DISSERTATION

TUMOR-ASSOCIATED MACROPHAGE RECRUITMENT AND REGULATION OF ANGIOGENESIS

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LANCE W. U'REN ENTITLED: "TUMOR-ASSOCIATED MACROPHAGE RECRUITMENT AND REGULATION OF ANGIOGENESIS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TUMOR-ASSOCIATED MACROPHAGE RECRUITMENT AND REGULATION OF ANGIOGENESIS

Tumors are no longer thought of as purely a mass of transformed cells. A major component of the cellular composition of a tumor is infiltrating immune cells. Macrophages can constitute a large proportion of infiltrating immune cells. In many cases, increased numbers of Tumor-Associated Macrophages (TAMs) can be associated with a poorer prognosis. TAMs can favor tumor progression by providing the tumor with angiogenic factors, growth factors, and by breaking down the tumor matrix allowing for tumor cell escape and potential metastasis.

Utilizing mice which lack a functional type I interferon receptor (IFN-α/βR-/-), we found that endogenous levels of type I IFNs control tumor growth and angiogenesis. We also determined that tumors grown in IFN-α/βR-/- mice have an increase in macrophage infiltrate. *In vitro* assays suggest that suppression of macrophage responsiveness to CSF-1 by type I IFNs was responsible for the increased macrophage accumulation in tumors of mice unable to respond to type I IFNs. These results indicate that endogenous production of type I IFNs by tumor cells or inflammatory cells may be an important means of suppressing the accumulation of TAMs and their subsequent induction of angiogenesis.

The ability of TAMs to produce VEGF is one of the major means by which TAMs are known to induce tumor angiogenesis. Since VEGF expression is in part induced by hypoxia, it has been speculated that the hypoxic tumor environment is responsible for driving TAM VEGF production. As an alternative possibility we suggest that the engulfment of apoptotic tumor cells can stimulate TAM production of VEGF.

We determined that the use of Liposome DNA-complex (LDC) therapy can induce anti-tumor immunity through the combined activation of systemic innate and adaptive immune responses. We show that LDC can traffic into macrophages and induce expression of activation markers. *In vitro* results show that LDC therapy can inhibit the production of VEGF by macrophages after their consumption of apoptotic cells, suggesting that LDC may be an effective way to circumvent the pro-tumor function of TAMs. Additionally, we determined that LDC combined with chemotherapy can be used as a safe and effective immunotherapy for the treatment of canine hemangiosarcoma. Taken together, these findings could uncover new avenues in which TAMs can be targeted and identified a novel immunotherapy as a potential candidate.

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INTERACTIONS BETWEEN THE IMMUNE SYSTEM AND CANCER

The Immune System as a Primary Defense Against Tumors

The immune system can act in two primary ways to prevent tumor formation. The first critical function is the prompt removal of pathogens which otherwise could lead to cellular transformation and tumorgenesis. This role is not limited to the removal of tumor initiating viruses but also includes the quick resolution of bacterial and parasitic infections as well(1). The second function of the immune system in cancer prevention is a process termed "tumor immune surveillance". Tumor immune surveillance was first hypothesized by Burnet and Thomas in 1957 and it exemplifies the importance of the immune system in cancer prevention(2, 3). In the context of tumor immune surveillance, the immune system can recognize and destroy cancerous cells before they are allowed the opportunity to cause harm to the host.

The tumor immune surveillance hypothesis only offers a "black or white" understanding of the influence the immune system has on tumor cell recognition and elimination. Tumor immune surveillance does not offer a complete explanation of how tumors can still form in the face of a seemingly competent immune system and how in some cases the immune system appears to promote tumor progression. Robert Schreiber developed the concept of "tumor immunoeditting", which offers a more complete understanding of how the immune system can protect from tumor development in some cases while failing to do so in others(4). Cancer immunoeditting divides the immune system's relationship with cancer progression into three distinct phases: First is the elimination phase, in which the immune system recognizes and destroys tumor cells as in the immune surveillance hypothesis. Next is the equilibrium phase, which the immune system more discriminately eliminates some tumor cells allowing for the propagation of tumor cell clones that are immuno-privilaged. Finally is the tumor escape phase, in which the tumor can grow in a uncontrollable manner even in a seemingly immunocompetent host(4).

The Impact of Inflammation on Cancer Progression

The association of *Helicobacter pylori* infection with the initiation of gastric carcinoma is an example of how inflammation can be involved in the initiating step of cellular transformation and ultimately cancer initiation. It is believed that the inflammation associated with *Helicobacter pylori* infection leads to the production of high levels of

free radicals and subsequent cellular transformation(5). However, the involvement of inflammation during the process of cancer is most often a result of the disease process itself and seldom to blame for cancer initiation. Inflammation can play a major role in both the progression of cancer and its resolution. There is a long standing debate over whether inflammation promotes tumor growth or if it is responsible for tumor regression. In the late 1800's William Coley was the first to show that the induction of inflammation can result in cancer cure. Coley successfully treated cancer through the induction of inflammation by injecting inoperable tumors with a brew of killed bacteria (later called "Coley's Toxin"). On the contrary, in 1863 Rudolf Virchow was the first to describe the presence of tumor infiltrating leukocytes and hypothesized the link between inflammation and cancer progression.

The formation of tumor stroma is in many ways similar to the wound healing process. Both processes are dependant upon the formation of a fibrous scaffold, establishment of an adequate blood supply, and require a microenvironment rich in growth promoting factors. These similarities prompted Harold Dvorak's eloquent description of tumors as "wounds that do not heal"(6). It is probable that the infiltrating leukocytes within most tumors are there to provide support functions as they would within the wound healing process. However, it is also possible that the appropriate activation of these infiltrating immune cells can switch them from a tumor supporting role into antitumor effecter cells as Coley likely did with the injection of his toxin.

TUMOR-ASSOCIATED MACROPHAGES

The Influence of Macrophages on Tumor Progression

Macrophages are instrumental in the wound healing process through the production of growth and angiogenic factors, tissue remodeling, and the clearance of dead or dying cells. It is apparent that a tumor can take advantage of these natural macrophage functions and promote neoplastic cell growth, tumor angiogenesis, and encourage tumor cell metastasis. Macrophages have been loosely grouped in two functional categories: M1 (classically activated) and M2 (alternatively activated). The M1 phenotype is induced by pro-inflammatory agents like IFN-γ and LPS, while anti-inflammatory cytokines like IL-4, IL-10, and TGF-β tend to encourage the M2 phenotype(7). M1 macrophages are more likely to be effecter cells that actively produce pro-inflammatory cytokines and are capable of killing tumor cells. TAMs are typically classified as the M2 phenotype which is toleragenic, pro-growth, and promotes angiogenesis(7).

Along with tumor cells themselves, the tumor micro-environment is comprised of many different tumor infiltrating cells. One of the more prominent infiltrating cell types is the macrophage. A recent review of the literature showed that in a majority of the studies an increase in Tumor-Associated Macrophages (TAMs) was correlated with a negative

prognosis(8). Most reviews of the subject support the opinion that TAMs tend to encourage tumor progression(9-12).

Recruitment of Macrophages to the Tumors

Virtually all TAMs present in a tumor originate from circulating blood monocytes. When a monocyte gains entry into a tumor the correct cytokine milieu has to be present to allow its differentiation into a macrophage, the most important factor being colony stimulating factor-1 (CSF-1)(13, 14). The recruitment of monocytes into tumors can occur through a variety of cytokines, chemokines, and growth factors, one major contributor is CCL2 (formally called Monocyte Chemoattractant Protein-1 or MCP-1) (15-17). CCL2 is a member of the C-C chemokine family which is secreted by tumor cells and macrophages themselves. CCL2 production by tumor cells hasbeen positively associated with TAM infiltration of breast and lung carcinoma(18, 19). Although it has been suggested that tumor cell production of CCL2 is the main determinant of macrophage content in tumors, it is unknown what may induce tumor cell CCL2 production(20). Aside from CCL2, osteopontin plays a major role in macrophage recruitment and may be important in the recruitment of TAMs(21). Additionally, vascular endothelial growth factor (VEGF) is a chemotactic signal for macrophages and thought to direct responses to hypoxic tumor regions(12, 22).

Regulation of Tumor Angiogenesis by Macrophages

Stimulation of tumor angiogenesis is one of the major means by which TAMs are thought to promote tumor growth(23, 24). The degree of tumor angiogenesis is often closely linked to the density of macrophage infiltration. Tumors with higher TAM density also produce higher levels of VEGF, a major stimulus for blood vessel growth(25-27). It has been speculated that the hypoxic tumor environment is responsible for driving TAM VEGF production(24, 28, 29). Much of this speculation is based on the observations that TAMs can be found within hypoxic tumor regions and *in vitro* experimentation has shown that hypoxia can induce VEGF production by the stabilization of hypoxia inducible factor 1-alpha (HIF1α).

Functional Capacity of Tumor-Associated Macrophages

TAMs are retarded in their ability to be effecter cells in that they have limited capacity to generate pro-inflammatory cytokines and thus have little tumorcidal activity. Instead TAMs are effective at taming immune responses, inducing tissue remodeling, scavenging debris, and promoting angiogenesis(7). The cytokine mileu which bathes TAMS (IL4, IL13, and IL10) is largely responsible for their inability to maintain an effecter phenotype. TAMs are poor at presenting antigen and through the production of prostaglandins, IL-10, and TGF-β they can inhibit T-cell proliferation and activation(30). Additionally, TAMs are defective in IL-12 and nitric oxide production. The molecular

mechanism behind this defect has been attributed to a constitutive nuclear localization of NF-κB inhibitor p50 homodimer(31-33).

Myeloid Derived Suppressor Cells

Related to TAMs are the recently described Myeloid Derived Suppressor Cells (MDSCs). Like macrophages, MDSCs can accumulate in the tumor and are often associated with the induction of angiogenesis and tumor progression(34). Unlike TAMs, MDSCs are often also found accumulating in the spleen or within the blood of tumor bearing hosts. In addition to the induction of angiogenesis(35), MDSCs can directly inhibit antitumor T cell responses in a contact dependant manner(36). MDSCs have a complex phenotype in that they can express macrophage, granulocyte, monocyte, or endothelial markers (eg F4/80, GR-1, CD115, and CD31 respectively)(37). Recently it has been identified that IL4Rα my be a more specific marker in identifying MDSC(36). The differentiation path of the MDSC is currently not clear and it is unknown how they specifically relate to macrophages or if they can be a progenitor cell to the macrophage(35). However, it is important to acknowledge that this population of cells does exist and it may function similarly to TAMs and contain a common phenotype.

INFLUENCE OF TYPE I INTERFERONS ON MACROPHAGE ACCUMULATION IN TUMORS AND TUMOR ANGIOGENESIS

Functions of Type I Interferons

Interferons (IFNs) were first identified, and hence named after, by their ability to interfere with viral replication(38-40). Beyond the 1950's, the understanding of interferons evolved with studies showing that interferon was not one distinct anti-viral protein but instead a group of related proteins(41, 42). The IFNs are now categorized into two distinct groups, Type I (IFNs α and β) and Type II (IFN-γ), based upon genetic, biochemical, and functional features(43). Type I IFNs are a family of related proteins consisting of 12 IFN-α species and one INF-β in mice(44, 45). All type I IFNs utilize the same receptor, which is comprised of a complex of two subunits (IFNAR1 and IFNAR2). Engagement of this receptor activates a signaling pathway involving the phosphorylation of Jak1 and Tyk2, nuclear translocation of a STAT1/STAT2 heterodimer, with the ultimate induction of genes containing IFN-stimulated response elements (ISRE) promoters(46-48).

Since their initial discovery it has become quite apparent that the function of type I IFNs is not limited to the interference of viral replication. Type I IFNs have been found to be an important part of the immune response to bacterial (including *Chlamydia trachomatis*, *Bordetella pertussis*, *Listeria monocytogenes,* and *Mycobacterium tuberculosis*(49-52)),

protozoal (Leishmania, Plasmodium, Toxoplasma, and Trypanosoma(53-56)), and helminth infections(57). In addition to being an important component of the immune system in protecting the host from foreign invaders, it is now beginning to be realized that type I IFNs also influence the progression of non-pathogenic disease processes such as autoimmunity and cancer.

Type I Interferons and Cancer

The ongoing study of the relationship between type I IFNs and cancer dates backs for more than 40 years(58). All type I IFN subsets signal through the same receptor system(46-48). However, caution must be taken in inferring that all type I IFN subtypes act similarly in all cancers, since it has been shown that different subtypes can act differently in individual model systems(59, 60). With this mind, the vast majority of what we know about type I IFNs and cancer is from studies involving IFN-α. IFN-α is the most commonly used cytokine therapy around the world(58, 61). The exogenous administration of IFN-α is a treatment option for many different cancer types including: hairy cell leukemia, chronic myeloid leukemia, some B and T cell lymphomas, melanoma, renal carcinoma and Kaposi's sarcoma(61).

Even with all of the clinical experience using IFN-α and the numerous animal studies that have been undertaken, little is known about the mechanism behind the antitumor response nor why success with the therapy seems to be highly selective by tumor

type(61). It was thought that the effects of IFN-α were mediated directly at the tumor cells themselves by influencing their function or proliferation. This became less accepted when it was understood that IFN-α had no effect on tumor cells proliferation for some of the most clinically responsive tumor types to IFN-α therapy, like melanoma(61-63). It is unclear how the exogenous administration of type I IFNs enhances immune mediated elimination of tumors, however, several mechanisms have been suggested including: increased NK cell function(58, 61), up regulation of MHC class I antigens on tumor cells(58, 61), macrophage activation(63, 64), enhanced differentiation of Th1 subsets(65, 66), the generation and activation of more cytotoxic T lymphocytes (CTLs)(67, 68), promoting the survival of CTLs(69, 70), and the maturation of dendritic cells, to name a few(71, 72).

Far less is known about the effects of endogenous type I IFNs on cancer progression. Understanding how the body tries to naturally control tumor growth is important in that it may open up avenues for therapeutic exploitation. The earliest studies examining the influence of endogenous type I IFNs in tumor progression were antibody depletion experiments. Experiments using xenogeneic transplantable tumors into athymic mice showed that depletion of type I IFNs could promote tumor growth and metastasis(73-75). Similar results were also found in type I IFN antibody depletion experiments using syngeneic transplantable tumors in immunocompetent mice(76, 77). Interestingly, these results were replicated in models using tumor cells resistant to type I IFNs, indicating that the effects of endogenous type I IFNs is most likely on host derived cells and not the tumor cells themselves(78, 79). In addition to antibody depletion experiments,

experimentation with mice lacking a functional type I IFN receptor have shown that endogenous type I IFNs are important in tumor protection using xenogeneic, allogeneic, and syngeneic mouse tumor models(80, 81).

Influence of Type I Interferons on Angiogenesis

Type I IFNs have been shown to inhibit angiogenesis but not by acting directly on endothelial cell proliferation or migration(82). Much of the influence that type I IFNs have on angiogenesis is mediated by their effect on pro-angiogenic molecules like VEGF, basic fibroblast growth factor (bFGF), and interleukin-8 (IL-8)(83-87). Through its ability to inhibit these pro-angiogenic molecules, type I IFNs can indirectly effect endothelial cell migration, proliferation, and survival. Type I IFNs can also influence angiogenesis by their ability to inhibit MMP-9 production(88). This inhibition of MMP-9 impairs angiogenesis by not allowing for tumor matrix breakdown and impedes vascular expansion.

In a therapeutic setting IFN-α has been shown to inhibit tumor angiogenesis(87, 89-91). IFN-β has also been found to be an important cytokine in the inhibition of angiogenesis by the down regulation of bFGF and IL-8 specifically(84, 92). In addition to studies which focus on the effects that exogenous type I IFN administration has on tumor angiogenesis, it has been shown that endogenous levels of type I IFNs can also inhibit tumor angiogenesis, although a mechanism has not been yet elucidated(81).

Effects of Type I Interferons on Monocytes and Macrophages

The predominant effect of type I IFNs on macrophages is the induction of a proinflammatory phenotype. Type I IFNs can induce macrophage expression of IL-1, IL-2, IL-6, IL-1 receptor, TNF, TNF receptor, and IFN-γ(93). Type I IFNs can also enhance macrophage antibody dependant tumorcidal activity(94). IFN-α can increase the bactericidal function of macrophages by increasing their production of iNOS(95). Even though type I IFNs most often induce macrophage proinflammatory functions, there are examples of type I IFNs inhibiting immune functions. For example, pre-incubation of macrophages with IFN-α can inhibit some of IFN-γ actions on macrophages, such as, MHC class II expression, the induction of apoptosis, and iNOS expression(95-97).

One of the most profound effects that type I IFNs have on monocytes is their ability to influence monocyte differentiation path or their ability to differentiate. The combination of IFN-α with GM-CSF can stimulate the differentiation of dendritic cells with enhanced antigen presenting capacities(72). On the contrary, it has been shown that type I IFNs can inhibit the ability of monocytes to respond to CSF-1 and block differentiation into macrophages(98, 99). Similarly, type I IFNs can also inhibit the differentiation of monocytes into osteoclasts(100). These results suggest that the induction of type I IFNs favor monocyte differentiation towards dendritic cells while inhibiting macrophage differentiation.

INFLUENCE OF MACROPHAGE CLEARANCE OF APOPTOTIC CELLS ON TUMOR ANGIOGENESIS

Mechanisms of Apoptotic Cell Clearance by Macrophages

Apoptosis is a form of programmed cell death that is characterized by cell shrinkage, membrane blebbing, nuclear fragmentation, and chromatin condensation(101). The clearance of apoptotic cells is largely regarded as an immunological quiescent, if not an immunosuppressive, process. Defects in apoptotic cell clearance can allow apoptosis to proceed into secondary necrosis resulting in a proinflammatory immune response. Macrophages are key players in the removal of apoptotic cells, although many other cell types can also participate in apoptotic cell clearance including: dendritic cells, fibroblast, epithelial cells, hepatocytes and others(102, 103).

