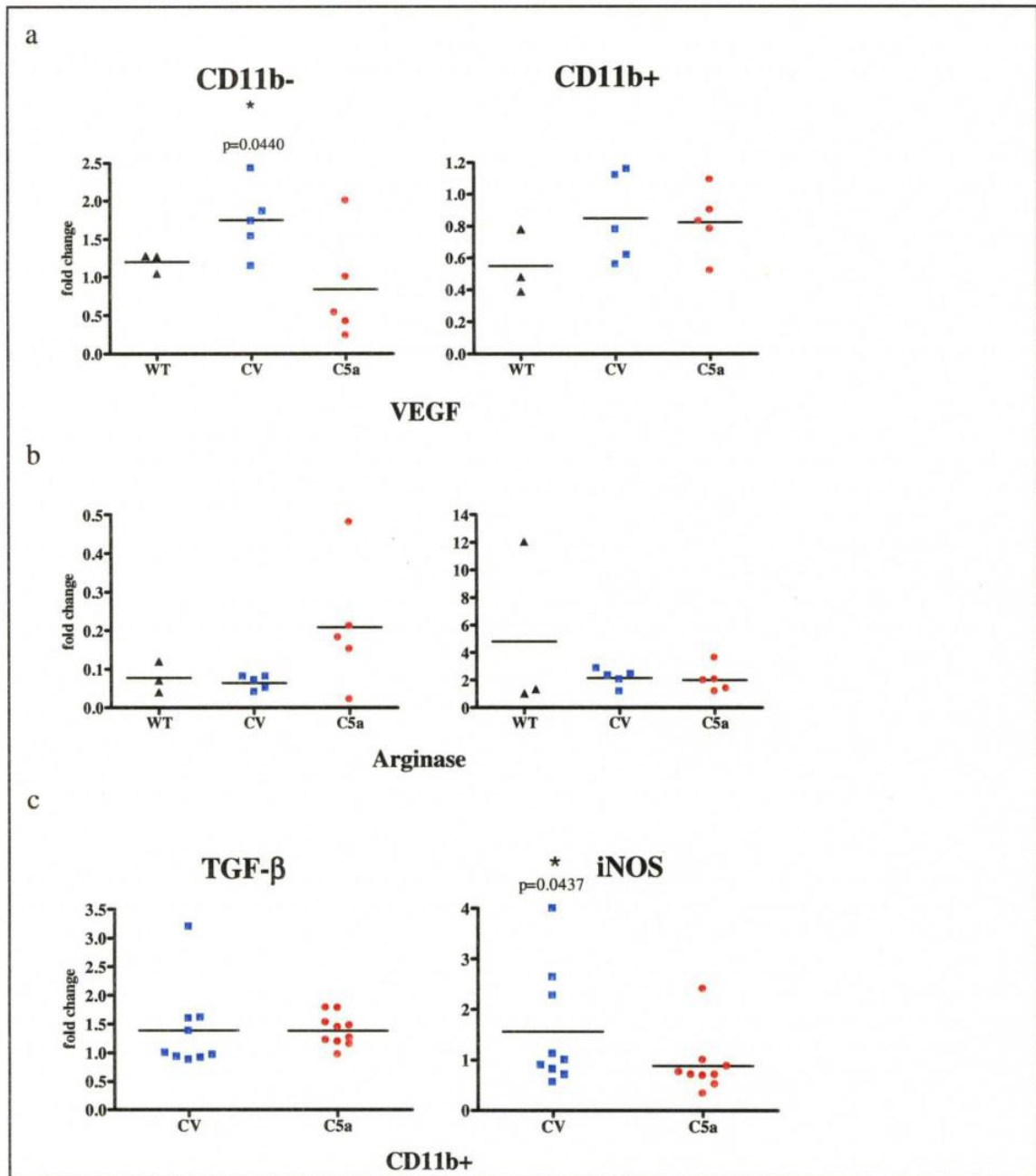


Figure 8: Immune cell infiltrates vs. tumor cell expression of mediators. Immune cells were sorted from tumor cells using a cell sorter by with the innate immune cell marker CD11b which is expressed on the majority SCID mouse innate immune cell subsets. (a) Confirmation of significantly greater levels of VEGF expression in SKOV-3 CV and WT samples, and demonstration that the tumor cells are the source of VEGF. (b) Arginase did not reach significantly different levels, but overall the CD11b+ cells produce higher detectable levels of arginase than tumor cells. (c) Two important mediators of immune function and tumor growth, TGF- β and iNOS, were also evaluated, and iNOS levels were determined to reach significantly higher levels of expression at the mRNA level in SKOV-3 CV tumors in the immune cell subset.

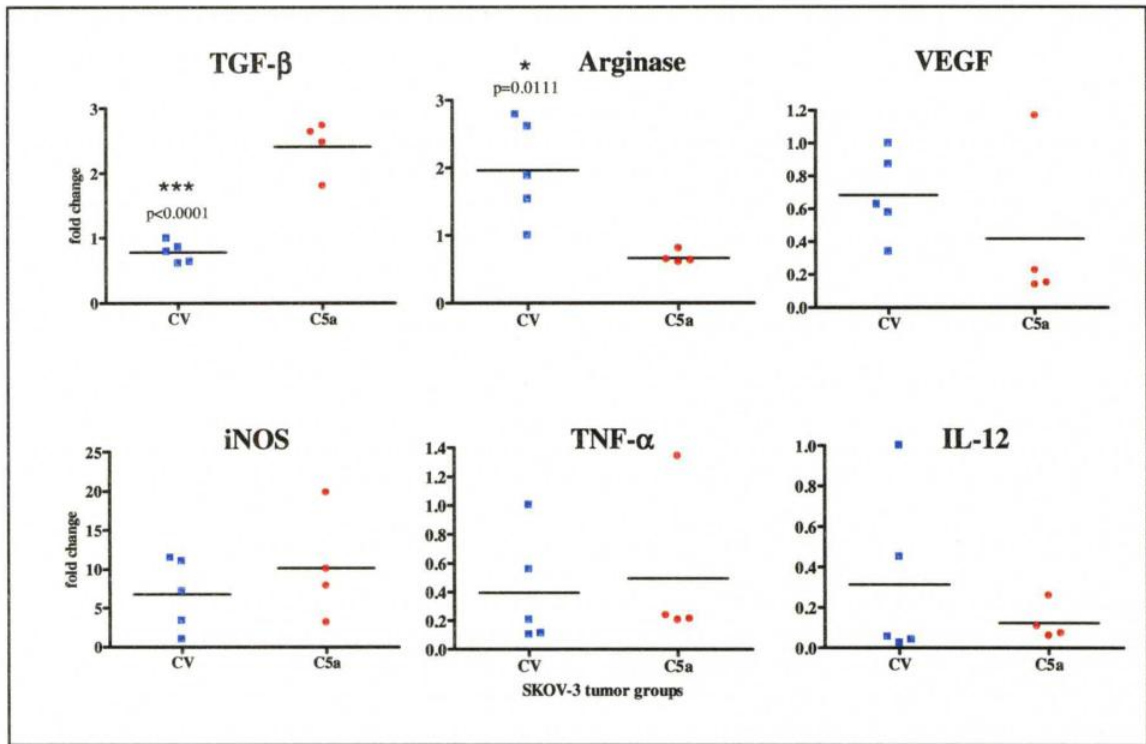


Figure 9: F4/80+ tumor infiltrates mediator expression profile. Further evaluation of F4/80+ infiltrating tumor cells by qRT-PCR separated out of *in vivo* grown tumors by cell sorting. In order to determine the tumor associated macrophage phenotype induced by C5a expression in the tumor, several cytokines and mediators were evaluated. F4/80+ immune cells infiltrating cells expressed significantly higher levels of TGF-β and significantly lower levels of arginase.

Table 7












	VEGF	arginase	TNF-α	iNOS	TGF-β
Total Tumor					
CD11b-Tumor cells			Ovarian Tumor cells		
CD11b+ Immune cells			NK cells N ϕ		
F4/80+ Mϕ					

Table 7: Summary table for SKOV-3 C5a tumors of qRT-PCR data collected for total and sorted tumor samples. In the tumor, VEGF mRNA levels were significantly decreased in SKOV-3 C5a total samples, and it was determined following sorting of tumor cells and infiltrating CD11b+ innate immune cells that tumor cell production of VEGF was responsible for this decrease. Arginase mRNA levels were also significantly decreased in the total microenvironment of SKOV-3 C5a tumors, and decreased production was found to be specifically a result of macrophage decreased arginase levels. One final cytokine, TNF- α , was found to be significantly decreased in total SKOV-3 C5a tumor samples compared to SKOV-3 CV and WT. Although it was determined following sorting that the macrophage production of TNF- α mRNA was not different between the tumor groups, it remains undetermined as to whether the change in production is a result of tumor cell or other innate immune cells, including NK cells or neutrophils, in the SKOV-3 C5a microenvironment. Production of mRNA levels of iNOS was significantly less in the infiltrating innate immune cells of SKOV-3 C5a tumors. As well, TGF- β mRNA levels were significantly less in macrophages isolated from SKOV-3 C5a tumors.