Macrophage clearance of apoptotic cells requires the interaction of signaling molecules on both the apoptotic cell and the macrophage. Phosphatidylserine (PS) externalization is the most recognized ligand on apoptotic cells for macrophage clearance(104). PS is an aminophospholipid that is normally found on the inner leaflet of the plasma membrane bilayer. During apoptosis the activation of phospholipid scramblase moves PS to the outer membrane while the concurrent inactivation of aminophospholipid translocase

prevents PS from returning to the inner leaflet(105, 106). This allows for the PS dependant recognition and clearance of apoptotic cells.

Recognition of PS by the phagocyte is a complex interaction that can involve the use of scavenger receptors, PS receptors, bridging molecules, receptors for complement or microbial components (CD14), and others(107, 108). Of the scavenger receptors, CD36 has been well studied for its ability to recognize PS(109). Macrophages can use the CD36-PS interaction to recognize and engulf apoptotic cells, however studies have shown the PS must be in the oxidized form(110, 111). Recently three different PS receptors have been identified, these include: 1) Tim4 (T-cell immunoglobin and mucin-domain-containing molecule)(112), 2) BHI1 (brain-specific angiogenesis inhibitor 1)(113), and 3) stabilin-2(114). In addition to scavenger and PS receptors there have been the identification of bridging molecules which bind to PS on apoptotic cells and then to their native receptors to initiate engulfment. Milk fat globule-EGF-factor 8 (MFG-E8) and developmental endothelial locus-1 (Del-1) are both bridging molecules that can bind to αv integrins for apoptotic cell clearance(115, 116). Similarly, Gas6 and protein S can stimulate the PS dependent uptake of apoptotic cells when they bind with their proper receptor(Mer tyrosine kinase) on the phagocyte(117).

As described above, there are multiple mechanisms by which apoptotic cells can signal for their clearance. It is also important to understand what keeps non-apoptotic, living cells from being eaten as well. After all, it is known that PS can be expressed transiently on the surface of live cells during activation, such as the case with some activated T cells

and B cells(118-121). The work of Gardai et al provides an explanation of why viable cells which express molecules marking them for phagocytosis are not eaten(122). They showed that live cells expressing calreticulin on their surface, which would normally mark them for engulfment by binding LDL-receptor-related-protein/CD91 on the phagocyte, were not eaten because live cells express a "don't eat me" signal (CD47) which inhibits phagocytosis by binding SIRPα on the phagocyte(122). This could explain why live cells expressing PS on their surface are not automatically cleared by phagocytes.

Phosphatidylserine Expression and Macrophage Responses

The clearance of apoptotic cells by macrophages is not just an immunological quiescent process regardless of the type of apoptotic cell target. This process actually induces the production of immunosuppressive cytokines. The cytokines most often implicated in this process including transforming growth factor-beta (TGF-β) and IL-10, although it appears PS is only directly associated with the production of TGF-β(123-126). This process is important in the context of tumor immunity because TGF-β significantly weakens anti-tumor immune responses via tolerance(123, 127).

In addition to the anti-inflammatory environment created by the clearance of apoptotic cells, macrophages are induced to secrete pro-growth factors as well. Removal of

apoptotic cells by professional and non-professional phagocytes induce their production of a variety of growth factors including: VEGF, TGF-β, and hepatocyte growth factor (HGF)(103, 125). It is easy to speculate that a tumor could take advantage of this growth factor milieu by enhancing tumor cell proliferation and tumor angiogenesis. We are particularly interested in VEGF because it has been well described as one of the main factors that supports tumor angiogenesis and tumors are dependant upon VEGF for survival(25-27). It is already known that TAMs produce VEGF *in vitro* and *in vivo*, and that they are associated with new vessel formation(24, 29). In this regards, it has been demonstrated that TAMs are more prevalent in hypoxic tumor regions where they enhance VEGF production for these poorly vascularized areas(12, 22).

Several *in vitro* models have demonstrated that macrophages respond to hypoxia(24, 28), presumably by inducing the transcription factor hypoxia inducible factor 1-alpha (HIF-1α). The induction of HIF-1α prompts the transcription of VEGF and other hypoxia inducible genes(128). However, it has never been shown *in vivo* that this is the sole mechanism by which TAMs induce VEGF transcription. We feel that it is just as likely that TAMs responding to hypoxic tumor areas are induced to produce VEGF as a result of apoptotic cell clearance within these hypoxic regions.

PS asymmetry appears to behave differently in tumor cells. Unlike their untransformed counterparts, some tumors cells will not externalize PS as a part of the process of apoptosis(102, 129). On the other hand, PS can be constitutively expressed on the outer leaflet of other tumor cell lines that are not undergoing apoptosis(127, 130-134). It

appears that the mechanism for this disregulation of PS in tumor cells is likely due to the constitutive inactivation of aminophospholipid translocase(129, 135). The advantage, if any, that the tumor gains through the disregulation of PS has not been specifically studied. One could speculate that the expression of PS on tumor cells would be detrimental to tumor growth due to recognition and removal of PS expressing tumor cells by macrophages. In fact, Utsugi showed that monocytes could kill tumor cells through the recognition of PS on the surface of the live tumor cell(131). However, this PS dependant killing could only be induced by first activating the monocytes with LPS and IFN-gamma. Even though TAMs are capable of removing apoptotic cells is a PS dependent manner, it has not been shown that TAMs are capable of PS dependant removal of live tumor cells *in vivo*(136).

CANCER IMMUNOTHERAPY

Introduction

The immune system eliminates the vast majority of developing tumors and the tumors that do develop have found a way to evade immune surveillance(137). In the past the goal of most cancer immunotherapies was to "re-educate" the immune system so that it no longer recognized tumor cells as "normal" and would hopefully generate an effective immune response against them. This was most often explored through the development

of tumor vaccines that relied on the generation of CTLs specific for an individual tumor cell antigens(138-142). Although there have been clinical successes reported with tumor vaccines, the overall sentiment of tumor vaccine development has been one of disappointment(143-145). It is therefore apparent that the current cancer vaccines that are being designed are not reaching their full potential and changes must be made before effective cancer immunotherapies are found(143, 146).

Although the generation of a strong CTL response against tumor antigens is an important component of cancer vaccines, it is obviously not the sole component that dictates vaccine success. In addition to strong CTL generation, there are many immunosuppressive mechanisms of the tumor immune response that proper immunotherapy must address including: TAMs, regulatory T cells (Tregs), and MDSCs. As described above, TAMs can influence cancer progression by the promotion of neoplastic cell growth, tumor angiogenesis, and by encouraging tumor cell metastasis. Tregs can inhibit the function of CTLs, dendritic cells, and NK cells(147-149). MDSCs can influence tumor progression by inducing Treg cell formation, directly inhibiting CTL function, and enhancing tumor angiogenesis(150, 151). With all of these inhibitory mechanisms ready to dampen cancer vaccine responses, it is important that immunotherapies, or vaccine adjuvants, control such mechanisms.

Cationic Liposome-DNA Complexes (LDC) as Cancer Immunotherapeutics

Cationic liposome-DNA complexes (LDC) were originally described as effective gene delivery vehicles for cancer or gene replacement therapies(152-154). It is now understood that the use of LDC made with non-coding, high CpG content, plasmid DNA has profound immunostimulatory effects and therapeutic potential by itself(155-157). The use of unmethylated CpG motifs (bacterial DNA) is an attractive candidate for macrophage activation because in addition to the translocation of NFκB thay also stimulate several mitogen-activated protein kinase pathways(158).

LDC are extremely potent activators of innate immunity and their systemic administration triggers a strong activation of innate immune responses and release of high concentrations of key Th1-biasing cytokines, including IL-12, IFN-γ, and type I IFNs (155, 156, 159). This effect is likely due to the ability of cationic liposomes to target innate immune cells and trigger an immune response to the unmethylated bacterial CPG motif within the plasmids. In addition, the systemic administration of LDC can elicit potent anti-tumor activities(155, 156, 160, 161).

Summary and Project Rationale

The composition of a tumor is much more than tumor cells alone. It consists of a complex microenvironment comprised of many different host derived cells, including macrophages. Although macrophages can carry out tumoricidal functions, within the suppressive tumor environment macrophages lose the ability to destroy tumor cells. Often tumor-associated macrophages (TAMs) carry out actions that are supportive of tumor growth including the enhancement of tumor angiogenesis, support for tumor cell proliferation, and augmentation of tumor cell escape for distant metastasis. Since TAMs have the potential of encouraging tumor growth and metastasis, there is intense investigation into methods of limiting their numbers or altering their function from one of a pro-tumor phenotype to an antitumor effecter cell. The overall goal of the research reported within this dissertation is to better understand the mechanism of macrophage accumulation within tumors, what potentially induces their production of pro-angiogenic factors, and to assess if they can be targeted by immunotherapy.

The work within this dissertation was first initiated by an observation made by our lab and others, that tumors grown in type I IFN receptor knockout (IFN-α/βR -/-) mice grew more rapidly and had an increase in vascularity. We were also able to show that tumors grown in IFN-α/βR -/- mice had an increase in tumor infiltrating macrophages. This led us to hypothesize that type I IFNs can inhibit the accumulation of TAMs and hence obstruct tumor angiogenesis **(Chapter 2: Inhibition of Tumor Growth and**

Angiogenesis by Endogenous Type I Interferons Is Associated With Suppression of Intratumoral Macrophage Accumulation). There are many different mechanisms which could allow for an increase in TAMs within the IFN-α/βR -/- mice. Type I IFNs could influence the cytokine profile of tumors in a way that discourages macrophage migration or type I IFNs could act directly on monocytes/macrophages by altering their ability to respond to a chemotactic gradient. It is also possible that type I IFNs could inhibit the ability of monocytes to differentiate into macrophages. Similarly, the difference in macrophage numbers could be a response to endogenous type I IFNs inhibiting macrophage survival or potentially proliferation.

Chapter 2 gives a detailed account of the methodologies used to examine the increased TAM frequency in IFN-α/βR -/- mice. *In vitro* assays were utilized to assess if type I IFNs could alter the ability of monocytes to differentiate into macrophages or if they affected macrophage survival. We also tested *in vitro* if type I IFNs affected the ability of macrophages to migrate across a chemokine gradient. Utilizing the techniques of flow cytometry, ELISAs, and RT-PCR we could also obtain an *in vivo* assessment of how type I IFNs may have effected macrophage accumulation. This included analysis of macrophage chemotactic factors produced by the tumors and the expression of relevant receptors on the surface of the macrophages themselves. Chapter 2 also explores the potential link between the increase frequency of TAMs within the IFN-α/βR -/- mice and the increase in microvessel density that was also observed. This includes analysis of both pro-angiogenic and anti-angiogenic factors which are commonly associated with macrophages.

Based on our observation in chapter 2 that TAM accumulation appeared to be related to increased angiogenesis, we became interested in better understanding the influence that the tumors had in driving macrophage production of angiogenic factors. It had been recently reported that the clearance of apoptotic cells by macrophages could drive macrophage production of VEGF(103). We thought that this observation could be of great importance of our understanding of how TAMs influence tumor angiogenesis. It was already known that TAMs enhanced tumor angiogenesis by their production of VEGF. *In vitro* experimentation suggested that TAMs are most likely stimulated to produce VEGF by the hypoxic tumor microenvironment driving Hypoxia Inducible Factor (HIF) pathways. We chose to study an alternative mechanism that may be responsible for driving TAM VEGF production. We hypothesized that phosphatidylserine (PS) on the surface of apoptotic tumor cells induces TAM production of VEGF.

Chapter 3 **(The Effects of Apoptotic Tumor Cell Engulfment on Macrophage VEGF Production)** introduces the concept that PS can drive TAM production of VEGF. This is an important concept to understand because most chemotherapies induce tumor cell apoptosis while not effecting the non-dividing TAM population. Although the induction of tumor cell apoptosis is often a necessary component of cancer therapies, we believe that interference of macrophage clearance of apoptotic cells could enhance the effectiveness of chemotherapies. It is likely that TAMs allow the tumor, or tumor metastases, to "rebound" from the tumorcidal effects of chemotherapy. We find it

plausible that TAMs can limit the effectiveness of chemotherapies through the production of growth factors following apoptotic cell clearance. Macrophage clearance of apoptotic cells can induce the production of TGF-β, hepatocyte growth factor (HGF), and VEGF(103). More relevant to the topic of this dissertation is how macrophage clearance of apoptotic cells could allow for a "rebound" of tumor vasculature through the production of VEGF. This is particularly important because tumor endothelial cells and vasculature are often abnormal and susceptible to death from chemo- and radiotherapies(162, 163). This suggests that macrophage production of VEGF could support these damaged endothelial cells or allow for the recruitment or proliferation of new ones.

The interference with macrophage clearance of apoptotic tumor cells as a method to augment antitumor therapies has been largely unexplored. Numerous studies have shown that depletion of TAMs can inhibit tumor growth and angiogenesis(164, 165), but it is unknown if the inhibition of apoptotic cell clearance plays any role in these observations. There has been the development of monoclonal antibodies which can bind to PS on the surface of tumor endothelial cells, so it may be feasible that a PS blocking antibody could be developed to inhibit apoptotic cell clearance(166). Along these lines, the blocking of bridging molecules or PS receptors could also be explored to block PS mediated apoptotic cell clearance. Broad spectrum caspase inhibitors could also be utilized to block the process of apoptosis and allow progression of cellular necrosis(167). Since the process of apoptotic cell clearance is largely anti-inflammatory and that of necrosis is

pro-inflammatory, allowing apoptotic cells to progress into necrosis may help generate a pro-inflammatory response to the tumor.

It is likely that the importance of PS inducing macrophage VEGF production extends beyond apoptotic cell clearance alone. Some tumors have been shown to constitutively externalize PS while they are alive and not undergoing the process of apoptosis(127, 130-134). These tumor cells also likely shed PS expressing vesicles which could act upon macrophages in much the same way as apoptotic cells.

The purpose of chapter 3 was to identify if apoptotic tumor cells could induce VEGF production and to determine if this response was reliant on PS. Using different cells lines which can or can not externalize PS during the process of apoptosis, we assessed the importance of PS on macrophage VEGF production. We also determined if it is only PS stimulation that was necessary for VEGF production or if it was the act of physical engulfment of the apoptotic cell that was important for macrophage VEGF production.

In chapters 2 and 3 of this dissertation, we explained mechanisms which can influence the accumulation of TAMs and enhance their ability to be pro-angiogenic. In chapter 4 **(Vaccination With Liposome-DNA Complexes Elicits Enhanced Anti-Tumor Immunity)** we studied the ability of cationic lipid-DNA complexes (LDC) immunotherapy to mount tumor specific T cell responses and its ability to target and effect macrophages. A focus of our lab as been the development and study of LDC as an immunotherapy for cancer and infectious diseases (156, 160, 168). LDC are made with

plasmid DNA that contain a rich unmethylated CpG motifs. Bacterial CpG are pathogen associated molecular patterns (PAMPs) that signal through TLR-9 to induce strong activation of innate immune responses and release of high concentrations of key Th1-biasing cytokines, including IL-12, IFN-γ, and type I interferons(155, 156, 159). The liposomal construction of LDC makes it likely that it would target immune cells with phagocytic capacities. The combined features of being able to deliver immunostimulatory CpG DNA directly to immune cells are what makes LDC an immunotherapy that warrants further study.

TAMs are attractive targets for cancer therapy because of their ability to promote cancer progression. Mouse models have shown that the depletion of TAMs may be an effective method of inhibiting their deleterious effects(164, 165). In human breast cancer, bisphosphonates have been used to deplete osteoclasts and have had some clinical success(169, 170). However, it is unclear how well systemic depletion of macrophages using reagents such as liposomal clodronate would be handled in the human. An alternative method to TAM depletion would be to activate TAMs and change them from a pro-tumor to a tumorcidal phenotype. It has been shown that mice treated with CpG DNA and IL-10 blocking antibody were able to switch TAMs from an M2 to a M1 phenotype(171). Since liposomes are more likely to target macrophages, it is likely that LDC immunotherapy may be a more effective means of creating a similar phenotype switch of TAMs.

We hypothesized that LDC could be used effectively as a DNA vaccine platform to enhance the generation of therapeutic anti-tumor immunity through the combined activation of systemic innate and adaptive immune responses. The four goals of the research within chapter 4 were to: 1) determine if LDC could induce an effective antitumor CD8+ T cell response, 2) determine if LDC could directly target macrophages, 3) assess if macrophages and other immune cells could acquire an activated phenotype from LDC administration, and 4) determine if LDC could inhibit the ability of macrophages to produce VEGF. To test our hypothesis we first immunized tumor-bearing mice with conventional plasmid DNA vaccines or with LDC vaccines and assessed antigen-specific $CD8^+$ T cell responses. We than fluorescently labeled LDC and tracked the immune components it targeted by flow cytometry and utilized flow cytometry to measure the expression of activation markers on macrophages. Finally, using the methods described in chapter 3, we determined if LDC treatment could inhibit the ability of macrophages to produce VEGF in response to apoptotic cell clearance. The research described in chapter 4 will not only assess the ability of LDC to induce antitumor T cell immunity but also help address if LDC therapy may be an effective therapy for targeting TAMs, activate them into an antitumor phenotype, and inhibit their ability to produce angiogenic factors.

Chapter 5 (**Evaluation of a Liposome-DNA Complex Vaccine in Dogs With Hemangiosarcoma**) extends upon the groundwork laid out in chapter 4 by evaluating the safety and immunologic responses of LDC in canine patients with hemangiosarcoma (HSA). LDC-based tumor vaccines have not been previously evaluated in dogs,

therefore, we conducted an open, phase I study of an allogeneic tumor vaccine based on the LDC adjuvant in 28 dogs with stage II HSA. Since the administration of immunotherapy together with chemotherapy has been reported to improve survival in dogs with HSA(172), we chose to study the combined treatment of an allogeneic tumor lysate LDC vaccine with doxorubicin. We hypothesized that LDC vaccination could be safely and effectively combined with doxorubicin chemotherapy for treatment of dogs with HSA. The research described in chapter 5 will provide a clinical assessment of the effectiveness and safety of LDC therapy for large animals with spontaneously occurring cancer and hopefully open the door for exploration of LDC based cancer vaccines for human patients.

It is hoped that the research encompassed within this dissertation will help highlight the importance of macrophages in the progression of tumors. This research will help clarify the mechanisms important for TAM accumulation and VEGF production and provide evidence that LDC immunotherapy may target TAMs and inhibit their ability to produce VEGF. Additionally, we show the LDC is a safe and effective cancer immunotherapy in canine patients with spontaneously occurring cancer. Ultimately, the goal of this dissertation is to provide a better understanding of tumor biology and provide the groundwork for a new cancer immunotherapy in humans.

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Chapter Two

Inhibition of Tumor Growth and Angiogenesis by Endogenous Type I Interferons Is Associated With Suppression of Intratumoral Macrophage Accumulation

ABSTRACT

Tumor-associated macrophages (TAMs) are very numerous in tumors and are now considered to be a major factor in promoting tumor growth. The recruitment and accumulation of TAMs in tumors is positively regulated by several molecules, including CSF-1 and CCL2. However, factors that suppress the accumulation of TAMs within tumors have not been previously identified. A previous study indicated that the anti-tumor effects of endogenously produced type I interferons (IFNs) were mediated by effects on hematopoietic cells, rather than by direct effects on tumor cells. Therefore, we tested whether the anti-tumor effects of endogenously produced type I IFNs might be mediated in part by effects on TAMs. In tumor models in IFN-α/βR -/- mice, we found that there were significantly more TAMs present in tumor tissues than in tumors of wild

type mice, as assessed by both flow cytometry and immunohistochemistry. Moreover, tumors in IFN-α/βR -/- mice grew significantly more rapidly and had significantly increased angiogenesis compared to tumors in wild type mice. The increase in TAM density in tumors of IFN-α/βR -/- mice was not due to tumor overproduction of CSF-1 or CCL2. Rather, *in vitro* assays indicated that the exposure to type I IFNs inhibited macrophage proliferation and survival. This inhibitory effect appeared to be due to antagonism of the stimulatory effects of CSF-1, as both IFN-α and IFN-β inhibited the macrophage stimulatory effects of CSF-1 to equivalent degrees. These results suggest that endogenous type I IFNs inhibit tumor growth and angiogenesis in part by decreasing accumulation of TAMs within tumor tissues.

INTRODUCTION

Type I interferons (IFNs) are a family of related proteins consisting of 12 IFN-α species and one INF-β in mice (1, 2). Both type I IFNs utilize the same receptor, which is comprised of a complex of two subunits (IFNAR1 and IFNAR2). Engagement of this receptor activates a signaling pathway involving the phosphorylation of Jak1 and Tyk2, nuclear translocation of a STAT1/STAT2 heterodimer, with the ultimate induction of genes containing IFN-stimulated response elements (ISRE) promoters(3, 4). Although they were initially identified based on their potent anti-viral activity, type I IFNs have since been recognized as important regulators of anti-bacterial and protozoal immunity as well(5-7).

It is also well-established that therapeutic administration of IFN-α is capable of significantly inhibiting angiogenesis, including tumor angiogenesis (8-11). These effects are mediated in large part by suppression of endothelial cell motility and survival through the inhibition of pro-angiogenic factors such as VEGF, basic fibroblast growth factor, and interleukin-8(8, 12-15). Although many studies have examined the effects of exogenous administration type I IFNs on inhibition of tumor growth, much less is known about the effects of endogenous production of type I IFNs on tumor growth and progression. In this most thorough report to date, McCarty et al utilized IFNα/β receptor knockout mice (IFNα/βR-/-) to demonstrate that endogenous production of type I interferons plays an important role in inhibiting tumor progression, metastasis, and angiogenesis (16). More recently, it was shown that the inhibitory action of type I IFNs on tumor growth was

mediated by their effects on hematopoietic cells, rather than on the tumor cells themselves(17). However, it was not established in these studies which hematopoietic cells were the targets for the tumor inhibitory effects of type I IFNs.

Increasingly it is clear that tumor-associated macrophages (TAMs), rather than helping control tumor growth as previously thought, actually contribute significantly to promoting tumor growth. For example, increased numbers of TAMs correlate with significantly reduced survival times in human cancer patients, including those with breast, prostate, and endometrial cancer (18-20). TAMs can promote tumor growth by stimulating tumor cell invasion into surrounding tissues, by increasing tumor cell growth and survival, by triggering tumor metastasis, and by stimulating angiogenesis(21). The propensity of TAMs to stimulate tumor angiogenesis may be related to the observation that macrophages are often located in hypoxic regions of the tumor (22, 23). In these regions, hypoxia leads to the induction of HIF-regulated genes such as VEGF, which in turn stimulate local angiogenesis. The recruitment of TAM precursor monocytes into the tumor parenchyma is thought to be mediated by chemokines such as CCL2, CXLC12, and CCL5 and others (24, 25). The survival and differentiation of these recruited monocytes is in turn critically dependent on local tumor production of CSF-1 (26). Both experimentally and clinically, CSF-1 production within tumors has been correlated with the degree macrophage infiltration and with decreased survival times (27, 28).

Previous studies indicated that low levels of type I IFN production can inhibit the response of bone marrow derived macrophages to CSF-1, as assessed by decreased

proliferative responses (29, 30). Therefore, we conducted studies to assess the role of endogenous production of type I IFNs in the regulation of macrophage accumulation, tumor growth, and angiogenesis. Specifically, we investigated tumor growth rates, tumor microvessel density, and numbers and phenotype of TAMs in wild type and IFN-a/bR -/- mice. We also assessed production of macrophage chemokines and pro-angiogenic cytokines by both tumor tissues and by purified TAMs. The effects of type I IFNs and tumor conditioned medium on proliferation and survival of bone marrow macrophages was also assessed. The results of these studies provide important insights into a previously undefined role for type I IFNs as negative regulators of the accumulation and survival of TAMs in tumor tissues.

MATERIALS AND METHODS

Animals

Breeding pairs of mice lacking the type I interferon receptor (IFNα/βR-/-) on the 129 Sv/Ev background were kindly provided by Dr. Philippa Marrack (National Jewish, Denver, CO) and Dr. Michel Auget (Swiss Institute for Biomedical Research, LuCerne). Age and sex matched wild type 129 Sv/Ev mice, containing a fully functional type I interferon receptor, were obtained from Taconic Laboratories (Germantown, NY).

Animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Tumor cell lines and tumor model

A syngeneic cell line from 129 Sv/Ev (wild type) mice was established by s.c. injection of methylcholanthrene (MCA) in peanut oil. The cutaneous fibrosarcoma tumor that developed was established *in vitro* and then passaged *in vivo* through two additional generations in wild type 129 mice. The resulting cell line was designated MCA 2.1. The tumor cells were maintained at low passage number in modified Eagle's complete medium with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA), and incubated at 37°C in 5% CO_2. To assure mycoplasma-free conditions, cells were routinely tested by MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA). Tumors were established in either wild type or IFNα/βR-/- mice by the intradermal inoculation of $1x10^6$ MCA2.1 cells over the lateral hip. Tumor diameters were measured every 2-3 days using calipers and mice were euthanized once the tumor diameter reached 1.0 cm.

Immunohistochemistry

Tumor tissues were embedded in Tissue-Tek OCT compound (TedPella Inc., Redding, CA) and snap-frozen in isopentane. Tissues were cryosectioned to a thickness of 4 μm and adhered to glass slides (Superfrost, VWR International, West Chester, PA). For assessment of macrophage infiltration, macrophage specific monoclonal antibodies F4/80 (clone BM8, eBiosciences) and CD68 (clone FA11, Serotec, Raleigh, NC) were utilized. After fixation in acetone and blocking nonspecific binding, tissues were incubated first with the primary antibody, followed by donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), then with streptavidin–HRP and AEC substrate (Vector Laboratories, Burlingame, CA), followed by hemtoxylin counterstain. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies.

For quantitation of tumor microvessel density, an antibody directed against murine CD31 mAb (clone 309, eBiosciences, San Diego, CA) was used for the identification of tumor microvessels and angiogenic endothelium. Staining of acetone fixed tissues was carried out as above. Four photomicrographs at 10X magnification were obtained by digital camera (Leica Microscope equipped with digital camera with SPOT Advanced Imaging software). Utilizing Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC), the photomicrographs were converted to binary images and the number of microvessels per section was determined digitally, as described previously(31). Microvessel density values for each tumor sample were calculated based on the average number of vessels for the four 10x fields.

Flow cytometry

Flow cytomety was used to assess and quantitate leukocytes infiltrating tumor tissues. Tumor tissue was collected, minced, digested for 20 minutes in 4 mls of 1.5 mg/ml collagenase, 65 μg/ml soybean trypsin inhibitor, 1.5 μg/ml DNase I (all from Sigma Chemical Co, St Louis, MO). Single cell suspensions from digested tumor tissues were prepared and the live cells were isolated by Ficoll gradient centrifugation (LSM Lymphocyte Separation medium; MP Biomedicals, Aurora, Ohio). Single cells suspensions were resuspended in FACS buffer (PBS with 2% FBS and 0.1% sodium azide) at a concentration of 2.5×10^5 to 1×10^6 cells per well. The cells were then immunostained with the following antibodies, all purchased from eBiosciences (San Diego, CA unless otherwise noted): anti-F4/80 (APC; clone BM8), CD11b (APC-Cy7 clone M1/70), Gr-1 (PE-Cy7; clone RB6-8C5), and NKG2D (biotin, clone CX5). Nonspecific binding of antibodies was blocked by pre-incubation of cells in normal mouse serum with 40% supernatant from rat anti-FcRIII hybridoma 24.G2, plus 0.2 mg/ml human immunoglobulin IgG. Staining was carried out at 4°C for 20 min, followed by washing in FACS buffer. In most cases, cells were fixed in 1% paraformaldehyde for 30 min and stored in FACS buffer at 4 °C before analysis. Flow cytometry was performed using a Cyan ADP flow cytometer (Dako, Ft Collins, CO). Data analysis was carried out using Summit software (Dako).

ELISA assays for cytokines

In some experiments, tumor conditioned medium was generated by collecting freshly harvested tumor tissues, sterile cutting(and weighing) the tumor tissues into approximately 1 mm cubes, and then placing 30 mg of tumor tissue in 1 ml of serum-free media (X-Vivo 15, Biowhittaker, Walkersville, MD) in a tissue-culture incubator for 24 hrs. The supernatants were than harvested and frozen at -70°C until ELISA analysis was preformed. The following cytokine assay kits were used to quantitate cytokine concentrations in tumor conditioned medium. Colony stimulating factor-1 (R&D Biosystems, Minneapolis, MN), CCL2 (BD Biosciences, San Jose, CA), VEGF (R&D Biosystems), KC (R&D Biosystems), IL-1b (R&D Biosystems) IFN-γ (R&D Biosystems) and M-CSF1 (R&D Biosystems). Commercial ELISAs were preformed as per manufacture's instructions. For detection of mouse IFN-α an ELISA was developed as previously described using a rat anti-mouse capture (PBL, Piscataway, NJ) and a rabbit anti-IFN-α detection antibody (US Biological, Swampscott, MA)(32, 33). ELISAs were developed with tetramethylbenzidine substrate (Sigma Chemical Co, St Louis, MO) and absorbance measured at 450 nm with an optical plate reader (Thermo Labsystems, Waltham, MA).

Migration assay

A transwell migration was preformed as previously described(34). Peritoneal macrophages (PECs) were lavaged from the abdomen of wt and IFNα/βR-/- mice with 5

mls of ice cold hanks. PECs were washed twice and resuspended in serum-free media at $2x10^6$ cells/ml. 100μL of cells were added to the upper chamber of a 24-well transwell with 8μm pore size (BD biosciences, Bedford, MA). Cells were allowed to equilibrate for 30 min at 37°C in 5% CO2. As a positive control, 100ng/ml CCL2 (BD biosciences, Bedford, MA) was used. Tumor conditioned medium, or medium alone, was also evaluated. Samples to be tested were added to the lower chamber of the transwell plate. After 3 h incubation at 37°C in 5% CO2, the cells remaining on the upper chamber were wiped off with a cotton swab. The cells which migrated to the lower filter of the membrane were fixed in 75% ethanol, washed with water, and stained with hemotoxylin. Migrated cells were quantified by averaging the counts of 5 random 200X fields.

Bone marrow macrophage culture

Bone marrow macrophage cultures were generated from wild type 129sv/ev or IFNα/βR-/- mice. Briefly, bone marrow was flushed from the femurs and tibias with HBSS plus 2% FBS, as described previously(35). Cells were washed and RBC were lysed using NH_4Cl solution. Cells were plated at 2 X 10^5/well in a 96 well plate (Corning lifesciences, Acton, MA) in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 0.2 mM Lglutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA), and incubated at 37°C in 5% CO_2.

Macrophage survival was assessed using MTT assay (36). Briefly, BMMAC from wild type and IFN-a/bR -/- mice were incubated in serial dilutions of tumor-conditioned medium. As a positive control, cells were also incubated with 50 ng/ml recombinant CSF-1 (PeproTech Inc, Rock Hill, NJ). To assess the effects of endogenous CSF-1 produced by tumor cell lines, we measured CSF-1 concentrations in tumor conditioned medium. We also neutralized endogenous CSF-1 in tumor conditioned medium by incubating the medium with 50 ng/ml CSF-1 neutralizing polyclonal Ab (goat anti-mouse, R&D Biosystems, Minneapolis, MN) or 50 ng/ml purified goat IgG as an isotype control (R&D). All cultures were incubated at 37°C in 5% CO_2 for 96 hrs. BMMAC survival/proliferation was measured using a 3-(4,5-dimethylthylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma). Culture supernatants were removed and replaced with 100μl of 0.5 mg/ml MTT in PBS supplemented with 2% heat inactivated FBS, cultured at 37°C in 5% CO_2 for 3 hrs, lysed with 100 μl of 0.1N HCl in isopropanol, and absorbance measured at 570 nm with an optical plate reader (Thermo Labsystems).

We also assessed the ability of recombinant type I IFNs to inhibit BMMAC responsiveness to CSF-1. Freshly prepared BMMAC from wild type mice were treated with 40 ng/ml recombinant mouse CSF-1 (R&D Biosystems) and 25 ng/ml recombinant mouse IFN-α or 25 ng/ml recombinant mouse IFN-β (both from PBL Biomedical Laboratories, Piscataway, NJ). Cultures were incubated at 37°C in 5% CO_2 for 96 hrs and assessed with MTT as above.

RT-PCR

The expression of mRNA was quantified via realtime RT-PCR. Tumor tissue was homogenized in lysis buffer using a rotostator tissue homogenizer. Total RNA was isolated using an RNeasy Mini Kit and an on-column DNase treatment was preformed (Qiagen, Valencia, CA). One μg of RNA was used to generate first strand cDNA using a MMLV reverse transcription kit as per manufactures protocol (Invitrogen). PCR was carried out in 25μl reaction volumes using iQ SYBR Supermix (Bio-Rad, Hercules, CA) and amplification was carried out using the iQ iCycler system (Bio-Rad). Negative controls were preformed with water and primer specificity was assessed by melt curve analysis. All PCR reactions were preformed in duplicate and the average Ct value was used to calculate relative expression when normalized to HPRT housekeeping gene for each sample. The primers that were used have already been described in previous reports and were specific for mouse c-fms (For: 5'-GACTGGAGAGGAGAGACCAGGAC-TATG, Rev: 5'-GTGCACCAGTTGGCATAGTAAATGTAGAGGCT), CCR2 (For: 5'-GAGCCTGATCCTGCCTCTACTTGT, Rev: 5'-CCTGCATGGCCTGGTCTAAGTGC), bFGF (For: 5'-ACTCCAGTTGGTATGTGGCACTGA, Rev: 5'-AACAGTATGGCCT-TCTGTCCAGGT), PDGF-A (For: 5'-GTCCAGGTGAGGTTAGAGG, Rev: 5'-CACGGAGGAGAACAAAGAC), PDGF-B (For: 5'-TGAAATGCTGAGCGACCAC, Rev: 5'-AGCTTTCCAACTCGACTCC), PDGF-C (For: 5'-AGGTTGTCTCCTGGTC-AAGC, Rev: 5'-CCTGCGTTTCCTCTACACAC), PDGF-D (For: 5'-CCAAGGAACC-TGCTTCTGAC, Rev: 5'-CTTGGAGGGATCTCCTTGTG), MMP9 (For: 5'-GGCGTGTCTGGAGAT, Rev: 5'-AGG GTCCACCTTGTTCACC), EGF (For: 5'-

TTAGCATACTCAGCGTCACAGC, Rev: 5'-TTCCATTGGGTCAATCCG), and HPRT (For: 5'-GCTTTCCCTGGTTAAGCAGTACA, 5'-CAAACTTGTCTGGAATT-TCAAATC)(37-44).