CHAPTER 7

C5A ENHANCES INNATE IMMUNE CELLS TO BE ANTI-TUMORIGENIC AT LOW DOSES

***In vitro* studies demonstrate the presence of C5a in transfected SKOV-3 cultures render naïve innate leukocytes more cytotoxic to tumor cells**

In vitro studies were enlisted to determine whether C5a in the culture could enhance the cytotoxicity of tumor cells by naïve leukocytes isolated from SCID spleens. Acea system technology was used to measure the amount of cytotoxicity produced to tumor cells by non-adherent naïve leukocytes, which includes neutrophils and NK cells. Enhancement of cytotoxicity of C5a expressing tumor cells was demonstrated. When compared to SKOV-3 CV and WT cells, SKOV-3 C5a cells were killed at a significantly higher percent in the presence of the naïve leukocytes (Fig. 10). These results indicate the C5a in the supernatant of the culture has activating potential to the added naïve white blood cells for superior effector function in destroying tumor cells.

Cytotoxicity of naïve, non-adherent leukocytes from SCID spleen were addressed against SKOV-3 WT in a subsequent study with cells sorted from SKOV-3 C5a and SKOV-3 CV tumors out of growth *in vivo*. Gr-1+CD11b+ cells were isolated from the tumors of endpoint mice, and these cells were cultured with naïve leukocytes in SKOV-3 WT culture, or only with SKOV-3 WT cells to determine the effect they have on immune cell function and tumor growth. The markers used in purifying out this population are widely used and accepted as markers for tumor infiltrating MDSCs. In the current study

Gr-1+CD11b+ cells have been labeled neutrophils determined by the decrease of this specific subset of cells in tumor bearing mice compared to naïve mice. However, in the setting of the tumor, infiltrating immune cells are known to possess tumor promoting immune suppressive functions. In fact, when Gr-1+CD11b+ cells from both SKOV-3 C5a and SKOV-3 CV tumors were co-cultured with the naïve leukocytes that had previously shown cytotoxicity to each tumor cell line (SKOV-3 C5a, CV, and WT) (Fig. 10) decreases in percent cytotoxicity of tumor cells occurred (Fig. 11a). Cells isolated from SKOV-3 C5a tumors had significantly lower suppressive effects on the naïve leukocyte cytotoxicity. In the absence of the naïve leukocytes, neither tumor isolated Gr-1+CD11b+ population led to SKOV-3 WT tumor cell destruction. The negative cytotoxicity demonstrated was interpreted as SKOV-3 WT cell growth in culture in the presence of Gr-1+CD11b+ tumor infiltrated cells (Fig. 10a). Isolated cells from SKOV-3 C5a tumors were less permissive of SKOV-3 WT *in vitro* growth (Fig. 11a). Remaining isolated Gr-1+CD11b+ cells and the CD11b+ cells also sorted from the tumor were evaluated microscopically following cyospin, hematoxylin, and eosin staining in order to characterize phenotype of these cells. The images of the Gr-1+CD11b+ cells (Fig. 11b) demonstrate that nearly all these cells are morphologically similar to neutrophils. In tumor studies of MDSCs, defined as more immature cells and heterogenous, whereas the population seen here is more homogenous. Additionally, CD11b+ cells were evaluated by the same means, and these cells demonstrate a predominant monocyte/macrophage phenotype, fairly homogenous as well (Fig 11c). Even though Gr-1+CD11b+ cells isolated from the tumor grown *in vivo* are

immunosuppressive and pro-tumorigenic in nature, the neutrophils isolated from SKOV-3 C5a tumors are rendered significantly less suppressive by C5a in the local tumor microenvironment to which they were exposed.

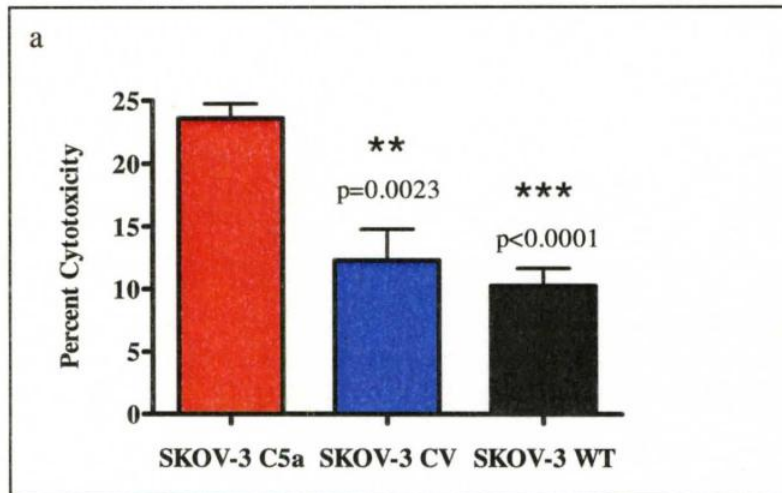


Figure 10: Innate immune cells from SCID show enhanced cytotoxicity to SKOV-3 C5a cells *in vitro*. Non-adherent immune cells isolated from SCID spleen samples including NK cells and neutrophils kill SKOV-3 C5a expressing cells significantly better than SKOV-3 CV and WT controls cells *in vitro* as evaluated by Acea assay system after 16 hours of co-culture.

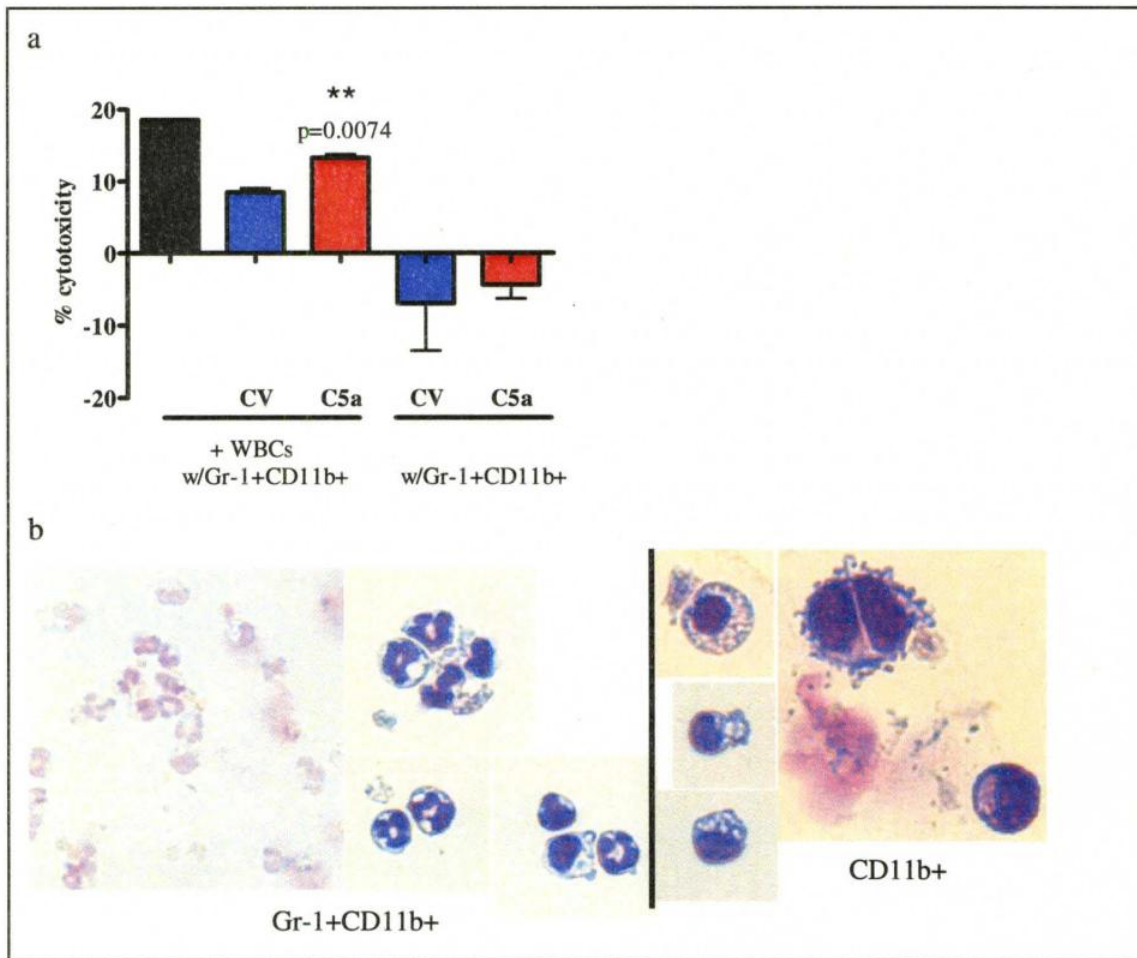


Figure 11: Function and phenotype of Gr-1+CD11b+ tumor infiltrates. Gr-1+CD11b+ tumor infiltrating cells were sorted from SKOV-3 C5a and SKOV-3 CV tumors following sacrifice of SCID mice at endpoint tumor growth and evaluated for cytotoxic or suppressive effects on SKOV-3 WT tumor cells in the presence or absence of naïve non-adherent immune cells isolated from SCID mouse spleens after 28 hours. Cell histology was also evaluated. (a) Cytotoxicity evaluation by Acea revealed the Gr-1+CD11b+ cells isolated from both tumors are immunosuppressive. In the presence of WBCs, there is an evident decrease in the cytotoxicity demonstrated by these cells. Additionally, in the absence of naïve innate immune cells, co-culture of SKOV-3 WT cells and Gr-1+CD11b+ sorted cells permit enhanced tumor cell growth. Gr-1+CD11b+ cells sorted from SKOV-3 C5a tumors are significantly less immunosuppressive and allow a more limited degree of enhanced tumor growth in the absence of naïve leukocytes. (b) Histological analysis of tumor sorted Gr-1+CD11b+ and CD11b+ cells revealed homogenous, differentiated neutrophils and monocyte/macrophages, respectively

Enhanced cytotoxicity of tumor cells by NK cells in the presence of C5a

C5a expression *in vivo* by SKOV-3 cells resulted in an increased percentage of NK cells infiltrating into these tumors, which proves valuable due to the cancer killing reputation of this cell subset [10, 54]. *In vitro* studies were performed to determine if the C5a could provide NK cells improved cytotoxic capabilities over tumor cells in addition to increased chemotaxis as shown by the *in vivo* migration into SKOV-3 C5a tumors. Indeed, *in vitro* cytotoxicity studies with Acea system technology revealed NK cells separated from naïve SCID spleen killed SKOV-3 cells releasing C5a significantly better than SKOV-3 CV cells (Fig. 12a).