Cell sorting

Macrophages were sorted by magnetic bead separation. Tumors were digested as above and further purified by Ficoll gradient centrifugation (LSM Lymphocyte Separation medium; MP Biomedicals, Aurora, Ohio). Single cell suspensions were resuspended in MACS buffer (PBS pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA) at a concentration of 1 X 10^7 cells per ml. The cells were then immunostained with 1μg/ml anti-F4/80 antibody (biotin; clone BM8; eBiosciences, San Diego, CA). Following immunostaining cells were washed twice with MACS buffer and incubated with 10 μl of Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA) per 10^7 total cells in 90μl MACS buffer for 15 minutes at 4°C. Cells were washed twice in MACS buffer and up to 10^8 cells were resuspended in 500μl of MACS buffer. F4/80 positive macrophages were than positively selected by being passed over an MS column attached to a MS separation magnet (Miltenyi Biotec). Purity of sorted macrophages was assessed by immunostaining with anti-CD11b (APC-Cy7 clone M1/70, eBiosciences) and performing FACS analysis. Sorted macrophages were found to be >80% in purity.

Statistical analyses

In experiments with multiple groups of mice, statistical differences between treatment groups were compared using ANOVA and Tukey's multiple means comparisons test. For comparisons between two treatment groups, Student's t-test was used. Statistical analyses were done using GraphPad software (San Diego, CA). A p value < 0.05 was considered statistically significant for these analyses.

RESULTS

Endogenous production of type I interferons suppresses tumor growth.

In a number of previous studies, it has been shown that treatment with IFN-α can therapeutically inhibit tumor growth and angiogenesis (8, 9, 45-47). However, in the present study, we wished instead to investigate the role that endogenous production of type I IFNs play in regulating tumor growth and macrophage accumulation. In a previous study, it was determined that growth of primary tumors, as well as tumor metastasis, was significantly accelerated in mice lacking type I IFN receptors(16). This study examined the growth of a human melanoma line grown in a nude mouse model. In the present study, we wished to follow-up on the original observations made by McCarty

et al and investigate mechanisms that might account for the increased tumor growth and angiogenesis observed previously in IFN-α/βR-/- mice.

Therefore, we first assessed the tumor growth kinetics in wild type 129 mice and 129 IFN-α/βR-/- mice injected with syngeneic MCA 2.1 cells. We found that the rate of tumor growth was significantly increased in IFN-α/βR-/- mice compared to wild type animals (**Figure 1**). Thus, in the MCA 2.1 tumor model as in the previously described tumor studies(16)(10), the inability of the host cells (but not the tumor cells) to respond to endogenously produced type I IFNs correlated with a significant increase in tumor growth rates.

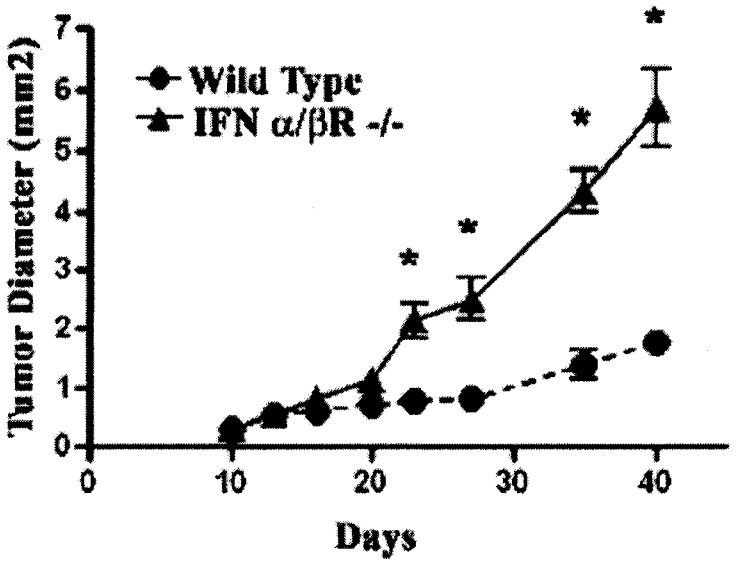

Figure 1: Tumors grow significantly faster in IFN-α/βR-/- mice. 1 X 10^6 MCA2.1 cells grown subcutaneously in wild type or IFN-α/βR-/- mice. Tumors growth was assessed by serial measurement of two tumor dimensions using calipers and the mean tumor diameter (± SEM) for each group was calculated. Tumor growth was significantly ($p < 0.05$) increased in IFN-α/βR-/- mice, compared to wild type mice, as assessed by repeated measures ANOVA. Five mice per group, mean +/- SEM. Data are representative of 3 experiments.

Endogenous levels of type I interferons suppresses tumor angiogenesis.

Given that tumors grew more rapidly in mice unable to respond to type I IFNs, we examined mechanisms that might account for this accelerated growth. First, we examined angiogenic responses in the IFN-α/βR-/- mice. We found that tumors in IFN-α/βR-/- mice had significantly higher microvessel density (MVD) than did wild type mice (**Figure 2**). This result is also in agreement with the result reported by McCarty et al, using a different tumor model in IFN-α/βR-/- mice(16).

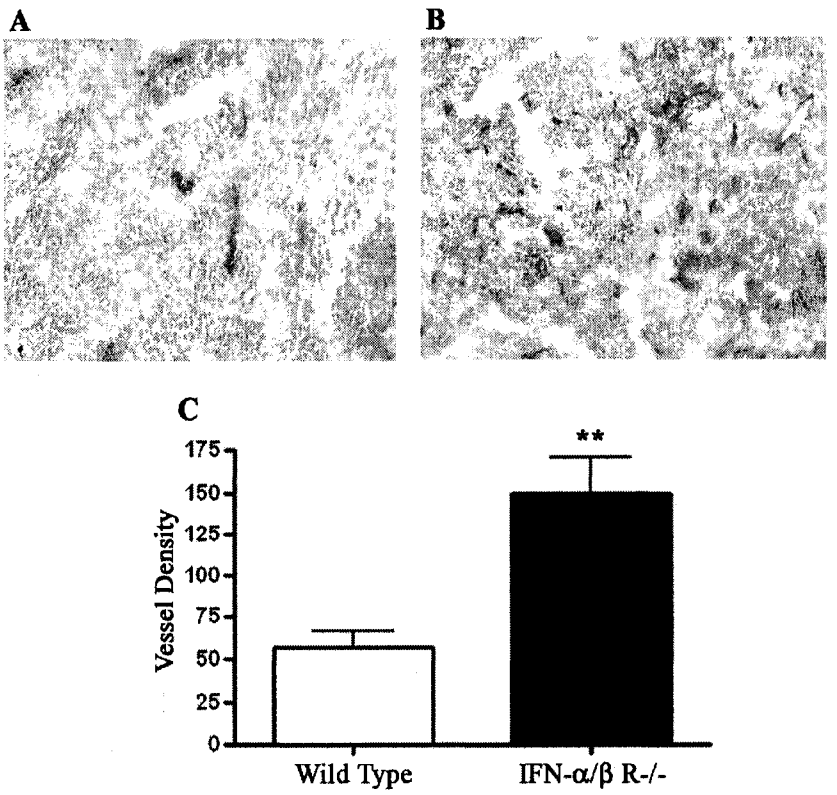

Figure 2: Comparison of the angiogenic response in MCA2.1 tumors grown in wild type versus IFN-α/βR-/- mice. IHC of tumor vasculature using CD31 mAb was carried out in 1 x 1 cm tumors and vessel number was quantified as described in methods for wild type (A) and IFN-α/βR-/- (B) mice. MCA2.1 tumors of IFN-α/βR-/- mice had a significantly higher MVD (C). Five mice per group, mean +/- SEM. **$p<0.001$ by student's t-test. Data are representative of 3 experiments.

Production of angiogenic cytokines by tumors in wild type and IFN-a/bR -/- mice.

One mechanism by which type I IFNs can inhibit angiogenesis is by inhibiting the release of pro-angiogenic cytokines, including IL-1β, VEGF, TNF-α, and IL-8. Therefore, we investigated whether concentrations of pro-angiogenic cytokines were significantly

different in tumor tissues from tumors growing in wild type mice versus IFN-α/βR-/- mice. To address this, tumor explant cultures from 1 X 1 cm tumors of wild type and IFN-α/βR-/- mice were placed *ex vivo* for 24 h and the concentrations of VEGF, TNF-α, IL-1β, and KC (mouse homolog to IL-8) in supernatants was determined by ELISA. We found that there were no significant differences in the concentrations in any of these 4 cytokines in supernatants from wild type versus IFN-α/βR-/- mice (**Figure 3**).

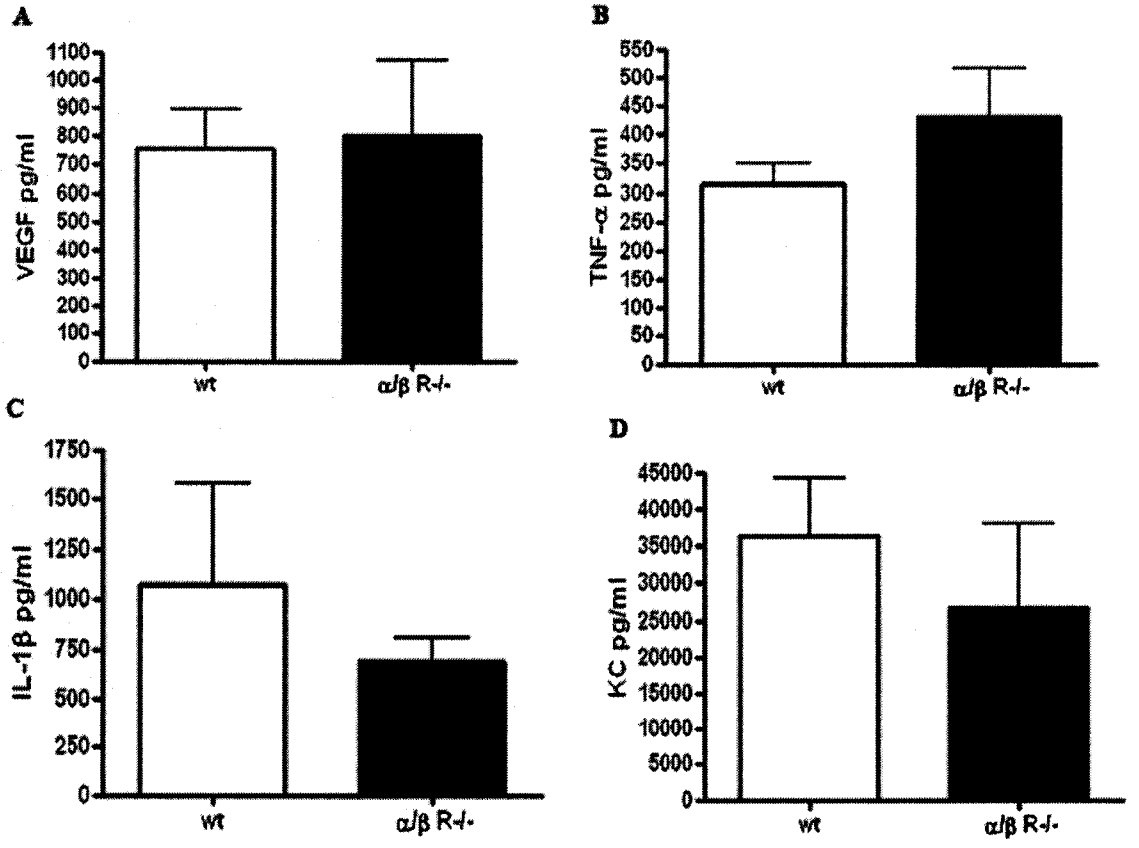

Figure 3: Analysis of the levels of pro-angiogenic factors of MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. VEGF (A), TNF-α (B), IL-1β (C), and KC (D) were measured in culture supernatants after incubating 30 mg of tumor tissue for 24 hrs in 1 ml of media. No statistical difference was found between any of the samples. Five mice per group, mean +/- SEM. Data are representative of 2 experiments.

To further address the issue, we also assessed the expression of genes known to be associated with angiogenesis directly in tumor tissues. Total RNA was extracted from tumor tissues of wild type and IFN-α/βR-/- mice (n = 4 per group) and subjected to amplification by quantitative real-time PCR. Primers were designed for amplification of bFGF, PDGFA-D, MMP-9, and EGF, based on published primer sequences (**Table 1**) (37-39, 43, 44). Using this analysis, we did not detect significant differences in the level of expression in any of these 5 genes, when RNA extracted from tumors of wild type and IFN-α/βR-/- were compared (**Table 1**). We conclude therefore that differences in intratumoral expression of bFGF, PDGF, MMP-9, and EGF cannot account for the increased tumor angiogenesis noted in tumors growing in the IFN-α/βR-/- mice.

Gene	Primers (5'to 3')	Fold Induction	
		Lysate	F4/80 Sort
bFGF	For: ACTCCAGTTGGTATGTGGCACTGA Rev: AACAGTATGGCCTTCTGTCCAGGT	-1.13	ND
PDGF-A	For: GTCCAGGTGAGGTTAGAGG Rev: CACGGAGGAGAACAAAGAC	-1.74	-1.03
PDGF-B	For: TGAAATGCTGAGCGACCAC Rev: AGCTTTCCAACTCGACTCC	1.03	-1.04
PDGF-C	For: AGGTTGTCTCCTGGTCAAGC Rev: CCTGCGTTTCCTCTACACAC	1.23	1.19
PDGF-D	For: CCAAGGAACCTGCTTCTGAC Rev: CTTGGAGGGATCTCCTTGTG	1.58	1.68
EGF	For: TTAGCATACTCAGCGTCACAGC Rev: TTCCATCTGGGTCAATCCG	1.0	1.19
MMP-9	For: GGCGTGTCTGGAGAT Rev: AGG GTCCACCTTGTTCACC	1.62	ND

Table 1: There was not a significant change in the mRNA expression of the pro-agngiogenic factors bFGF, PDGF A-D, EGF or MMP-9 between whole tumor lysate or F4/80 sorted macrophages between MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. Quantitative PCR analysis was performed in duplicate on 4 mice in each genotype or pooled samples from the 4 mice in each genotype. The mRNA level in each sample was then normalized to its corresponding HPRT level. Realtime RT-PCR reaction was preformed as described in methods. A significant change in mRNA expression was set at a 2 fold difference. Data is representative of 1 experiment.

Macrophage infiltration is significantly increased in IFN-α/βR-/- mice.

Since macrophages and their cytokines are one of the critical factors regulating angiogenic responses in tumors, we next assessed and compared the density of TAMs in tumors from wild type and IFN-α/βR-/- mice. By flow cytometry, we found that the number of F4/80 positive macrophages was significantly increased ($p<0.05$) in IFN-α/βR-/- mice compared to wild type animals (**Figure 4**). This finding was confirmed

using immunohistochemistry with the F4/80 antibody (**Figure 5A and 5B**). Immunohistochemistry using the macrophage antibody CD68 also corroborated this finding (**Figure 5C and 5D**).

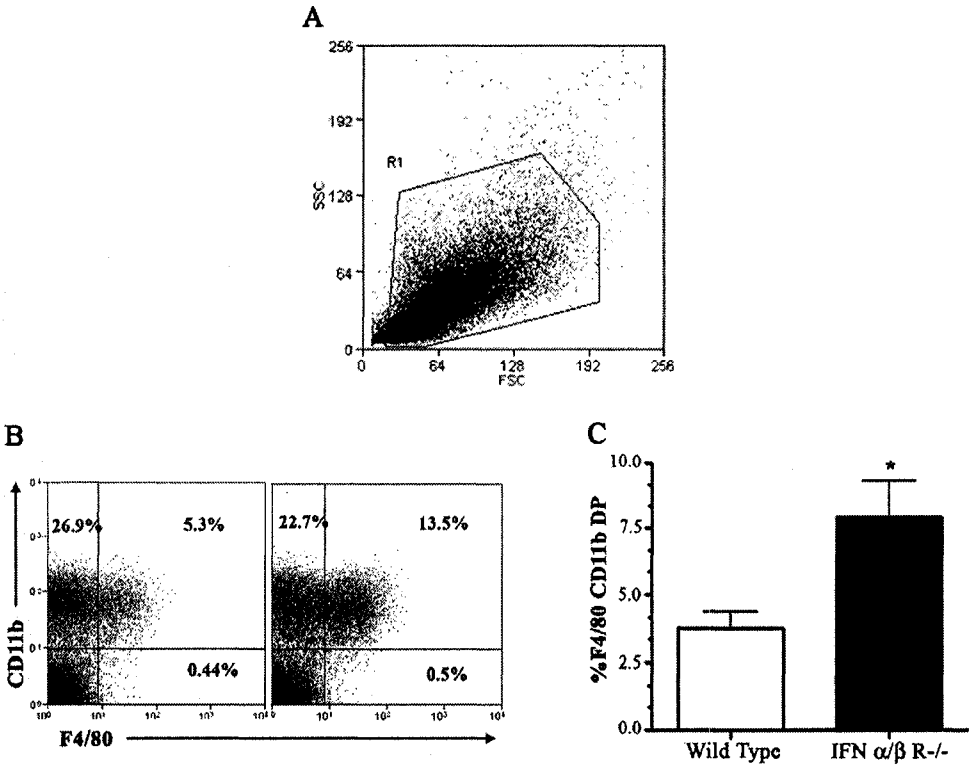

Figure 4: Quantification of macrophage infiltrate in MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. Example of FSC X SSC gating of MCA2.1 tumor digest (A). Flow cytometry was used to quantify F480/CD11b double positive macrophages (B). Tumors derived in IFN-α/βR-/- mice had a significant increase in F480/CD11b double positive macrophages (C). Five mice per group, mean +/- SEM. * $p<0.05$ by student's t-test. Data are representative of 3 experiments.