In an attempt to ascertain levels of C5a that improved cytotoxicity of NK cells against tumors, *in vitro* studies utilizing NK specific target tumor cells, Yac-1 Luciferase (Yac-1 Luc) cells, were used. Luciferase expression by these cells was utilized for measuring specific tumor cell lysis for *in vitro* studies with varying concentrations of recombinant mouse C5a (250 ng/mL-0.5pg/mL). These studies demonstrated that the addition of C5a to the Yac-1/NK cell culture mediated changes in NK cells to improve the levels of specific lysis of the target cells, but only at low levels in the pg/mL range (Fig. 12b). No enhancement of cytotoxicity observed at 250-50 ng/mL ranges (data not shown). These studies were critical to understanding potential levels of SKOV-3 C5a release of C5a into the tumor microenvironment. Through ELISA experiments for C5a during screening and re-evaluation of C5a expressing transfected cell lines, it was identified that the transfected SKOV-3 cells produced levels of C5a in the supernatants between 1200-250 pg/mL levels. The levels of C5a released by the cells *in vivo* is probably similar to slightly lower as a result of enzyme degrading activity, and is

confirmed by NK cell cytotoxic improvement at low rather than high levels of C5a (Fig. 12).

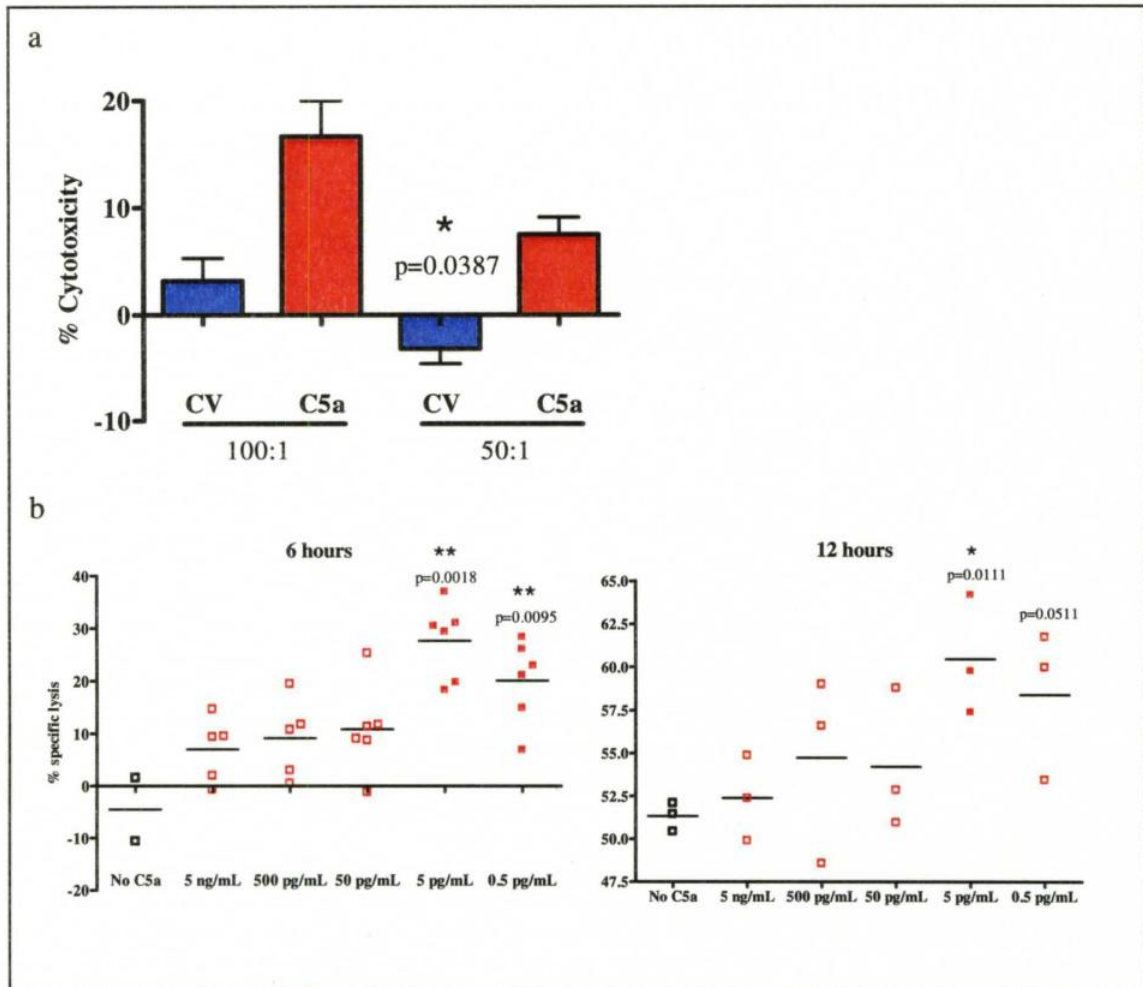


Figure 12: C5a promotes NK cell cytotoxicity of tumor cells. C5a present in the culture supernatants as a result of either transfected cell production or the addition of exogenous recombinant protein enhances cytotoxicity of tumor cells by NK cells. (a) NK cell cytotoxicity of SKOV-3 C5a expressing cells is greater than SKOV-3 CV cells *in vitro*. (b) The presence of low levels, and not high levels, of recombinant mouse C5a added to co-culture of NK cells and Yac-1 Luc cells enhances NK cell cytotoxicity of the tumor cells.

CHAPTER 8

EXOGENOUS C5A EXPRESSION FROM TUMOR HAS A LOCAL, NON-SYSTEMIC EFFECT DESPITE ENDOGENOUS CRP EXPRESSION BY TUMOR CELLS

C5a effect in the tumor is local:

Thus far, the role of C5a in the tumor microenvironment has been inconclusive, with previously published studies drawing opposite conclusions, determining either that C5a release from the tumor resulted in reduced growth and tumor regression [35] or that C5a enhances immune suppression to support tumor growth [36]. We have demonstrated here in the SKOV-3 xenograft model of ovarian carcinoma support of a pro-immunogenic, anti-tumor role for C5a released in the tumor microenvironment. Both *in vitro* and *in vivo* data suggest that C5a is acting on host cells/immune cells and indirectly on the tumor cells to alter the cytokine milieu and enhance tumor infiltration and cytotoxic function of innate immune effector cells to produce the reduction in tumor progression.

Spleen and peripheral blood data collected from tumor study mice indicated the effect of C5a release in the tumor elicits minimal changes in the periphery, and C5a produces most changes to the tumor microenvironment cell populations and milieu. Observing the absence of a significant systemic response in C5a tumor bearing mice demonstrates this potent chemoattractant produced within the tumor has a local and limited effect (data not shown). Further studies involving the use of C5a in tumor therapy

is attractive because the systemic effects are minimal and delivery of C5a directly to the tumor site, for instance via conjugation to monoclonal anti-tumor antibodies [4], may lead to reduced tumor growth and tumor cell destruction by enhancing the recruitment and activity of effector innate immune cells to the site. Determining appropriate levels of C5a to deliver to the tumor environment is still required to obtain the desired effects on recruitment of immune cells and infiltrating cell activity.

Circumventing CRP expression effects on complement inhibition:

Despite the abundant expression of CD55 by SKOV-3 cells [20], C5a generation by transfected SKOV-3 cells appears to successfully by-pass inhibitory effects of CD55 by releasing its target product directly into the environment. Eliminating blockade of CD55 on many cells in the body with antibody therapy and risking healthy tissue damage to accomplish a similar goal is attractive. In previous studies, CRPs were identified to be highly expressed by tumor cells and led to interference with tumor therapy efficacy [6, 7, 64]. Specifically Li et al. demonstrated CD55 expression by SKOV-3 WT cells negated the positive effects of β -glucan enhancement of anti-tumor monoclonal antibody therapy as a result of CD55 activity inhibiting C5a release and subsequent chemoattraction of neutrophils into the tumor. SKOV-3 C5a expressing cells demonstrated enhanced recruitment of both NK cells, macrophages, and a correlation of enhanced recruitment of neutrophils to smaller C5a expressing tumors.

Demonstrated by the *in vitro* growth data (Fig. 4c) and by flow data collected showing lack of C5aR expression on SKOV-3 cells (Fig. 3c), altered *in vivo* growth of SKOV-3 tumor cells expressing C5a is not a result of the activity of C5a directly on the

tumor cells. Reduced tumor growth of SKOV-3 C5a cells is a result of the response by host innate immune cells to C5a released into the tumor microenvironment or the culture supernatant (Fig. 5a, 10, 12a). Also as demonstrated by the *in vitro* cytotoxicity assay with innate leukocytes from SCID mouse spleen, the C5a released by the SKOV-3 cells enhances the effector functions of neutrophils and NK cells *in vitro* and renders them more cytotoxic to the tumor cells (Fig. 10). SKOV-3 cells expressing C5a in the absence of leukocytes continue to grow, and SKOV-3 CV and WT cells are killed significantly less well by the same leukocyte preparation. Taken together, these data suggest that once immune cells are recruited to the tumor microenvironment, the presence of C5a locally can activate and enhance the immune cell cytotoxic functions against the tumor and opposes the immunosuppressive environment the tumor generally establishes.

CHAPTER 9

C5A ENHANCED RECRUITMENT OF IMPORTANT INNATE IMMUNE CELL SUBSETS TO THE TUMOR AND ACTIVATION OF ANTI-TUMOR EFFECTS BY THESE RECRUITED CELLS

C5a enhancement of recruitment and anti-tumor effects on NK cells *in vivo* and *in vitro*:

C5a release from SKOV-3 cells *in vivo* enhanced the recruitment of innate immune cells to the tumor as demonstrated by flow cytometry and immunofluorescent staining on tumor samples excised from SCID mice (Fig. 6). A significantly increased percentage of the DX5+CD11b+ NK cell population was found in the C5a expressing tumors (Fig. 6a). Enhanced recruitment of the DX5+CD11b+ NK cell subset into the tumor microenvironment is ideal because of the tumor cytotoxic and immune enhancing potential of NK cells in an immune activating setting [10]. NK cells have been long established as innate immune cells that recognize and kill tumor cells [38, 65]. NK cell deficiencies in animals have demonstrated a severe increase in tumor susceptibility, more so than the lack of T cells [38].

Increased infiltration of NK cells is desirable due to the potent tumor cell killing functions of these cells without a prerequisite priming step by an antigen presenting cell (APC) limiting the mode of activation [10]. In solid tumors, NK cell penetration is noted as a positive prognostic factor, but most solid tumors demonstrate inferior NK cell infiltration [10]. Often NK cells only group in surrounding stromal tissue without closely

interacting with the cancerous cells as is required for NK cell cytotoxicity, providing explanation for NK cell enhancing anti-tumor therapies to be most effective for blood-borne metastases and hematological cancers [10]. C5a increases the percentage of infiltrating NK cells, and as seen by immunofluorescence more NK cells enter beyond stromal association (Fig 6a).

Perforin, granzyme B, TRAIL, and IFN- γ have all been associated with NK cell killing and tumor growth suppression [10, 38]. Perforin-mediated cytotoxicity is considered one of the most important mechanisms enlisted by NK cells to eradicate tumor cells; however, when total tumor sample perforin and granzyme B mRNA levels were evaluated, no enhancement of either was observed in SKOV-3 C5a tumors, but there are additional mechanisms by which NK cells may be targeting SKOV-3 C5a cells for destruction [65, 66]. Another mechanism by which NK cells may be cytotoxic to SKOV-3 cells *in vivo* is through CD40-CD40L interactions [66]. NK cell expression of CD40L by human NK cells rendered these effector cells increasingly cytotoxic to P815 mastocytoma cells transfected with CD40 [66], and SKOV-3 tumor cells demonstrated CD40 expression upon flow cytometric analysis following *in vivo* growth (observation).

NK cells can also promote other immune cell functions through the release of potent inflammatory cytokines including IFN- γ and TNF- α . SKOV-3 C5a and SKOV-3 CV and WT tumors were evaluated by qRT-PCR for the expression of both these critical cytokines *in vivo*. No significant differences in IFN- γ expression were noted between the groups, and TNF- α expression at the mRNA level was significantly decreased from the total SKOV-3 C5a tumor microenvironment as compared with SKOV-3 control tumors. This may indicate a negative role for TNF- α to the immune response to the tumor, as has

been demonstrated previously [55, 56]. NK cells are a potential source of the tumor TNF- α produced at significantly increased levels in SKOV-3 CV and WT tumors, and C5a expression in the tumor may in fact stimulate NK cells to decrease expression of TNF- α in this setting.

C5a enhancement of recruitment and anti-tumor effects on macrophages *in vivo* and *in vitro*:

Macrophages have been demonstrated numerous times over, to play a dominant role in influencing other immune cells and tumor growth depending on macrophage phenotype determined by release of characteristic sets of factors [13, 17]. Two extreme ends of macrophage polarization have been characterized based on the stimulating factors used. M1 macrophages stimulated with IFN- γ and/or LPS are described as classically activated, proinflammatory, and anti-tumorigenic macrophages. To the contrary, M2 macrophages are stimulated with M-CSF, IL-4 and IL-13, or TLR ligands and are described as alternatively activated, immunosuppressive in function, and pro-tumorigenic [13, 46]. In most settings, including the tumor, rather than extreme polarized macrophages occurring, several subpopulations reside in the environment and possess characteristic features of both polarized types with some variation, and usually have a stronger profile toward one extreme or the other [13, 17]. Important interactions occur between cells of the innate immune system, including macrophage and NK cell interactions that are important in shaping the initial and adaptive inflammatory response. Macrophage phenotype, dependent on the stimulating factors in the local environment are

critical to inducing NK cell activation against an offending agent, or rather NK cell lysis of an unproductive, polarized, autologous macrophage [67].

C5a potential enhancement of anti-tumor effects and recruitment of neutrophils *in vivo* and *in vitro*:

As a result of the study involving β -glucan mediated monoclonal antibody therapy in the SKOV-3 model identifying CD55 expression by the tumor cells as responsible for eliminating C5a release, and as a result drastically decreasing the infiltration of β -glucan primed neutrophils to kill targeted cancer cells [20], engineering SKOV-3 cells that expressed C5a was expected to negate the effects of CD55 and lead to enhanced neutrophil recruitment without anti-CD55 therapy. However, C5a expression from the tumor did not appear to increase the percentage of the neutrophil subset over SKOV-3 CV and WT as it did NK cells and macrophages as expected. Percentage of neutrophil infiltration into C5a tumors was however, in fact related to the size of the tumor (Fig. 6c). This interesting correlation may have been related to the concentration of C5a produced from that SKOV-3 C5a specific tumor microenvironment. Smaller SKOV-3 C5a tumors may have produced lower local concentrations of C5a during initial growth and therefore had a greater impact on neutrophil recruitment.

In the Lambris group's study of C5a production in the TC-1 tumor microenvironment, Gr-1 and CD11b surface markers were used to identify the highly suppressive, immature MDSC. In this study, we refer to cells positive for the same markers as neutrophils. Terminology differences stem from higher levels of Gr-1+CD11b+ cells observed in the spleens of naïve SCID mice when compared with tumor

bearing mice (data not shown). These data indicated cells expressing high levels of these two markers in SCID mice recognized the granulocyte population as opposed to the MDSC population, as MDSCs are known not to be expanded or suppressive in naïve animals [68]. Additionally, the spleens of naïve and tumor-bearing mice from both SKOV-3 C5a and control tumors weighed equal amounts, indicating there was not significant splenomegaly of tumor-bearing mice as a result of enhanced cellular components, namely MDSCs in tumor mice (data not shown). Lastly, cellular morphology of Gr-1+CD11b+ cells sorted from SKOV-3 C5a and SKOV-3 CV tumors revealing cells in this population were predominantly large polymorphonuclear cells (Fig. 11b). Interestingly, despite neutrophil (Gr-1+CD11b+) expression of the most abundant levels of C5aR, there was no significant difference in the percentage of these cells infiltrating C5a or control tumors in either group. Neutrophils may also demonstrate varied kinetics for tumor entry and identification of the amount of this innate immune cell infiltrating may be complicated by their short life span, even though C5a exposed neutrophils are less susceptible to apoptosis [5].

In the TC-1 study, MDSCs were further classified into two subsets based on levels of Gr-1 and CD11b expression. The Gr-1^{hi}CD11b^{hi} population of MDSCs are considered polymorphonuclear-MDSCs (PMN-MDSCs) because of the similarities with immature neutrophils, and the monocytic-MDSCs expressing Gr-1^{int}CD11b^{int} (MO-MDSCs) with a striking resemblance to monocytes [69]. Similar to the current model, no enhanced infiltration of tumors by PMN-MDSCs occurred although a significant correlation was demonstrated in each model showing a relationship between the amount of infiltrating cells (Gr-1+CD11b+ (SKOV-3 tumors) and CD11b+ (TC-1 tumors) and

tumor size. TC-1 tumors demonstrated that larger tumors were more greatly infiltrated by CD11b+ cells; contrastingly in the SKOV-3 model more Gr-1+CD11b+ cells migrated into smaller tumors. The MO-MDSC infiltrated significantly more in C5a responsive host, likewise F4/80+ macrophages infiltrated SKOV-3 C5a expressing tumors to a greater extent.