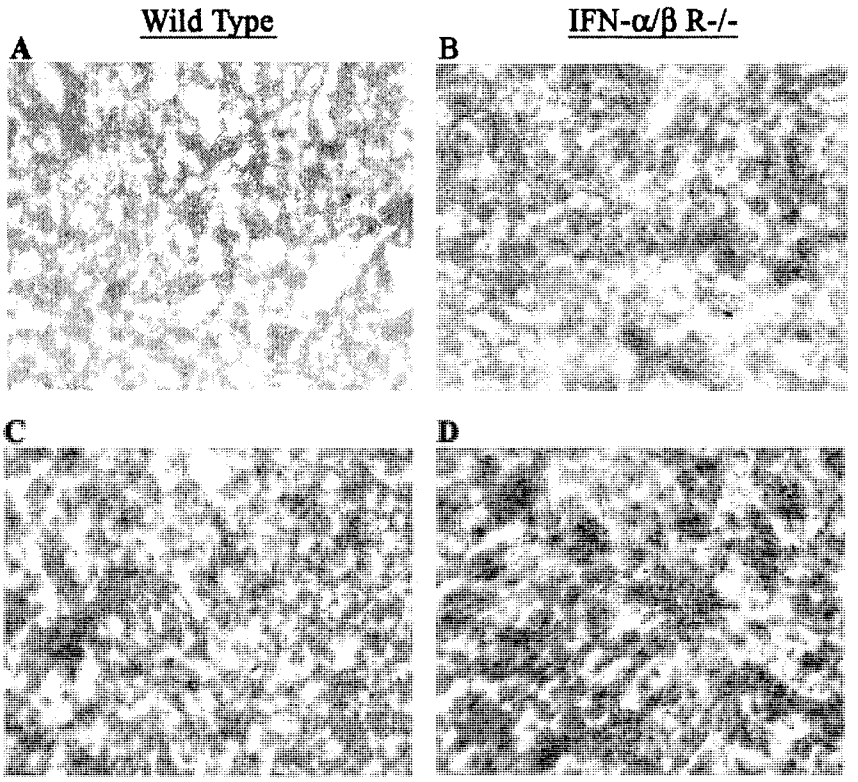

Figure 5: IHC staining for macrophages within MCA2.1 tumors established in wild type or IFN-α/βR-/- mice. There is increased staining of tumors grown in IFN-α/βR-/- mice for the macrophage specific markers F4/80 (A&B) and CD68 (C&D). These images are representative of findings in tumor tissue of 5 different mice per group.

In addition, the numbers of other tumor infiltrating leukocytes was also assessed and compared between wild type and IFN-α/βR-/- mice using flow cytometry. When the numbers of infiltration $CD8^+$ T cells was assessed, significant differences were not noted (**Figure 6D**). We also used flow cytometry to assess the numbers of $CD11c^+/CD11b^+$ dendritic cells and again significant differences were not noted (**Figure 6C**). Finally, the number of $CD11b^+/Gr\text{-}1^+$ neutrophils and $NKG2D^+$ NK cells was also not significantly different between wild type and IFN-α/βR-/- mice (**Figure 6A and 6B**). Thus, the only

significant difference between wild type and IFN-α/βR-/- mice in terms of leukocyte infiltration was found in tumor infiltrating macrophages.

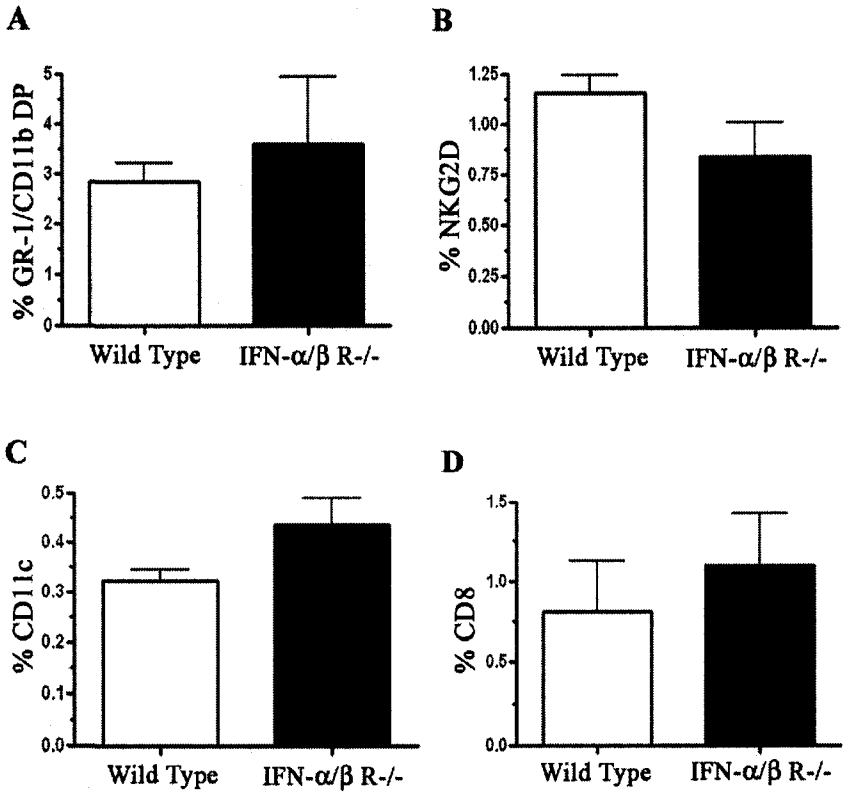

Figure 6: Flow cytometric analysis of other immune infiltrating cells in MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. Tumor digest was gated for FSC X SSC as depicted in Figure 4. There was no significant difference in infiltrating Gr-1/CD11b double positive neutrophils (A), NKG2D positive NK cells (B), CD11c positive dendritic cells (C), or CD8 positive T cells between tumors of wild type and IFN-α/βR-/- mice. Five mice per group, mean +/- SEM. Data in A, B, and C are representative of 2 experiments, and data in D is representative of 1 experiment.

Increased expression of CCL2 or CSF-1 or their receptors are not responsible for increased macrophage numbers in tumors of IFN-α/βR-/- mice

We sought next to elucidate a mechanism that might account for the increased numbers of macrophages found within tumors of IFN-α/βR-/- mice. CCL2 is a key chemokine regulating the recruitment of monocytes into tumor tissues, followed by their differentiation into macrophages(24, 25). Therefore, we measured CCL2 concentrations in tumor tissues, using in tumor conditioned media. The CCL2 concentration (**Figure 7a**) was not significantly different. In addition, when CCL2 concentrations in lysates prepared from freshly collected tumor tissues were measured we again did not detect significant differences.

Therefore, we next assessed the concentration of CSF-1 in tumor tissues, since CSF-1 is a major positive regulator of macrophage survival and differentiation in tissues(26). When CSF-1 concentrations in tumor lysates or short-term tumor conditioned medium were assessed, we again did not observe significant differences between tumors in wild type and IFN-α/βR-/- mice (**Figure 7b**).

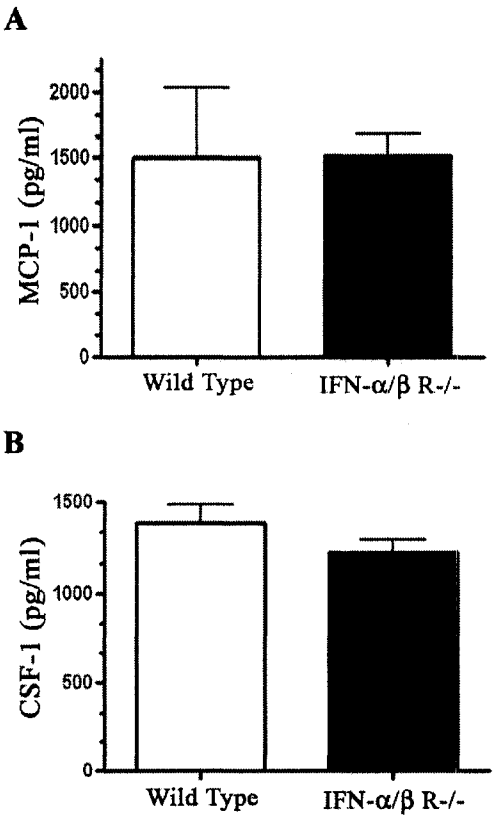

Figure 7: Analysis of CCL2 and CSF-1 levels of MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. CCL2 was measured in culture supernatants after incubating 30 mg of tumor tissue for 24 hrs in 1 ml of media (A). CSF-1 was measured in protein lysate prepared from tumor tissues (B). Five mice per group, mean +/- SEM. Data are representative of 2 experiments.

We also wanted to understand if macrophages from tumors of wt and IFN-α/βR-/- mice could have different expression of receptors that mitigate their response to CSF-1 and CCL2. To assess this we purified macrophages from tumors grown in wt and IFN-α/βR-/- mice and performed quantitative RT-PCR for the CSF-1 receptor (c-fms) and for the CCL2 receptor (CCR2). We found that transcription of the c-fms and CCR2 genes was not significantly different between wild type and IFN-α/βR-/- mice (**Table 2**). Taken

together, these results suggest that tumor production of CCL2 or CSF-1 did not account for the observed differences in TAMs in tumor of wild type versus IFN-α/βR-/- mice.

Gene	Primers (5'to 3')	Fold Induction
c-fms	For: GACTGGAGAGGAGAGACCAGGACTATG Rev:GTGCACCAGTTGGCATAGTAAATGTAGAGGCT	-1.04
CCR2	For: GAGCCTGATCCTGCCTCTACTTGT Rev: CCTGCATGGCCTGGTCTAAGTGC	1.71

Table 2: There is not a significant change in the mRNA expression of c-fms or CCR2 in F4/80 sorted macrophages between MCA2.1 tumors grown in wild type and IFN-α/βR-/- mice. Quantitative PCR analysis was performed in duplicate on 4 mice in each genotype or pooled samples from the 4 mice in each genotype. The mRNA level in each sample was then normalized to its corresponding HPRT level. Realtime RT-PCR reaction was preformed as described in methods. A significant change in mRNA expression was set at a 2 fold difference. Data is representative of 1 experiment.

Monocyte migration in wild type and IFN-α/βR-/- mice.

The increase in TAMs in tumors of IFN-α/βR-/- mice could also result from increased emigration of monocytes into the tumors from the bloodstream. To indirectly assess the migratory potential of macrophages from wild type and IFN-α/βR-/- mice, we tested the transwell migration of peritoneal macrophages from wild type and IFN-α/βR-/-mice. Although peritoneal macrophages from both groups of mice migrated towards a CCL2 gradient, there was no difference in the responsiveness of wt or IFN-α/βR-/- PECs (**Figure 8**). Moreover, tumor conditioned medium also did not elicit migration differences in wild type and IFN-α/βR-/- mouse macrophages. These results indicated

that macrophages from wild type and IFN-α/βR-/- mice were equally responsive to chemokine mediated migratory stimuli.

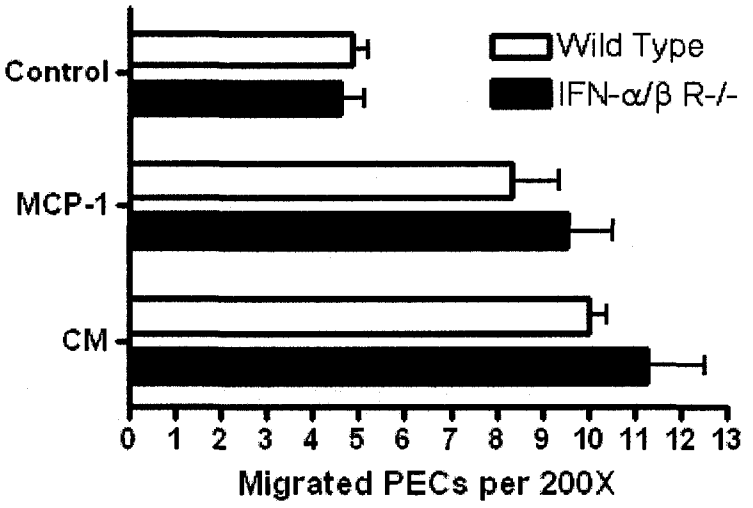

Figure 8: Assessment of the ability of macrophages from a wt or IFN-α/βR-/- mouse to migrate across a transwell. The was no significant difference in the ability of wt or IFN-α/βR-/- PECs to migrate toward 100ng/ml CCL2 or tumor cell conditioned media. Three replicates per group, mean +/- SEM. Data are representative of 2 experiments.

Type I IFNs inhibit macrophage responsiveness to CSF-1.

A previous report described the observation that type I IFNs appeared to limit the responsiveness of bone marrow macrophages to the proliferative and growth-promoting effects of CSF-1(29, 30). However, those effects had never been assessed previously in a tumor model, nor had the specific effects of recombinant IFN-α or IFN-β been assessed separately. Therefore, we investigated these effects in our tumor system.

First, we assessed whether the MCA 2.1 tumor cells produced type I IFNs. By ELISA, we found levels of IFN-α to be less than 5 ng/ml in supernatants of MCA2.1 tumor tissues cultured *ex vivo*. The concentrations of IFN-β were below the limit of detection by ELISA. However, by RT-PCR we were able to detect low levels of expression of both IFN-α and IFN-β in tumor tissues grown in wild type mice.

Next, we set up *in vitro* assays to assess the effects of the low levels of type I IFNs produced by tumor cells on macrophage proliferation and survival. Bone marrow adherent cells were isolated from wild type or IFN-α/βR-/- mice by brief adherence to tissue culture plastic, then the cells were incubated with serial dilutions of supernatants from cultured MCA 2.1 tumor cells. Using this technique, we found that after 96h in culture that > 80% of the adherent cells were $CD11b^+/F480^+$ macrophages. Therefore, we incubated adherent bone marrow macrophages with tumor cell conditioned media and quantitated the number of viable cells using MTT assay, as described in Methods. We found there were significantly fewer ($p<0.0001$) viable macrophages in cultures derived from wild type mice compared to IFN-α/βR-/- mice (**Figure 9**). To determine the role of endogenous CSF-1 produced by the MCA 2.1 tumor cells in inducing macrophage proliferation and differentiation in this assay, we used a neutralizing antibody to deplete CSF-1 from tumor supernatants (**Figure 9**). This treatment abrogated the responsive effect of conditioned to macrophages in culture, indicating that the major stimulatory factor present in the supernatants was in fact CSF-1.

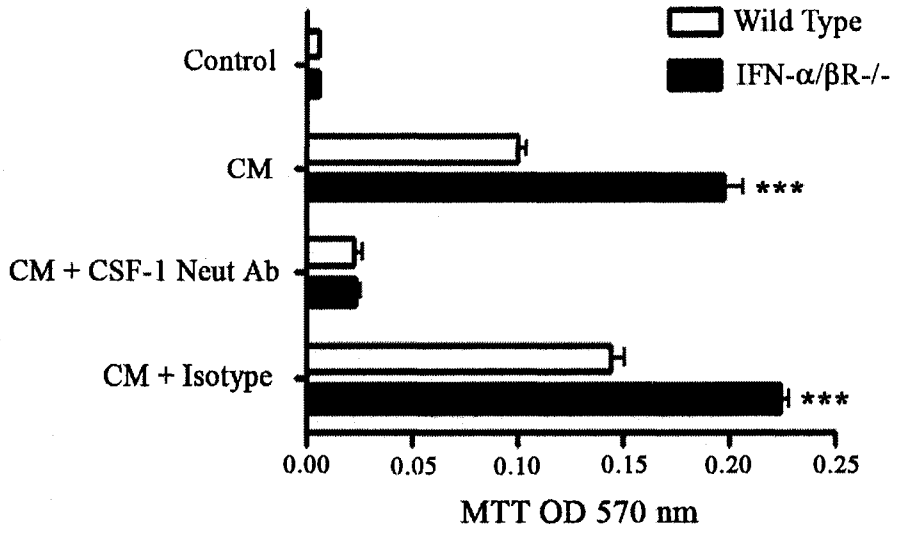

Figure 9: Comparison of wild type versus IFN-α/βR-/- bone marrow macrophage (BMMAC) responsiveness to CSF-1 in conditioned media. BMMAC were cultured for 96 hrs in the presence of MCA2.1 CM with or without 50 ng/ml CSF-1 neutralizing Ab, BMMAC numbers were measured via MTT assay. Four replicates per group, mean +/- SEM. ***$p<0.0001$ by student's t-test. Data are representative of 3 experiments.

Finally, we also used the macrophage *in vitro* assay to directly assess and compare the inhibitory effects of recombinant murine IFN-α and IFN-β on macrophage responsiveness to recombinant CSF-1. The degree of macrophage proliferation induced by recombinant CSF-1 was similar in wild type and IFN-α/βR-/- bone marrow cells **(Figure 10)**. Incubation of wild type macrophage cultures with recombinant CSF-1 (25 ng/ml) and recombinant IFN-α (25 ng/ml) resulted in 67% inhibition of macrophage survival. In addition, incubation with recombinant IFN-β (25 ng/ml) also resulted in 87% inhibition of macrophage survival. However, the inhibition elicited by IFN-α or IFN-β was not observed in macrophage cultures from IFN-α/βR-/- mice. These results indicate

therefore that low concentrations of type I IFNs (both IFN-α and IFN-β) are able to interfere with the macrophage stimulatory effects of CSF-1.