CHAPTER 10

SKOV-3 C5A TUMORS DEMONSTRATE A SIGNIFICANTLY ALTERED TUMOR MICROENVIRONMENT, IMMUNE AND TUMOR GROWTH MEDIATORS ALTERED AS A RESULT OF C5A EXPRESSION FROM SKOV-3 TUMOR CELLS IN VIVO

C5a modulates expression of important pro-tumorigenic factors

The significantly reduced tumor growth observed in SKOV-3 C5a tumors may result from the increased degree of infiltration by innate immune cell subsets leading to increased tumor cell death; however, other data indicates it is likely C5a acts locally on the infiltrating cells. Evidence exists suggesting that C5a leads to events that alter a local tumor environment away from being highly immunosuppressive as seen in several changes in the cytokine milieu. Significant changes in five important tumor and immune regulating factors exist between SKOV-3 C5a and SKOV-3 control tumors: TNF- α , iNOS, VEGF, arginase, and TGF- β .

TNF- α is an important proinflammatory cytokine playing a key role in immunity and inflammation, as well as mediating communication between cells [55, 56]. This mediator is a type II transmembrane protein, part of a superfamily of ligands, is soluble and possesses two different receptors, TNFR1 and TNFR2. It is generated in the cell as a pro-peptide that is membrane bound. Following cleavage of the pro-peptide by a specific activating enzyme, TNF- α is secreted in its soluble active form, but the pro-peptide form also has limited functional activity upon cell-cell contact. Active TNF- α can regulate

several separate cell functions by initiating signaling through multiple pathways including pro-apoptotic, anti-apoptotic, and NF- κ B pathways[55]. The cytokine is produced predominantly by macrophages, but has also been found to be produced in other cells including tumor cells and NK cells [10, 55]. During the early 20th century, surgeon Dr. William Coley developed a systemic treatment for his cancer patients containing bacterial endotoxin known as ‘Coley’s mixed toxins’ that was highly effective in treating patients with various types of tumors. Later it was discovered the mechanism by which Coley’s mixture was effective in leading to tumor death was a direct result of endotoxin stimulated macrophage production of TNF- α .

Ovarian tumors have been demonstrated by in situ hybridization and IHC to express TNF- α in tumor cell islands, not infiltrating immune cells. In this setting, the constitutive expression of TNF- α was found to be pro-tumorigenic and associated with poor patient survival and therapy resistance [56]. TNF- α in other cancer studies was found expressed by tumor associated macrophages or MDSCs, production by these cells led to apoptosis of activated anti-tumor T cells and enhanced tumor growth [70]. In our tumor studies, TNF- α is observed in elevated levels in SKOV-3 CV and WT tumors but significantly lower levels in SKOV-3 C5a expressing tumors, as seen by qRT-PCR. Total tumor was processed for the qRT-PCR data acquired, consequently the cellular source of the TNF- α is not specifically identified. However, as a result of subsequent qRT-PCR experiments on sorted tumor populations, F480+ cells from SKOV-3 C5a and SKOV-3 CV tumors appear to have generated similar quantities of TNF- α at the mRNA levels. These results indicate elevated TNF- α may be generated instead by the tumor cells themselves, NK cells, or neutrophils [10, 56, 71]. TNF- α release may be restricted

through a C5a mediated effect on innate immune cells, or an indirect effect of C5a may exist on tumor cells resulting in the near elimination of TNF- α expression via a C5a-mediated mechanism resulting in an infiltrating immune cell activity or expression of factors that acts on tumor cell TNF- α production. The current SKOV-3 C5a model may demonstrate the capacity of C5a treatment to reduce the tumor promoting effects of TNF- α by reducing its expression from innate immune cells or cancer cells *in vivo*.

iNOS is induced in some tumor cell lines by elevated levels of TNF- α or other cytokines [57, 72]. In SKOV-3 CV and WT tumors, increased TNF- α levels in the microenvironment may have stimulated cells in the infiltrating immune population (CD11b+ cells Fig. 8c) to generate iNOS which amplifies the pro-tumorigenic effect of TNF- α [57]. Nitric oxide (NO) produced in tumors can have two main sources: it may be continuously expressed by tumor or stromal cells as cNOS (constitutive nitric oxide synthase) or as iNOS by infiltrating myeloid cells. A distinct isoform of nitric oxide synthase, iNOS, is uniquely produced as a result of stimulation by cytokines, is expressed in nearly every cell type, and is capable of producing micromolar concentrations of NO for extended amounts of time [72]. NO is a lipophilic molecule and easily migrates across cell membranes to act intracellularly, and signaling and post-translational modifications lead to anti-apoptosis and increased cell growth of recipient cells [72]. NO is a short-lived molecule; however, iNOS can induce sustained levels of NO, expanding the duration and levels of NO in the environment [57].

Increased iNOS through TNF- α induction is a characteristic of gynecological, solid tumors, such as ovarian cancer [56]. The initial publication of elevated iNOS detected from gynecological tumors demonstrated the expression was localized to tumor

cells without any identified in surrounding normal cells [73]. Further studies in malignant CNS tumors revealed a strong association between high tumor grade and elevated iNOS levels, localized to tumor regions of hypoxia near necrotic areas [57]. Other clinical studies evaluating melanoma patients for iNOS expression revealed tumor iNOS levels were related to survival, with tumor expression of iNOS resulting in significantly decreased survival, compared to iNOS negative tumor patients [57]. Additionally, a clinical study of breast cancer including 104 specimens demonstrated increasing iNOS expression with increasing disease severity. No iNOS was observed in benign lesions; however, over 3/4 in situ carcinomas and nearly 2/3 of invasive disease grade tumors stained positive for iNOS [74].

Tumor promoting mechanisms of iNOS are likely mediated through the production and activity of NO promoting increased growth of tumor and stromal cells, as well as increasing VEGF and vascular activity [57, 58]. iNOS levels present in SKOV-3 CV and WT tumors potentially promote tumor vascularization through induction of VEGF expression in the tumor. Also, NO is a major macrophage effector molecule stimulated to be released under inflammatory conditions. Macrophage NO can be cytotoxic to tumor cells; however, NO produced by the infiltrating macrophage can also be cytotoxic to anti-tumor lymphocytes [72]. Immune inhibition by iNOS was found to be stronger than tumor cell cytotoxic effector functions; therefore macrophage NO had dramatic negative effects on the survival of anti-tumor CD8+ T cells in a murine tumor model. SKOV-3 CV and WT potentially generated NO, through elevated levels of iNOS, may enhance tumor growth by promoting angiogenesis and invasion or the preferential destruction of anti-tumor immune cells [72].

In the absence of C5a release in the tumor, TNF- α , iNOS, and VEGF production can be exploited by the tumor to promote growth. As previously acknowledged, TNF- α produced at low levels in tumors can lead to increased expression of iNOS [55] and both of these factors have been shown to lead to proangiogenic factor VEGF induction [55, 75]. VEGF and its role in angiogenesis and tumor neovascularization is exploited by the tumor and is absolutely critical to its growth [76]. Within the tumor microenvironment, VEGF has been shown to promote angiogenesis *in vivo* [75, 77], promoting tumor growth by enhancing nutrient delivery to developing parts of the cancer. Tumor cells themselves produce VEGF, and factors released from host cells in the tumor can enhance tumor expression of VEGF [77]. Studies involving the administration with anti-VEGF antibodies in two xenograft models of human colon and colorectal cancer (SW480 and DLD-1 cell lines) revealed the proangiogenic effect in the tumors was predominantly mediated by VEGF [77].

In other studies, tumor associated cells stimulated with various inflammatory cytokines, such as TNF- α , can lead to enhanced VEGF production and release in the tumor microenvironment resulting in increased tumor growth via angiogenic enhancement in a murine colon cancer model [75]. Sorting experiments in the current model reveal that it is the tumor cell population and not the immune infiltrating CD11b+ cells that express significantly more VEGF (Fig. 7a). C5a release in SKOV-3 tumors enhances an immune response that inhibits the promotion or baseline production of VEGF by the tumor. Due to the lack of C5aR expression on tumor cells, the mechanism of C5a is indirect, but likely mediated through significantly decreased levels of TNF- α

and/or iNOS release by tumor cells or infiltrating C5aR expressing innate immune cells activated by local C5a.

The lack of TNF- α and iNOS in SKOV-3 C5a tumors may be sufficient to inhibit VEGF signaling in the tumor cells; however, there is an additional mechanism by which C5a may be acting to inhibit tumor VEGF. C5a has previously been shown to play an important role in negative regulation of neovascularization and angiogenesis as a result of hypoxia [78]. In a murine model for retinopathy of prematurity (ROP), the Lambris group demonstrated C3 and C5aR deficiency, as well as C5 blockade and C5aR antagonism led to enhanced pathological angiogenesis. In this model, macrophages were found responsible for the anti-angiogenic effect of C5a rather than endothelial cells of the vasculature which also express C5aR. C5a stimulation of macrophages resulted in increased secretion of soluble VEGFR1 (sVEGFR1) [78]. Release of sVEGFR1 inhibits VEGF signaling important in angiogenesis. Depletion of macrophages in C3 or C5aR deficient mice reversed angiogenic enhancement demonstrated in undepleted complement deficient mice [78]. Additionally, administration of C5a peptide led to a decrease in pathological neovascularization in WT ROP mice, as well as C3 deficient ROP mice that previously showed significantly more angiogenesis [78]. Other cells of the innate immune system, including neutrophils have been shown to be important in the regulation of angiogenesis in tumors [71]. C5a inhibition of TNF- α and iNOS from tumor or innate immune cells, or induction of sVEGFR1 release may be alterations of the tumor microenvironment occurring to reshape the tumor promotion of the VEGF response in the microenvironment of a SKOV-3 CV or WT tumor without sufficient C5a present.