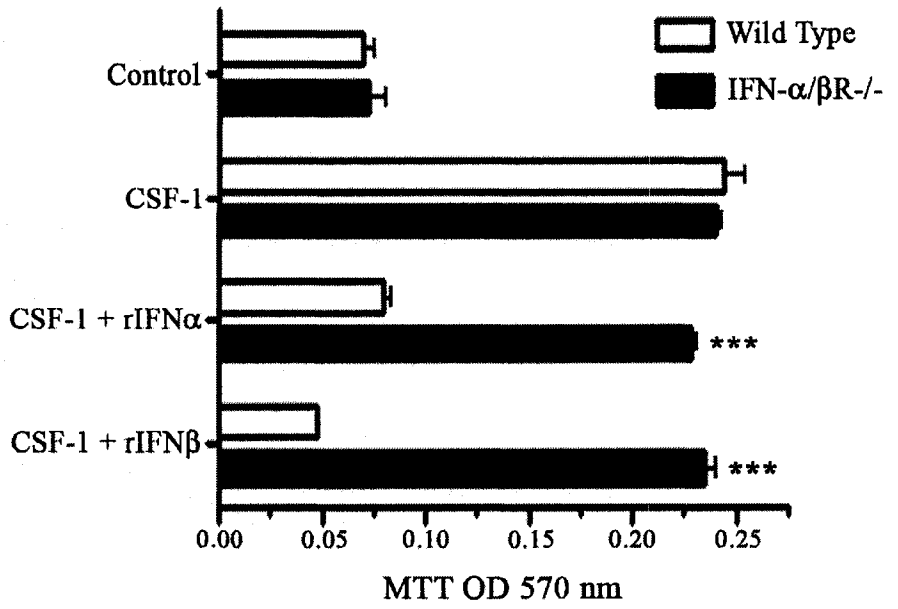

Figure 10: Comparison of wild type versus IFN-α/βR-/- bone marrow macrophage (BMMAC) responsiveness to CSF-1 in the presence of type I interferons. BMMAC were cultured for 96 hrs in the presence of recombinant CSF-1 (40 ng/ml) with recombinant IFNα (25 ng/ml) or IFNβ (25 ng/ml). BMMAC numbers were measured via MTT assay (B). Four replicates per group, mean +/- SEM. ***p<0.0001 by student's t-test. Data are representative of 3 experiments.

DISCUSSION

TAMs are beginning to be recognized as important players in tumor progression. It is thought that the cytokine milieu (particularly IL-4, IL-10, and IL-13) within the tumor

environment is responsible for driving TAMs into the "alternatively activated", pro-tumor phenotype(48, 49). One mechanism by which TAMs can promote tumor progression is by enhancing the tumor's angiogenic responses. Often implicated in this pro-angiogenic response is the ability of macrophages to produce VEGF, IL-8, bFGF, angiopoietin, and others(21, 50). We have shown that tumors grown in IFN-α/βR-/- mice have approximately a 2 fold increase in the number of TAMs (Figure 4). We suggest that it is this increase in TAMs that is responsible for the increased tumor growth and angiogenesis in the IFN-α/βR-/- mice (Figures 1 and 2). We found that low levels of type I IFNs spontaneously released by tumor cells were sufficient to inhibit the macrophage stimulatory properties of CSF-1. Thus, endogenously produced type I IFNs appear to be important negative regulators of the generation of TAMs in tumors.

With the realization of the importance of TAMs on tumor progression, they are under intense investigation as a therapeutic target. Studies are underway to understand if the depletion or genetic modification of TAMs can inhibit tumor progression and angiogenesis(39, 51, 52). With the understanding of the influence TAMs can have on tumor growth, it is important to study mechanisms which may inhibit their differentiation and survivability. We show here that the endogenous levels of type I IFNs in tumor cell supernatants can inhibit the differentiation and survivability of macrophages (Figure 9). We further show that this inhibition is a result of the ability of type I interferons to inhibit macrophage responsiveness to CSF-1 (Figures 10). This finding is similar to that of Hamilton et al, who showed that inhibition of LPS and TNF-α on bone marrow macrophage proliferation is largely the result of such treatments to induce type I IFN

production and subsequent inhibition of CSF-1 responsiveness(29, 30). Similar to our *in vivo* findings, Listeria experimentation using IFN-α/βR-/- mice showed an increase in macrophage numbers in the spleen and liver of infected IFN-α/βR-/- versus infected wt controls(53).

Previous work has shown that endogenous levels of type I IFNs can accelerate tumor growth, metastasis, and angiogenesis(16). With the use of a syngeneic tumor model and IFN-α/βR-/- mice we validate the observation that endogenous levels of type I IFNs are important in suppressing the tumors angiogenic response and thus tumor growth. We go on to show that the immune response to the tumor is markedly different in the IFN-α/βR-/- mice. IFN-α/βR-/- mice respond to the tumor with a significantly higher number of macrophages.

The findings herein, show that endogenous levels of type I IFNs can inhibit tumor angiogenesis and growth. We propose that this response is a result of an increase in TAM numbers in tumors grown in IFN-α/βR-/- mice and an increase in TAM induced angiogenesis. We further show that the inability of IFN-α/βR-/- macrophages to respond to type I IFNs releases them from the inability to respond to CSF-1 resulting in increase macrophage differentiation or survivability. These finding are important in that they describe a new mechanism by which type I IFNs may be responsible for inhibiting tumor progression and angiogenesis.

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Chapter Three

The Effects of Apoptotic Tumor Cell Engulfment on Macrophage VEGF Production

ABSTRACT

Tumor-associated macrophages (TAMs) play a deleterious role in tumors by promoting tumor progression, metastasis, and tumor angiogenesis. However, it is not well understood how tumors may trigger TAMs to produce factors that stimulate blood vessel growth, such as vascular endothelial cell growth factor (VEGF). Using *in vitro* experimentation we tested if apoptotic tumor cells could stimulate macrophage VEGF production. We further attempted to ascertain the importance of the expression of phosphatidylserine (PS), a molecule associated with apoptosis, in regulating macrophage secretion of VEGF. Through *in vitro* experimentation, we found that apoptotic tumor cells can stimulate macrophage production of VEGF. This induction of VEGF can be repeated using different apoptotic tumor cell targets and with different mouse macrophage sources. We can also provide evidence that the physical engulfment of the

apoptotic targets is necessary for macrophage VEGF production and that this process is most likely reliant on the expression of PS on the apoptotic cell target. These finding suggest that apoptotic cell engulfment is important in macrophage VEGF production. It is an important finding since most traditional cancer therapies induce tumor cell apoptosis while leaving TAMs unharmed, possibly allowing macrophages to produce a microenvironment that is conducive to tumor bed repair and reoccurrence.

INTRODUCTION

Phosphatidylserine (PS) is an aminophospholipid that comprises a substantial portion of the inner leaflet of the plasma membrane bilayer. Upon induction of apoptosis, PS translocates from the inner to the outer leaflet of the cell membrane bilayer. This transition in PS expression occurs due to the combined activation of a putative scramblase enzyme and inhibition of an aminophospholipid transferase enzyme that normally keeps PS expressed on the inner leaflet of the cell membrane(1, 2). Surface exposed PS is a potent signal for macrophages and other cell types to engulf and remove apoptotic cells(3, 4). When macrophages encounter surface-expressed PS, they are also triggered to produce anti-inflammatory cytokines (eg, TGF-β, IL-10)(3, 5, 6). Interestingly, PS expression is disregulated in many tumors(7-9). For example, some tumor cells spontaneously express PS on their outer membrane leaflet even when not undergoing apoptosis, while other tumors fail to externalize PS even when apoptotic(4, 7-13). The consequences of disregulated PS on promotion of tumor growth and angiogenesis are largely unexplored.

Stimulation of tumor angiogenesis is one of the major means by which TAMs are thought to promote tumor growth(14, 15). The degree of tumor angiogenesis is often closely linked to the density of macrophage infiltration. Tumors with higher TAM density also produce higher levels of VEGF, a major stimulus for blood vessel growth(16-18). It has been speculated that the hypoxic tumor environment is responsible for driving TAM

VEGF production(15, 19, 20). It had been recently reported that the clearance of apoptotic cells by macrophages could drive macrophage production of VEGF(21). We feel that macrophage clearance of apoptotic cells may be an alternative mechanism to drive TAM VEGF production, other than hypoxia initiation. The studies within this chapter will provide a greater understanding of the mechanism that induces macrophage VEGF production following apoptotic cell clearance and provide further context for its importance in tumor biology.

MATERIALS AND METHODS

Animals

C57Bl6 and outbreed ICR mice were purchased from Taconic (Germantown, NY). Peritoneal macrophages were elicited by the IP administration of 1.5 mls of 4% Brewers Thioglycollate Medium (Sigma Chemical Co, St Louis, MO). All procedures with animals were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Cell culture

The human leukemia T cell line Jurkat and human myelomonoblastic PLB 985 cells (both gifts from Peter Henson) were maintained in RPMI 1640 with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). The A20 cell line is a BALB/c B cell lymphoma line and EL4 is a T cell lymphoma line from a C57Bl6 background. A20 and EL4 cells were maintained in modified Eagle's complete medium supplemented as above. All cells were incubated at 37°C in 5% CO2. To assure mycoplasma-free conditions, cells were routinely tested by MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA).

Induction of apoptosis and necrosis

Apoptosis of Jurkat cells and PLB 985 was induced by UV exposure (405 nM) for 10 mins, followed by a 3 hr incubation at 37°C in 5% CO2. Apoptosis of EL4 and A20 cell was induced by UV exposure for 15 mins, followed by a 6 hr incubation at 37°C in 5% CO2. Necrosis of all cells was induced by 2 cycles of rapid freezing at -80°C and slow thawing at RT. Cells were than resuspended in Dulbecco's modified Eagle's medium at a concentration of $1x10^7$/ml. The induction of apoptosis was assessed by quantifying the loss of PS asymmetry and PS externalization by an annexin-V-fitc binding assay (BD biosciences, Bedford, MA). The annexin-V assay was preformed exactly as described in

the manufacture's protocol. Evaluation of secondary necrosis was assessed by both the use of PI staining and trypan blue positivity. Apoptotic cells used for these experiments showed >70% annexin-V+/PI- staining. The induction of necrosis was considered adequate if the proportion of Annexin-V+/PI+ cells was > 70%. Since PLB 985 does not externalize PS while undergoing apoptosis(4, 10, 22), apoptosis was assessed by nuclear condensation, membrane blebbing, and changes in forward and side-scatter characteristics while staining with PI and trypan blue was < 10%. Flow cytometry was performed using Cyan MLE cytometer (Dako-Cytomation, Ft Collins, CO). Data analysis was carried out on Summit software.

In vitro peritoneal macrophage assays

Three days following Thioglycollate stimulation, mice were euthanized by CO2 asphyxiation and peritoneal exudate cells (PECs) were harvested by IP lavage with 7mls of ice cold HBSS supplemented with 2% FBS. PECs were washed twice with HBSS/FBS and resuspended at $2x10^6$ cells/ml in DMEM with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin. 100μl of cell suspension was added to the wells of a 96 well flat bottomed plate. The PECs were incubated for 2 hrs at 37°C in 5% CO2 and wash gently twice with 200μl of prewarmed HBSS to remove non-adherent cells. 200 μl of fresh DMEM was than added and the PECs were allowed to rest for 36 hrs at 37°C in 5% CO2. Target cells ($1x10^6$ in 100μl DMEM) were than fed to the PECs and incubated

for 1 hr at 37°C in 5% CO2. Unphagocytozed target cells we removed by gently washing twice with 200µl of pre-warmed HBSS. PECs were than fed with 125µl of fresh DMEM and incubated for 36 hrs at 37°C in 5% CO2. Supernatants were harvested and stored at -70°C until analysis. Each treatment group was carried out in quadruplicate wells.

Amiloride Treatment

In some experiments amiloride (Sigma Chemical Co, St Louis, MO) was used to inhibit macrophage phagocytosis of apoptotic cells. Amiloride was solubalized in DMSO and diluted to the indicated concentrations in DMEM. Following 15 minutes of amiloride treatment the cells were washed twice with HBSS and the in vitro phagocytosis assay was preformed as described above.

ELISA assays

Supernatants were than harvested and frozen at -70°C until ELISA analysis was preformed. The VEGF ELISA kit was purchased form R&D Biosystems (Minneapolis, MN). ELISAs were then preformed as per manufacture's instructions. ELISAs were developed with tetramethylbenzidine substrate (Sigma Chemical Co, St Louis, MO) and absorbance measured at 450 nm with an optical plate reader (Thermo Labsystems, Waltham, MA).

Proliferation assay

PEC numbers were measured using a 3-(4,5-dimethylthylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma). Culture supernatants were removed and replaced with 100μl of 0.5 mg/ml MTT in PBS supplemented with 2% heat inactivated FBS, cultured at 37°C in 5% CO_2 for 3 hrs, lysed with 100 μl of 0.1N HCl in isopropanol, and absorbance measured at 570 nm with an optical plate reader (Thermo Labsystems).

Statistical analyses

For comparisons between two treatment groups, Student's t-test was used. Statistical analyses were done using GraphPad software (San Diego, CA). A p value < 0.05 was considered statistically significant for these analyses.

RESULTS

Apoptotic tumor cells induce macrophage VEGF production

We sought out to determine the effect that apoptotic cell engulfment would have on macrophage VEGF production. From an outbred ICR mouse $2x10^5$ thioglycollate elicited peritoneal exudate cells (PECs) were plated into a 96 well plate and allowed to rest for 36 hours. Apoptotic Jurkat cells were prepared by treating with 10 minutes of UV irradiation, followed by 3 hours of incubation at 37°C in 5% CO2. Necrosis of Jurkat cells was induced by 2 cycles of rapid freezing at -80°C and slow thawing at RT. Adequate induction of apoptosis or necrosis was assessed by staining with annexin-V and PI. Apoptosis induction was considered adequate if the proportion of Annexin-V+/PI- cells was greater than 70% and the induction of necrosis was considered adequate if the proportion of Annexin-V+/PI+ cells was greater than 70% **(Figure 1).**

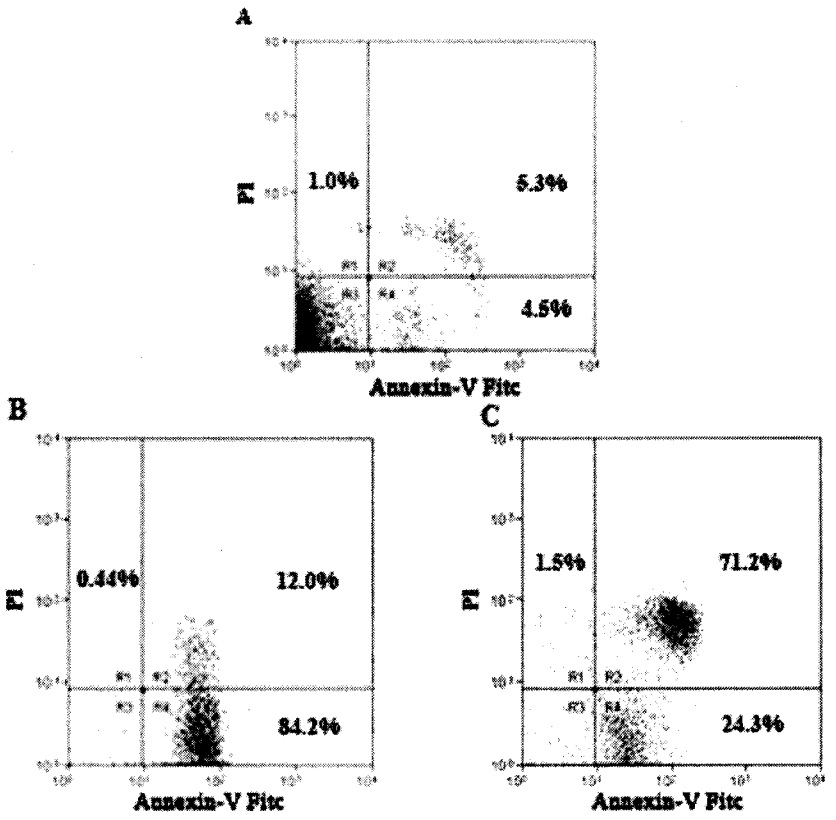

Figure 1: Annexin-V/PI staining of Jurkat cells following induction of apoptosis or necrosis. Live Jurkat cells were stained with annexin-V and PI (A). Apoptosis of Jurkat cells was induced by 10 minutes of UV irradiation, followed by 3 hours of incubation at 37°C in 5% CO2 (B). Necrosis of Jurakat cells was induced by 2 cycles of rapid freezing at -80°C and slow thawing at RT (C).

PECs were treated with target cells at a ratio of 1:5 (effector:target) for one hour and unphagocytosed target cells were washed away. After a 36 hour incubation a mouse VEGF ELISA was preformed on culture supernatants. We found that apoptotic Jurkat target cells could induce a significant increase in PEC VEGF production **(Figure 2).** Importantly, we determined that only apoptotic cells induced this response and VEGF production was not induced by live or necrotic Jurkat cells.

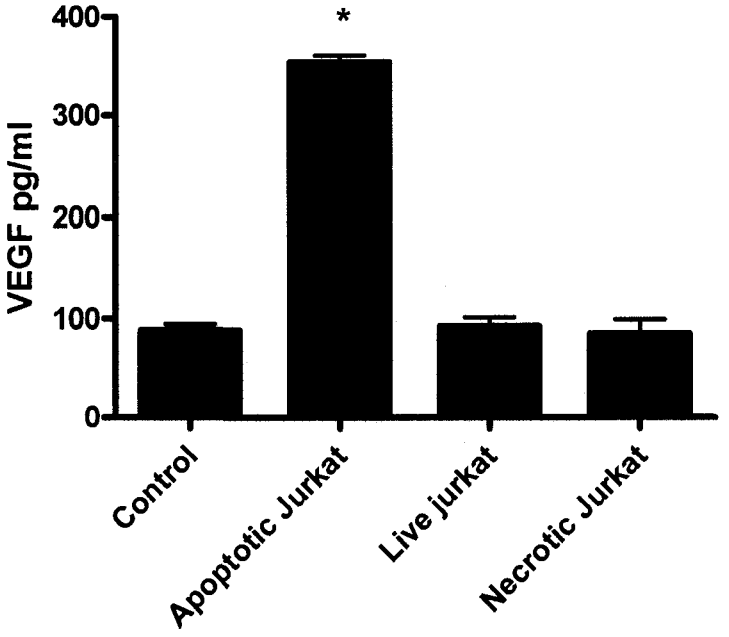

Figure 2: Apoptotic cells induce macrophage production of VEGF. VEGF production by PECs after being stimulated with either apoptotic, live, or necrotic Jurkat cells. Four replicates per group, mean +/- SEM. *p<0.0001 by student's t-test. Data are representative of 3 experiments.

Macrophage VEGF response is not specific to a single macrophage source or target population

We tested whether our observation was specific to a single macrophage source or target cell population. In addition to experimenting with PECs from an outbred ICR mouse, we also tested PECs from the inbred C57BL6 mouse strain. We found the PECs from both stains of mice could produce significantly more VEGF when treated with apoptotic Jurkat target cells **(Figure 3).** We also found that this response was not specific to a single

apoptotic target cell population. In addition to finding VEGF induction by the human Jurkat cell line, we also found that apoptotic mouse tumor lines EL4 and A20 could produce a similar induction of VEGF **(Figure 4).**

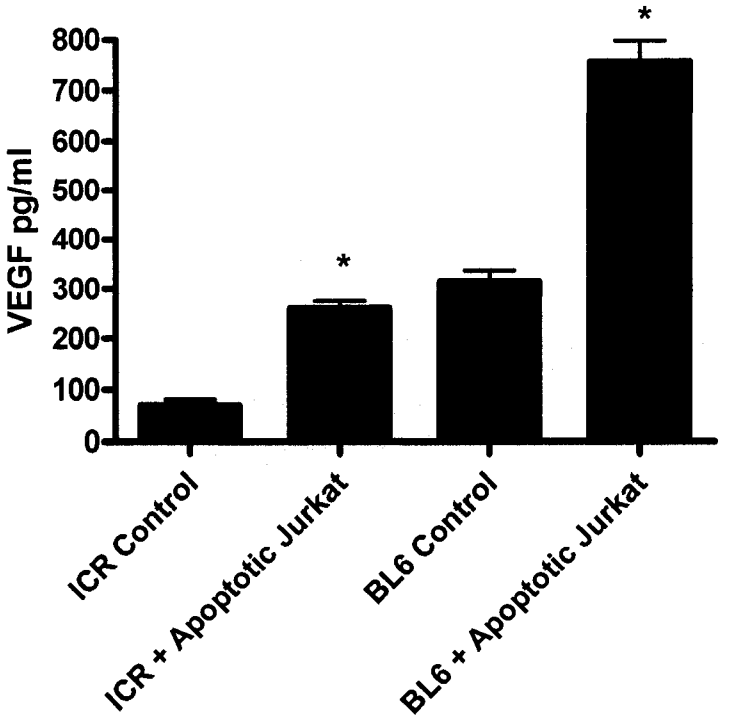

Figure 3: PECs from multiple sources can be stimulated with apoptotic cells to produce VEGF. Thioglycollate elicited PECs from ICR and C57BL6 mice were treated with or without apoptotic Jurkat cells and VEGF was measured in culture supernatants after a 36 hour incubation. Four replicates per group, mean +/- SEM. ***$p<0.0001$ by student's t-test. Data are representative of 2 experiments.

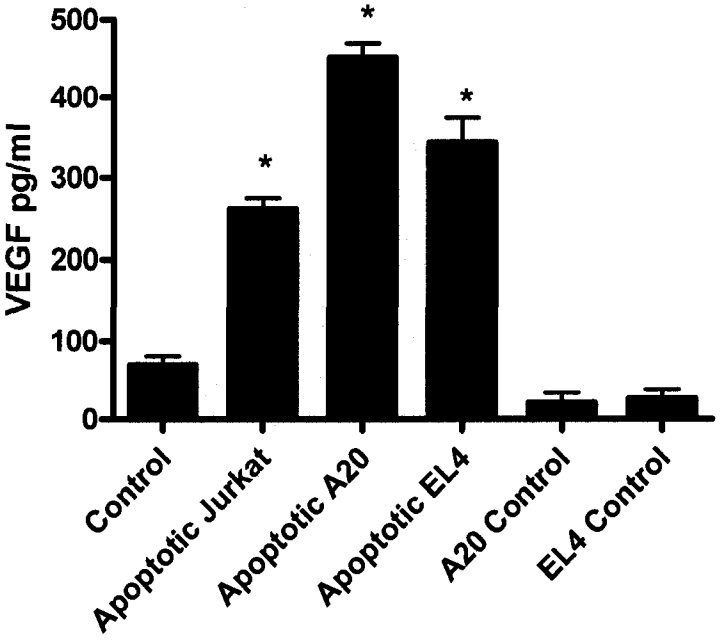

Figure 4: Multiple apoptotic target tumor cells can induce macrophage VEGF production. ICR PECs were treated with either apoptotic Jurkat cells, EL4, or A20 tumor cells for 36 hours and VEGF was measured in culture supernatants. Four replicates per group, mean +/- SEM. ***$p<0.0001$ by student's t-test. Data are representative of 3 experiments.

Apoptotic cell engulfment is necessary for macrophage VEGF response

Since it is well known that macrophages are a very important cell type in the removal of apoptotic cells, we wanted to test whether it was the act of macrophage engulfment and clearance of the apoptotic cell that stimulated VEGF release or was just having contact with the apoptotic cells enough of a stimuli. Amiloride is an inhibitor of the Na^+/H^+ antiporter and a selective inhibitor of the process of macropinocytosis(23-25). Since the process of phagocytosis of apoptotic cells has been shown to be an amiloride-sensitive

endocytic process(26), we used amiloride to inhibit phagocytosis of apoptotic cells in our system. We found that amiloride can inhibit the ability of apoptotic cells to stimulate macrophage VEGF secretion in a dose dependant matter **(Figure 5).** An MTT assay was preformed to ensure that macrophage cell death was not responsible for the decrease in VEGF production and a DMSO vehicle control was also preformed **(Figure 5).** These results indicate that the process of phagocytosis is responsible for macrophage VEGF production in response to apoptotic tumor cells.

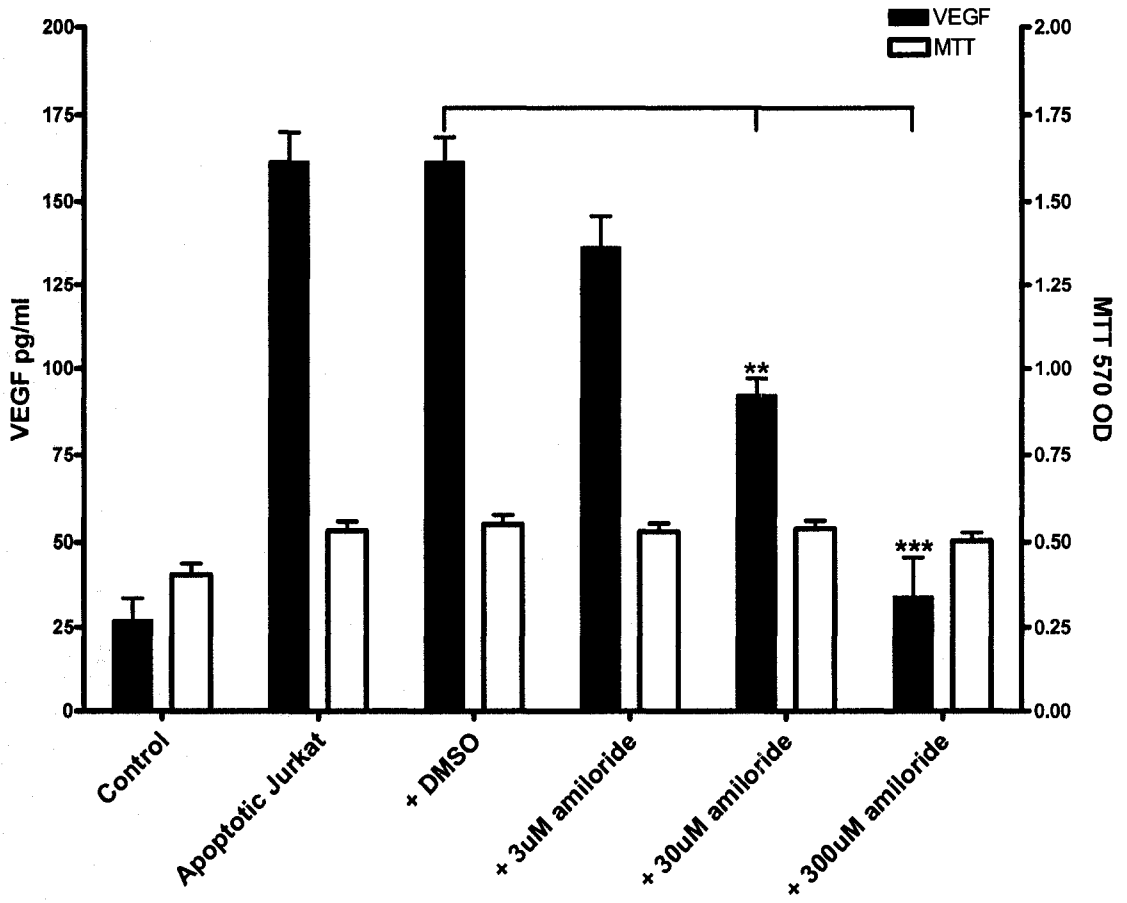

Figure 5: Amiloride abrogates the macrophage VEGF response to apoptotic tumor cells. ICR thioglycollate elicited PECs pretreated with amiloride for 15min prior to the addition of apoptotic jurkat target cells. Following a 36 hr incubation VEGF was measured in culture supernatants by ELISA (Black Bars). MTT was also preformed to insure that the results were not an artifact of cell viability (White Bars). Four replicates per group, mean +/- SEM. **p<0.001, ***p<0.0001 by student's t-test. Data are representative of 2 experiments.

Phosphatidylserine expression is necessary for macrophage VEGF response

It is known that phosphatidylserine (PS) externalization on the surface of apoptotic cell is a potent signal to stimulate macrophage clearance of such cells(3, 4). We wanted to determine if PS was an important signal in regulating the response of macrophage VEGF release following exposure to apoptotic cells. To help us ascertain the importance of PS in our system, we utilized a myelomonoblastic cell line (PLB 985) which does not externalize PS during the process of apoptosis(4, 10, 22). Since PLB 985 does not externalize PS during apoptosis, we were unable to use annexin V staining as our method of determining cellular apoptosis. Apoptosis for the PLB 985 line was assessed by nuclear condensation, membrane blebbing, and changes in forward and side-scatter characteristics while showing absent staining with PI and trypan blue. We found that while the PS expressing apoptotic Jurkats cells could stimulate macrophage VEGF production the non-PS expressing PLB 985 cell line was unable to do so **(Figure 6).** This result provides evidence that PS externalization is an important component of the response that allows macrophages to produce VEGF following exposure to apoptotic cells.

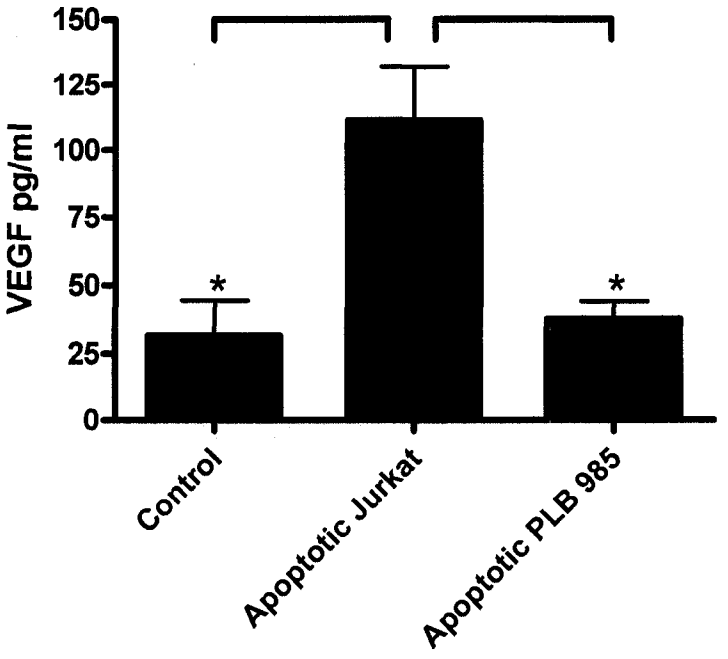

Figure 6: PS expression on the apoptotic target cell is needed for macrophage VEGF production. ICR thioglycollate elicited PECs treated with apoptotic Jurkat or PLB 985 targets. PS expressing apoptotic Jurkat cells could stimulate macrophage VEGF production while the non-PS expressing PLB 985 cell line was unable to do so. Following a 36 hr incubation VEGF was measured in culture supernatants by ELISA. Four replicates per group, mean +/- SEM. *$p<0.01$ by student's t-test. Data are representative of 3 experiments.

DISCUSSION

It was first described by Golpon et al that when macrophages encountered apoptotic cells it stimulated their production of growth factors, including VEGF(21). The purpose of the studies described in this chapter was to expand upon the findings of Golpon et al and explore its significance in terms of tumor biology. We also wanted to clarify the

mechanism by which apoptotic cells were stimulating macrophage VEGF, specifically the role of PS expression.

Since macrophages are an important constituent of the tumor microenvironment and can enhance tumor angiogenesis, we sought to determine the importance of apoptotic tumor cells on macrophage VEGF production. Golpon et al showed that the apoptotic epithelial cell line HC-11 could induce macrophages to produce VEGF but did not expand this finding with the use of apoptotic tumor cells(21). We are able to show that apoptotic, but not live nor necrotic, Jurkat leukemia T cells could induce macrophage production of VEGF (Figure 2). We go on to show that this response can be replicated using the mouse lymphoma lines A20 and EL4 (Figure 4) and that this response is not specific to a single mouse macrophage source (Figure 3). These findings give *in vitro* evidence that apoptotic tumor cells may be an important mechanism in driving tumor-associated macrophage VEGF production.

PS is an important signaling molecules in the process of macrophage clearance of apoptotic cells (3, 4). By utilizing a cell line that does not externalize PS during the process of apoptosis, we were able to determine that PS is an important constituent of the macrophage VEGF response (Figure 6). It is important to know that PS is an important molecule in this process because it could encourage the development of PS blocking reagents that could potentially block the macrophage VEGF response. Antibodies are currently being developed which block PS externalization on tumor endothelial cells

resulting in decreased tumor vasculature(27, 28). It is therefore possible that such antibodies could block macrophage recognition of apoptotic tumor cells.

We were also able to determine that in order for macrophages to produce VEGF in response to apoptotic cells, they must physically engulf the apoptotic cells. We determined this by using amiloride to block endocytosis and found that amiloride could diminish the macrophage VEGF response in a dose dependant manner (Figure 5). This is also an important finding because it could allow for the exploration of molecules other than PS which would block macrophage uptake of apoptotic cells. Potential candidates could include scavenger receptors such as PS receptor or CD14, bridging molecules such as MFG-E8, thrombospodin. collectins or many others(29).

We have been able to show ample *in vitro* evidence that the clearance of apoptotic tumor cells can cause macrophages to produce VEGF, it will be important to show that this observation can be replicated *in vivo.* It may be possible to modify a previously published PEC model that was used to show that macrophage clearance of apoptotic cells induced macrophage TBF-β production(5). A more direct approach may involve looking at TAM VEGF production within the tumor after either inducing or inhibiting tumor cell apoptosis. This could be carried out by analyzing sorted TAMs and determining VEGF expression following the induction of tumor cell apoptosis with chemotherapy administration. Reciprocally, broad spectrum caspase inhibitors could be utilized to inhibit the induction of apoptosis by chemotherapy administration and the inhibition of TAM VEGF production could be assessed.

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Chapter Four

Vaccination With Liposome-DNA Complexes Elicits Enhanced Anti-Tumor Immunity

ABSTRACT

Cationic liposomes have been shown to markedly potentiate the ability of plasmid DNA to activate innate immune responses. We reasoned therefore that liposome-DNA complexes (LDC) could be used to produce more effective plasmid DNA vaccines for cancer. To test this hypothesis, tumor-bearing mice were vaccinated with conventional plasmid DNA vaccines or with LDC vaccines encoding model tumor antigens and $CD8^+$ T cell responses and anti-tumor activity was assessed. We found that although plasmid DNA vaccines generated large increases in antigen-specific $CD8^+$ T cells, they failed to elicit significant anti-tumor immunity. In contrast, LDC vaccines elicited large numbers of antigen-specific $CD8^+$ T cells and also generated significant anti-tumor activity against established tumors. The anti-tumor activity elicited by immunization with LDC vaccines was mediated primarily by $CD8^+$ T cells. We also studied which compartments of the immune system that LDC trafficked into and how treatment altered the activation of the immune system by examining cytokine production and cell surface activation markers.

We found that the majority of labeled liposomes were detected in $CD11b^+/Gr\text{-}1^-$ cells (macrophages), $CD11b^+/Gr\text{-}1^+$ cells (neutrophils), and $CD11c^+$ cells (dendritic cells). Studies of the interaction of LDC with antigen presenting cells found that LDC triggered dendritic cell production of IL-12 and IFN-γ production by NK cells *in vivo*. Activation by LDC was also accompanied by upregulation of co-stimulatory molecule expression on macrophages and dendritic cells. Since tumor associated-macrophages (TAMs) can promote tumor growth by enhancing angiogenesis through VEGF production we wanted to specifically address whether LDC treatment could inhibit macrophage production of VEGF in response to apoptotic cell exposure. We found that LDC could inhibit the ability of macrophages to produce VEGF in response to apoptotic cells in a dose dependant manner. Taken together, these findings suggest that by concurrently activating strong systemic innate immune responses and generating CTL responses, LDC may be used to increase the effectiveness of therapeutic plasmid DNA vaccination for cancer. Furthermore, our LDC immunotherapy may target TAMs, activate them, and inhibit their proangiogenic potential.