A striking observation consistently occurred during each *in vivo* tumor study with SKOV-3 C5a and SKOV-3 CV and WT cells. The majority of SKOV-3 CV and WT tumors would become outwardly necrotic with the production of hardening and scabbing (Fig. 5d). Necrotic tissue possesses tumor promoting and proinflammatory properties different from apoptotic cells. Unlike the apoptosis pathway of cell death, necrotic cells have been demonstrated to result from an underlying genetic mechanism, rather than by random means [9]. Necrotic cells release proinflammatory signals and actively direct immune cells to the site of necrosis in the tumor microenvironment [9]. Cancer cell death by necrosis may seem useful in eliminating tumor cells; however, subsequent events indicate death by necrosis may end up benefiting the tumor [9]. Another group reported necrotic tumor cells stimulated MDSCs in the tumor to release molecules that induce apoptosis in anti-tumor T cells [70]. Arginase was an important component of this negative effect on the anti-tumor T cells produced by the infiltrating MDSCs [36, 70].

Arginase is an enzyme produced by innate immune cells that depletes arginine, an amino acid required for the activation of adaptive immune cells. Upregulation of arginase expression to high levels in tumor associated macrophages has been demonstrated [79]. SKOV-3 CV and WT tumors contain significantly higher levels of arginase mRNA than SKOV-3 C5a tumor cells. The F4/80+ infiltrates in SKOV-3 CV tumors are the source of elevated arginase. Arginase expressed by MDSCs to inhibit anti-tumor responses at the tumor edge, the site at which growth occurs has been observed [14]. Being that a major role in the suppressive function of arginase is arginine depletion of proliferating and activating anti-tumor T cells, the negative role in the SCID mouse must include another mechanism. MDSC suppressive factors predominantly include arginase and iNOS [14],

and the production of arginase is critical to generating the suppressive molecules of MDSCs, reactive oxygen and nitrogen species (ROS and RNS) [36]. Arginase dependent generation of reactive species can result in the enhanced mutagenesis in the tumor and progression.

Interestingly in the SKOV-3 CV mice with increased tumor growth, TNF- α and iNOS are elevated. These two factors released by macrophages are commonly associated with the M1 anti-tumor phenotype of macrophages. Even though TNF- α was previously considered anti-tumorigenic, it has been demonstrated to promote tumor growth by working on macrophages and destroying the mechanism by which these cells respond to suppressive cues, resulting in an acute, and sometimes chronic inflammatory response generated to which tumors respond well [13]. Macrophages are a critical component to the pro-tumorigenic inflammation established as a result of the generation of NO (through iNOS) and its mutagenesis of adjacent cells perpetuating tumor progression [13]. Increased infiltration of tumors by macrophages has been associated with poor survival in tumor patients many times over [80, 81]. Macrophage generation of TNF- α has been shown to lead to iNOS production, and iNOS is capable of producing a wide range of NO concentrations in the local environment that can last anywhere between seconds to days to sustain tumor growth and promote VEGF production and tumor vasculature [57]. As well, the increased levels of TGF- β detected in SKOV-3 C5a tumors are more often associated with the immunosuppressive phenotype of M2 pro-tumorigenic macrophages.

The final immune and tumor modulating factor that was recognized as having altered levels in the SKOV-3 model following introduction of C5a was TGF- β , however;

this typically immunosuppressive mediator was generated in significantly higher levels in the SKOV-3 C5a tumors. TGF- β has been found to have tumor stage dependent effects [62]. Expression in tumor early during development has a cytostatic/tumor suppressive effect and is proinflammatory, leading to the production of cytokines such as IL-6, IL-1, TNF- α and IL-17 [63]. Mohammed et al. looked at the role of TGF- β in benign premalignant tumors by overexpression of the protein in the skin of epidermal squamous lesions using inducible TGF- β transgenic mice. In this study, they found expression of TGF- β led to a proinflammatory response, increased infiltration of inflammatory cells into the lesion and tumor draining lymph nodes, resulting in tumor regression. However, the proinflammatory response was not completely responsible for the regression seen, and it was determined that the cytostatic program elicited by TGF- β overexpression was also having an effect directly on the tumor cells to reduce tumor development [63].

Additional evidence exists suggesting TGF- β in the tumor does not equate immunosuppression and tumor development. TGF- β anti-proliferation effects on tumor cells, a result of prevention of Retinoblastoma protein (Rb) phosphorylation or by suppression of c-myc, block cell cycle advancement through G1 [76]. Some tumors evade suppression by this mechanism by downregulating or altering TGF- β receptor expression [76]. The TGF- β II receptor is commonly mutated and functionally inactivated in colon and gastric carcinomas lacking replication error-repair. Germline mutations in the gene encoding TGF- β II receptor occurs in hereditary nonpolyposis colorectal cancer resulting in non-respondent tumor cells to the cytostatic effects mediated by TGF- β [60]. Lastly, in human cell studies involving activin A which is a member of the TGF- β superfamily, was shown to stimulate macrophages toward an M1 phenotype [82]. In the SKOV-3 model,

TGF- β may be predominantly working against SKOV-3 C5a cell growth by negatively regulating advancement of tumor cells through the cell cycle.

CHAPTER 11

CONCENTRATION OF C5A RELEASED/PRESENT IN THE TUMOR MICROENVIRONMENT IS CRITICAL IN THE DETERMINATION OF TUMOR GROWTH OR SUPPRESSION

Concentration levels critical to other mediators shown to be important in tumor growth outcome:

Previous studies have shown benefit to TNF- α in eliminating tumor cells [55], and iNOS is a key mediator produced by macrophages polarized toward an M1 phenotype thought to have anti-tumor characteristics [13], but in the SKOV-3 model, both are associated with increased tumor progression (Fig 5). Important to the specific effect of an immune cell and tumor growth mediator is concentration levels in the tissue. The highest levels of iNOS correlated with most poorly differentiated tumor cells (≥ 250 pmol/min/g of tissue) [73]. The concentration of NO produced by iNOS remains in question; however, based on evidence from previous studies, apparently intermediate ranges of NO that are not prolonged in the microenvironment provide ideal tumor assistance [57]. Levels of NO concentration ranging between 50-100 nM possess positive survival influences on tumor cells [57]. *In vitro* study shows when those levels are breached and overwhelming amounts of NO are produced surpassing 400 nM concentrations, tumor cell promoting effects are lost [57]. Additionally, prolonged low levels of NO, in the range of 1 nM, also appear to be detrimental to tumor cell survival [57].

Ranges existing within the significantly larger control SKOV-3 tumors promote tumor cell survival or initiate the other pro-tumorigenic features of iNOS including transformation of epithelial cells and iNOS and TNF- α activation of oncogenes [55]. Results of targeting the mediator of 'Coley's mixed toxins' led to trials and treatment of cancer patients with TNF- α ; however, successful TNF- α treatment of patients was found to only be sufficient in high doses, which was associated with serious adverse effects. Contrastingly, low dose TNF- α is a feature of tumor cell cytokine production, and in the setting of the tumor microenvironment, TNF- α at lower levels promotes tumor cell survival, proliferation and protection from apoptosis, immune evasion, and angiogenesis [55, 56, 76].

C5a concentration effects on tumor growth and the infiltrating innate immune cells:

Even though similar infiltration patterns exist between TC-1 and SKOV-3 tumors in MO-MDSC and macrophage as well as PMN-MDSC and neutrophil subsets, we hypothesize tumor growth outcomes differ tremendously due to C5a concentration levels generated in the two models and the impact this has on surface C5aR expression and cell activity in response to C5a signaling. As a G-protein coupled receptor, C5aR is continuously recycled from surface expression to an internalized form based on the level of stimulation. Important findings of C5aR internalization in tumor infiltrating MDSCs in the TC-1 model reveal that none of the C5aR expressed by tumor MDSCs is expressed on the surface, as it is only seen following permeabilization of these cells before staining [36]. Fairly high percentages of C5aR was still found to be expressed on Gr-1+CD11b+ and CD11b+ cells infiltrating SKOV-3 tumors (Fig 6c). Gr-1+CD11b+ surface

expression of C5aR pertains to C5a levels and effects cell functionality; levels of C5a and function of responding cells are directly related to the sensitivity and ability of expressing cells to respond [5]. However, the observation that more neutrophils are found in smaller C5a expressing tumors may highlight an important role and degree of C5aR expression responsiveness or sensitivity to C5a signaling for these effector cells in recruitment to tumors to suppress maximal growth (Fig. 10a). The difference in neutrophil percentages found in smaller tumors vs. larger tumors expressing C5a may be related to the actual concentration of C5a in the environment which may vary once *in vivo* during tumor establishment.

During sepsis, an over-activated complement system, namely the release of high levels of C5a, disables innate immune cells, decreasing phagocytic function and resulting in an overall immunosuppressive state [37]. Due to the high levels of C5a released during a septic response, the amount of surface C5aR on cells is critical to the susceptibility of that cell to the over-activating levels of the anaphylatoxin [5]. Neutrophils express abundant C5aR and these levels make the cell increasingly sensitive, while monocytes/macrophages express lower amounts of the receptor and are, therefore less susceptible and overall more resistant to over-activation by C5a [37].

Additionally studies of *in vivo* sepsis models demonstrated higher levels of C5aR expression on neutrophils was positively correlated with survival because the mice are more sensitive to C5a stimulation. It is not clear; however, whether the increased surface expression of C5aR was a result of lower C5a concentrations generated during sepsis of these mice or if the neutrophils possessed a receptor recycling efficiency advantage [5]. In the compared model systems, it is arguable that C5a concentrations produced by

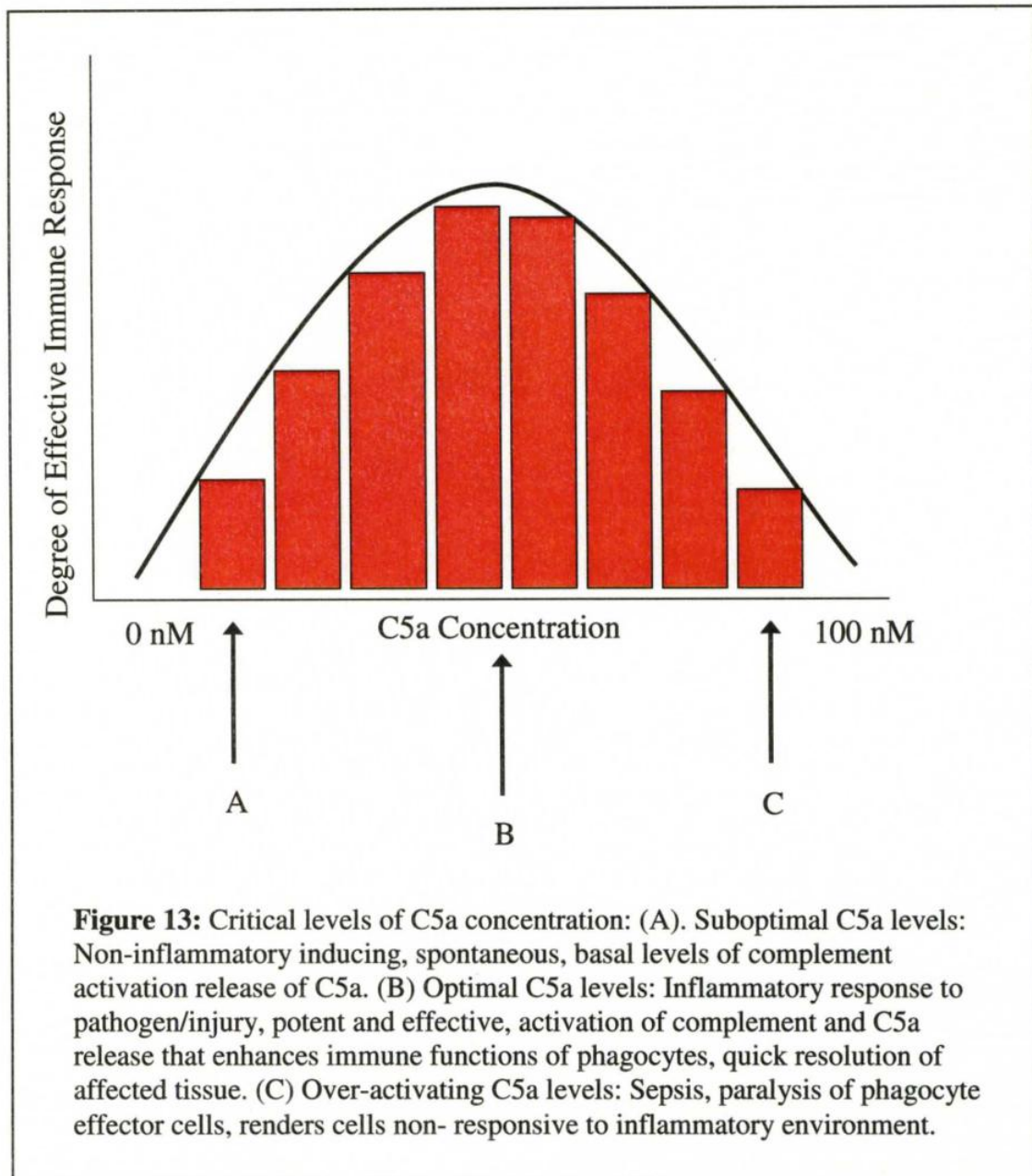
SKOV-3 C5a tumors are lower, but effective because enhanced in an isolated area. While TC-1 tumor cell growth *in vivo* may not produce over-activating levels of C5a throughout the host, these cells express Human Pappiloma Virus (HPV-16) protein E7 which presumably mounts a large antibody response in the foreign host that results in the classical activation of complement reported. This may actually lead to relatively higher local concentrations of C5a being generated, potentially leading to over-activation of infiltrating cells by C5a.

Quantitation of the chemoattractant and how quickly it is degraded by enzymes in the environment complicates pinpointing a clear role for C5a in the tumor and the critical concentration threshold. It has been demonstrated to this point that C5a expression from tumor cells can lead to the extreme opposite effects on tumor growth, but taken together the evidence generated between the tumor C5a studies (and the current work) support the hypothesis that C5a concentration holds the key to the response generated to tumor C5a released locally *in vivo*. During the generation of C5a expressing transfected SKOV-3 cells, we showed by ELISA for unconcentrated culture supernatants from these cells following *in vivo* passage to contain low ng/mL-pg/mL levels of C5a (<1.2 ng/mL).

According to the study using the C5a transfected EMT6 cells, low and high levels of C5a released from the tumor cells yields opposite results on tumor growth. However, this study never legitimately quantified C5a concentration released by the transfected tumor cells or the number of cells from which the supernatant had been collected and based concentrations of C5a on the extent of J774 macrophage cell line recruitment to culture supernatants. The chosen transfected cell line for the studies with identified low levels of C5a released, based on the chemotaxis assay, elicited nearly 10 times the

number of J774 cells migrating to the same clone's supernatant following only one additional day of tumor cell culture. SKOV-3 C5a transfected cells demonstrated changes in C5a concentration over the course of culturing *in vitro*. C5a concentration, as measured by ELISA went from 766 pg/mL to 1140 pg/mL over two days which may be sufficient a change to produce chemotactic variation in responder cells. These results complicate concentration concerns, especially when considering unknown changes that may occur *in vivo*.

It appears, at least *in vitro*, C5a may increase in culture supernatant overtime. Whether this is due to the accumulation of C5a over the incubation time period, more cells present over more time, or cells under certain growing conditions such as increased confluency or more acidic media may produce more C5a. C5a accumulation is less likely, even *in vitro*, as a result of enzymes in the serum that likely inactivate C5a to the C5a *desArg* degradation product. Another explanation lies in the activity of C5a *desArg* in chemoattracting cells *in vitro* [3]. Recalling the receptor is internalized to regulate the response of receptor expressing cells based on available ligand, the migration response curve of J774 cells or innate immune cells to C5a concentrations should be Bell-shaped with intermediate concentrations producing the greatest migration (Fig. 13). It is difficult to determine levels of C5a the clone used for the EMT6 studies consistently expressed *in vitro* or if variations day to day/passage to passage occurred. Additionally, with the release of C5a *in vivo*, the rapid cleavage to C5a *desArg* occurs and even though it has demonstrated chemotactic stimulation of C5aR expressing cells, this cleavage product possesses less abundant proinflammatory role, maintaining only 10% of activity [5].

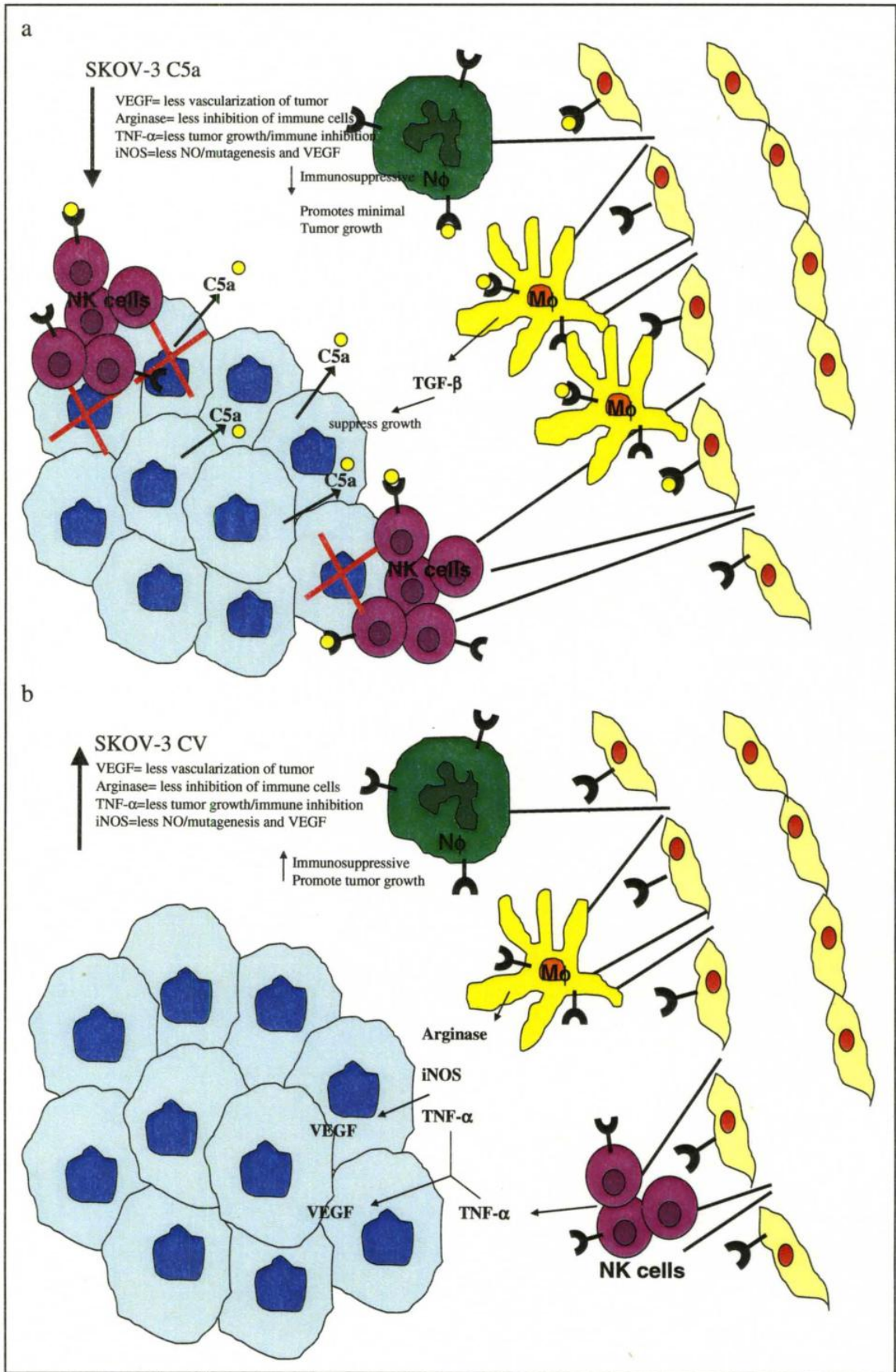


As is the case for $\text{TNF-}\alpha$ and iNOS leading to NO production in tumor studies, concentration levels of these factors possess opposing effects on tumor growth. The contradicting work we and others [35, 36] have demonstrated in animal models involving tumor C5a and the effects it has on tumor growth and infiltrating cells may be due to the levels of C5a in the environment (Fig. 14). High levels (such as ng/mL or higher) may lead to enhancement of an inflammatory setting that favors generation of factors by the

tumor and infiltrating cells to perpetuate an environment ideal for promoting the tumor growth, angiogenesis, and suppressing the anti-tumor response. Conversely, low levels (such as pg/mL) of the complement protein may enhance infiltration of immune cells, and upon entry into the environment, C5a at low levels stimulates a more powerful anti-tumor immune response (Table 2 and Fig. 1).

As a result of the inconsistencies generated over a definitive role for C5a in the tumor setting, further studies are imperative in order to dissect what clearly leads to an anti-tumor response mediated by C5a. Usage of C5a is attractive due to the fact that it is a small molecule and could be easily incorporated into an existing therapeutic protocol such as monoclonal antibody therapy. Additionally, as shown here, little adverse side effects would be anticipated as to its limited local effect and rapid degradation to a significantly less potent form *in vivo*. By linking C5a to Herceptin, for example in the treatment of breast cancer, following administration into patients, there are several concerns to consider. There may be a systemic response to C5a when it is present in the circulation leading to adverse events. It may also be degraded by serum enzymes, and upon binding of the Herceptin-C5a complex in the tumor, C5a *desArg* may be less effective, or it may still be beneficial in improving therapy [4]. Additionally, rather than using a form of C5a that can be cleaved, the proven stable peptide agonist of C5a [83] may be linked to a therapeutic antibody and administered, maintaining C5a functional activity. However, this could also have adverse effects. In some cases, C5a may be locally administered directly into the tumor so the local effect observed in the tumor model may be duplicated in treatment of patients, but this method likely will not be useful in metastatic disease.

It is also important to consider the effects, yet unclear on the adaptive immune response, as a result of the effects of C5a on the innate immune response and directly on C5aR expressing adaptive immune cells. In the setting of an immunocompetent mouse, the decrease in the levels of TNF- α and iNOS may remain beneficial in reducing tumor growth. Alternatively, whereas low levels of TNF- α may negatively affect the activation status of infiltrating T cells, the reduction in iNOS may be protective. In addition, the elevated levels of TGF- β observed in the SKOV-3 C5a tumor infiltrating macrophage population, if maintained in the immunocompetent mouse may be tremendously detrimental by enhancing the T regulatory cell and immunosuppressive response. Future directions of C5a study in the tumor are imperative to define the effects on the adaptive immune response in the tumor and should focus on understanding quantitative analysis *in vitro* and *in vivo* and defining a critical threshold for the anti-tumorigenic effects of C5a as well.



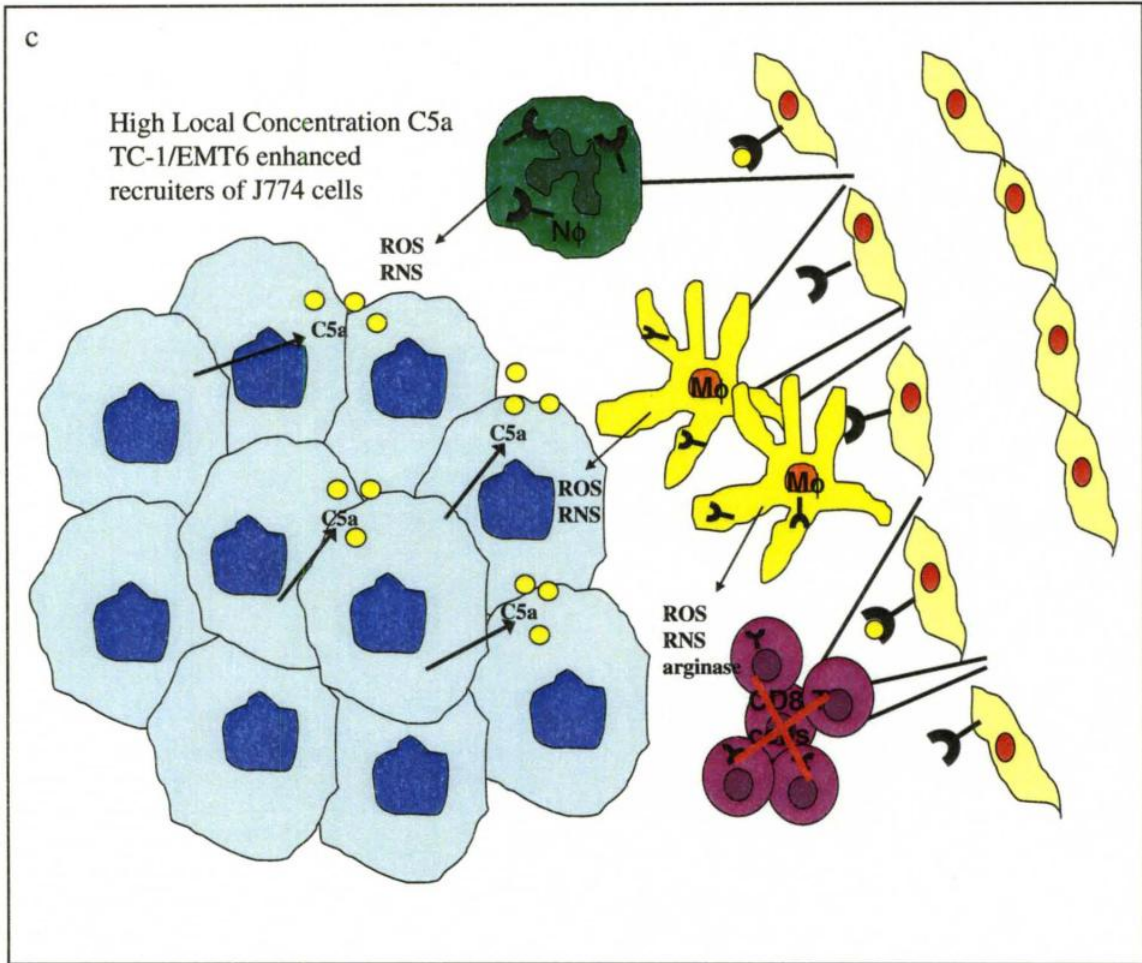


Figure 14: Role of C5a in the tumor microenvironment is concentration dependent. Schematic model of current and published studies revealing critical role for C5a in the tumor microenvironment emphasizing the critical dependence on concentration levels the effect the protein may have. (a) SKOV-3 C5a tumor microenvironment hypothesized to release and maintain consistent low (ng-pg/mL) levels of C5a, significantly enhances infiltration of effector NK cells and anti-tumor macrophages. Recruited NK cells are activated to induce superior cytotoxicity of SKOV-3 tumor cells. Additionally, increased numbers of recruited macrophages generate significantly higher levels of TGF- β to promote anti-tumor inflammation and effectively arrest tumor cell division. (b) Conversely, in the SKOV-3 CV microenvironment, lack of C5a results in significantly increased levels of VEGF, TNF- α , iNOS, and arginase which have all been shown to play important roles in tumor progression and immune suppression. (c) In order to rationalize discrepancies in the literature over the role of C5a in the tumor, a critical component that may offer an explanation of identifying opposite functions of an immune mediator is drawn from its local concentration. Generating or releasing abundant C5a may result in an immune over-activating phenotype, paralyzing responding cells from functioning in a productive anti-tumor response as a consequence of overstimulation.

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