INTRODUCTION

Cancer vaccines continue to hold a great promise because of their potential to not only control established tumors but to also prevent tumor metastases (1-5). Impressive clinical responses to cancer vaccines have been observed in a few recent human clinical trials, though the overall track record for cancer vaccines is less impressive (6-8). It is apparent therefore that a number of hurdles remain and there is still a strong need for improvements in tumor vaccine design before they realize their full potential (8, 9).

Improvements in tumor vaccine delivery systems are an important component of current research efforts. Previous tumor vaccine strategies have included immunization with whole tumor cell or tumor lysate vaccines, peptide vaccines, dendritic cell vaccines, viral vectored vaccines, and plasmid DNA vaccines (5, 10-20). Plasmid DNA vaccines are attractive because they are relatively easy to engineer and produce and are safe to administer to humans (1, 5, 21-24). A number of studies have reported the use of plasmid DNA-based vaccines for eliciting anti-tumor immunity in mice (25-30). However, induction of therapeutic anti-tumor activity, especially against tumor metastases to the lungs, has been more difficult to achieve using DNA vaccines. In some cases, co-administration of high doses of recombinant cytokines can increase the efficacy of plasmid DNA vaccination (29, 31). However, in human clinical trials, plasmid DNA vaccines have generally failed to elicit significant anti-tumor activity in patients with established tumors (8, 32, 33). Thus, despite the theoretical advantages of plasmid DNA

vaccination, there are still significant unresolved issues in the application of plasmid DNA vaccination for therapeutic induction of anti-tumor immunity.

The current generation of plasmid DNA-based cancer vaccines may fail to elicit effective anti-tumor immunity because they do not trigger sufficient systemic activation of innate immunity. One solution to this problem is to couple vaccination with systemic immunotherapy. For example, systemic administration of IL-2 and IL-12 significantly improved the efficacy of DNA vaccines in mouse tumor models (29). However, an alternative approach that would avoid the need to administer recombinant cytokines would be to modify the plasmid DNA delivery system to achieve a greater activation of innate immunity during vaccination. Complexing cationic liposomes to the plasmid DNA offers a relatively simple means of accomplishing this, since our group and others have shown that liposome-DNA complexes (LDC) are extremely potent activators of innate immunity (34-36). For example, systemic administration of LDC triggers strong activation of innate immune responses and release of high concentrations of key Th1-biasing cytokines, including IL-12, IFN-γ, and type I interferons. In addition, systemic administration of LDC can elicit potent anti-tumor activity (34, 36-38). Therefore, we hypothesized that LDC could be used effectively as a DNA vaccine platform to enhance the generation of therapeutic anti-tumor immunity through the combined activation of systemic innate and adaptive immune responses.

To address this hypothesis, we immunized tumor-bearing mice with conventional plasmid DNA vaccines or with LDC vaccines and assessed antigen-specific $CD8^+$ T cell

responses, generation of anti-tumor activity, and activation of antigen presenting cells (APC). We found that immunization with LDC administered i.v., even using very low doses of plasmid DNA, induced superior T cell responses and anti-tumor immunity compared to other methods of plasmid DNA immunization. In particular, LDC vaccination elicited large numbers of functionally active, tumor-specific $CD8^+$ T cells infiltrating tumor tissues. We also show which components of the immune system are targeted by LDC and using intracellular cytokine staining identify the immune cells which are producing the Th-1 biasing cytokines. Macrophages are one of the immune cells targeted and activated by LDC. Additionally, we provide *in vitro* evidence that LDC can inhibit macrophage VEGF production in response to apoptotic cell clearance. We conclude that LDC cancer vaccines administered systemically may be a uniquely effective means of generating therapeutic anti-tumor immunity, while inhibiting TAM proangiogenic function.

MATERIALS AND METHODS

Animals and tumor inoculations

Experiments with CL25 tumors were done in female BALB/c mice, 8-12 weeks of age, obtained from Harlan-Sprague-Dawley Laboratories (Indianapolis, IN) and experiments with B16-ova tumors were done in female C57Bl6 mice obtained from the same vendor.

Cutaneous tumors were established by intradermal (i.d.) injection of 2 X 10^5 tumor cells. Treatments were initiated 7 days post-tumor inoculation (B16-ova tumors) or 12 days post-tumor inoculation (CL25 tumors), at a time when tumor nodules were palpable. Tumor diameters were measured using calipers every 2-3 days once treatments were initiated. Mice were euthanized once the tumor diameter reached 1.5 cm. Pulmonary metastases were established by intravenous injection of 2.5 X 10^5 tumor cells and treatments were started 3 days later. The lung tumor burden was quantitated by counting the number of lung metastases as described previously (39). C57Bl6 and outbreed ICR mice were purchased from Taconic (Germantown, NY). Peritoneal macrophages were elicited by the IP administration of 1.5 mls of 4% Brewers Thioglycollate Medium (Sigma Chemical Co, St Louis, MO). Animal protocols for these experiments were approved by the Institutional Animal Care and Use Committees at the National Jewish Medical and Research Center and at Colorado State University.

Cell culture

The human leukemia T cell line Jurkat (gift from Peter Henson) and maintained in RPMI 1640 with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). All cells were incubated at 37°C in 5% CO2. To assure mycoplasma-free conditions, cells were routinely tested by MycoSensor PCR Assay Kit (Stratagene, La Jolla, CA).

DNA vaccinations and preparation of liposome-DNA complexes

Mice were immunized by the i.d., i.m., or i.v. routes with plasmid DNA alone or with plasmid DNA formulated as LDC. For immunization with plasmid DNA alone, mice received 50 ug plasmid DNA dissolved in 200 ul saline (i.m. immunization) or in 100 ul saline (i.d. immunization). For i.m. immunization, equal amounts of DNA were injected bilaterally in the tibialis muscles of anesthetized mice. For i.d. immunization, mice were injected in the skin of the base of the tail, using a #27 needle. Mice received 2 immunizations, administered 7 days apart. LDC was prepared from sterile 10 mM solutions of the cationic liposome DOTIM {octadecenoyloxy{ethyl-2-heptadecenyl-3-hydroxyethyl} imidazolinium chloride and cholesterol were prepared in a 1:1 molar ratio as described previously(40). Briefly, DOTIM and cholesterol were dissolved in chloroform, and then dried down to a thin film in round-bottomed tubes in a vacuum desiccator. Liposomes were prepared by rehydration in a 10% sucrose solution, followed by filtration trough a final filter diameter of 200 nm. Liposome-DNA complexes were formed just prior to injection by gently mixing cationic liposomes with plasmid DNA (non-coding plasmid DNA with <0.05 EU/ml endotoxin content; Althea Technologies) at a ratio of 16 nmol lipid per 1 ug DNA in 5% dextrose in water at room temperature. The final plasmid DNA concentration in the complexes was 100 ug DNA per ml. For i.v. immunization with LDC, mice received 5 ug plasmid DNA in 50 ul of LDC, administered via the lateral tail vein, followed by a second immunization 7 days later.

For i.d. and i.m. immunization with LDC, mice were injected with 50 ug total DNA in LDC in a total volume of 200 ul 5% dextrose in water, in the skin of the tail base or bilateral tibialis muscles, respectively.

Cytotoxicity assay

Cytotoxic lymphocyte (CTL) responses to vaccination were quantitated using a ^{51}Cr-release assay, as described previously (41). Briefly, spleen cells were harvested and restimulated *in vitro* in 24-well plates by pulsing with 1 uM ova8 (SIINFEKL) peptide for 5 days. The cell culture medium was supplemented with 1% concanavalin-A stimulated rat spleen cell supernatants beginning on day 2 in culture. At 5 days, the cells were harvested and assayed at decreasing effector-to-target ratios for their ability to lyse ova8 peptide-pulsed MHC-matched, ^{51}Cr-pulsed EL-4 target cells. Controls included incubation of spleen effector cells with ^{51}Cr -labeled EL-4 cells that were not pulsed with peptide. Target EL-4 cells were pulsed for 1 hour with ^{51}Cr and then 5 X 10^3 labeled target cells were added to each well of a Linbro plate, followed by addition of effector cells, beginning at an effector-to-target ratio of 50:1. After 4 hours incubation at 37C, supernatants were harvested and assayed for release of ^{51}Cr by automated gamma counter. The percentage specific lysis was calculated using the following formula: observed Cr minus spontaneous Cr release, divided by maximum Cr release minus spontaneous Cr release. The mean specific lysis detected in 4-5 animals per treatment group was plotted.

Flow cytometry

Directly conjugated antibodies used for flow cytometric analysis were purchased either from Pharmingen (San Diego, CA) or from eBiosciences (San Diego, CA). The following antibodies were used: anti-CD8a (APC; clone 53.6.7), anti-CD4 (APC; clone RM4-5), anti-CD44 (FITC: clone IM7), anti-CD62L (PE/Cy5; clone Mel-14), anti-CD69 (PE/cy7; clone H1.2F3), anti -I-A/ I-E (MHC II, biotin or PE; clone M5/114.15.2), followed by either SA-pe/cy5 or SA-Alexa-350 (Molecular Probes, Inc, Eugene, OR), anti-CD11b (PE-Cy5 or APC-Cy7; clone M1/70), anti-CD11c (PE or APC; clone N418), anti-Gr-1 (PeCy7; clone RB6-8C5), B220 (APC-Cy7; clone RA3-6B2), anti-NK1.1-biotin (clone PK 136) or anti-CD3 (APC-Cy7; clone 145-2C11). Non-specific binding of antibodies was blocked by pre-incubation of cells in normal mouse serum with 40% supernatant from rat anti-FcRIII hybridoma 24.G2, plus 0.2 ug/ml human IgG. Antibodies were diluted in FACS buffer (PBS with 2% FBS and 0.1% sodium azide) for staining. Staining (except for tetramers) was done at 4C for 20 minutes, followed by washing in FACS buffer. In most cases, cells were fixed in 1% paraformaldehyde for 30 minutes and stored in FACS buffer at 4C prior to analysis. Flow cytometry was performed using either a BD FACSCalibur cytometer (BD, Pleasanton, CA) for 4 color analysis or a Cyan MLE cytometer (Dako-Cytomation, Ft Collins, CO) for 6 to 7-color analysis. Gates for analysis of $CD8^+$ T cell responses were drawn to include live lymphocytes, based on forward and side-scatter characteristics of spleen cells, whereas larger gates were selected for analysis of antigen-presenting cells. For most experiments, the total $CD8^+$ cell population was analyzed to determine the percentage of tetramer

positive events as a percentage of all $CD8^+$ T cells. A minimum of 200,000 total events were collected for tetramer experiments. Data analysis was done using either CellQuest software or Summit Software.

MHC-peptide tetramer analysis

$H\text{-}2K^b$ MHC class I tetramers containing the ova8 peptide (SIINFEKL) were produced as previously described (42). The $CD8^+$ T cell response to DNA vaccination against ovalbumin was assessed in C57Bl6 mice with established cutaneous B16-ova tumors. Single cell suspensions of lymph node and tumor tissues were prepared by pressing through nylon mesh, followed by lysis of erythrocytes using ammonium chloride. Spleen mononuclear cells were prepared by dissociation of spleens, followed by NH4Cl lysis of erythrocytes. Tumor tissues were weighed prior to preparation of single cell suspensions and cell numbers after dissociation were determined by Coulter counter. For tetramer staining, single cell suspensions (approximately 5×10^5 to 1×10^6 cells per well of 96-well plates) were incubated for 1.5 hours at 37^C in 100 ul tissue culture medium with the appropriate tetramer concentration, as determined by staining of positive control ova-TCR Tg OT-1 cells (41). After incubation with tetramers, cells were incubated with anti-CD8-APC (clone 53-6.7.), anti-CD44 FITC (clone IM7; both from Pharmingen, San Diego, CA), and with biotinylated anti-$I\text{-}A^b$ antibody (clone Y3P), followed by incubation with streptavidin PE-Cy5 conjugate (Pharmingen). For tetramer analysis, at least 200,000 to 500,000 total events per sample were collected. Total $CD8^+$ T cells were

gated and then analyzed for tetramer binding and CD44 expression. I-A^{b+} cells were excluded from the analysis to reduce non-specific staining. The total number of tetramer-positive cells in tumor, spleen, and lymph node tissues was determined by counting the total number of live mononuclear cells analyzed and multiplying by the percentage of tetramer-positive cells. In the case of tumor tissues, the number of ova8-specific $CD8^+$ T cells was also expressed as cells per gm tumor weight in order to adjust for variability in tumor sizes.

Tracking distribution of labeled LDC

Fluorescently-labeled liposomes were used to track uptake of LDC in the spleen following i.v. injection. Liposomes were labeled by incorporating BODIPY-labeled cholesterol (Molecular Probes, Eugene, OR) during preparation of the liposomes, as described previously (43). At various time points after i.v. injection of labeled LDC, spleens were collected, digested for 20 minutes in 1 mg/ml collagenase, then single cell suspensions were prepared and immunostained with panels of antibodies specific for antigen presenting cells and lymphocytes. Cells were analyzed using a Cyan MLE multicolor flow cytometer, with data analysis done using Summit software. The mean percentage of $BODIPY^+$ cells of different cell types was determined for individual mice and the mean value for groups of 3-4 mice per group was determined. Controls included untreated mice and mice injected with unlabeled LDC. For activation of antigen presenting cells, the mean percentage of macrophages or dendritic cells (DC) expressing

CD86 was determined by comparison to untreated control mice (3-4 animals per treatment group).

Intracellular cytokine staining

Spleen cells were immunostained for detection of intracellular cytokines as described previously (44). Briefly, cells were collected at various time points after injection of DNA or LDC and then placed in FACS buffer in the presence of 10 ug/ml berfeldin-A. After 4-5 hours incubation, the cells were immunostained for detection of cell surface antigens, then fixed and permeabilized and stained for intracellular cytokines. Antibodies used for intracellular cytokine staining included anti-mouse IL-12 (PE; clone C17.8) and anti-mouse IFN-γ (PE; clone XMG1.2), both from eBiosciences. For each experiment, controls included cells from untreated mice and conjugated mAb matched to the isotype of the cytokine antibodies. The percentage of cells expressing a given cytokine was determined for each group of mice and the mean (± SE) of the cytokine-positive populations was calculated.

Cytokine release assay

Antigen-specific generation of IFN-γ by $CD8^+$ T cells was assessed by culturing single cell suspensions of spleen cells from Ova DNA vaccinated mice overnight at a

concentration of 2 X 10^6 cells/ml in complete medium in the presence or absence of 1 uM ova8 peptide. Supernatants were collected 18 h later and assayed for release of IFN-γ, using a commercial ELISA assay (R&D Systems. Minneapolis, MN). Controls included spleen cells from non-immunized mice and spleen cells from immunized mice incubated without peptide.

Antibody depletion of effecter cells

Antibody depletion was initiated 24 hours before vaccination and was repeated once weekly. $CD4^+$ T cells were depleted by i.p. administration of 50 ug purified rat-anti mouse CD4 IgG (clone GK1.5), $CD8^+$ T cells were depleted using 50 ug of rat anti-mouse CD8 IgG (clone 2.43), and NK cells were depleted using 50 ug purified rabbit anti-asialo GM1 antiserum (Wako Bioproducts, Richmond, Va). The doses of depleting antibodies used for to inducing efficient depletion of selected cell populations was determined by *in vivo* titration and depletion was confirmed by flow cytometric analysis.

Induction of apoptosis

Apoptosis of Jurkats was induced by UV exposure (405 nM) for 10 mins, followed by a 3 hr incubation at 37°C in 5% CO2. Cells were than resuspended in Dulbecco's modified Eagle's medium at a concentration of $1x10^7$/ml. The induction of apoptosis was assessed

by quantifying the loss of PS assymety and PS externalization by an annexin-V-fitc binding assay (BD biosciences, Bedford, MA). The annexin-V assay was preformed exactly has described in the manufacture's protocol. Evaluation of secondary necrosis was assessed by both the use of PI staining and trypan blue positivity. Apoptotic cells used for these experiments showed >75% annexin-v staining and < 10% typan staining. Flow cytometry was performed using Cyan MLE cytometer (Dako-Cytomation, Ft Collins, CO). Data analysis was carried out on Summit software.

In vitro peritoneal macrophage assays

Three days following Thioglycollate stimulation, mice were euthanized by CO2 asphyxiation and peritoneal exudate cells (PECs) were harvested by IP lavage with 7mls of ice cold HBSS supplemented with 2% FBS. PECs were washed twice with HBSS/FBS and resuspended at $2x10^6$ cells/ml in DMEM with 10% FBS, 0.2 mM L-glutamine, 1 mM HEPES buffer, and 0.1 mM nonessential amino acids, 50 IU/ml penicillin and 50 μg/ml streptomycin. 100μl of cell suspension was added to the wells of a 96 well flat bottomed plate. The PECs were incubated for 2 hrs at 37°C in 5% CO2 and wash gently twice with 200μl of prewarmed HBSS to remove non-adherent cells. 200 μl of fresh DMEM was than added and the PECs were allowed to rest for 36 hrs at 37°C in 5% CO2. Before the addition of apoptotic cells, PECs were incubated with LDC (as prepared above) at a 1:100 dilution in DMEM or DMEM alone (control) for 1 hr at 37°C in 5% CO2. LDC treatment was than gently was away with pre-warmed HBSS. Target

cells ($1x10^6$ in 100µl DMEM) were than fed to the PECs and incubated for 1 hr at 37°C in 5% CO2. Unphagocytozed target cells we removed by gently washing twice with 200µl of pre-warmed HBSS. PECs were than fed with 125µl of fresh DMEM and incubated for 36 hrs at 37°C in 5% CO2. Supernatants were harvested and stored at -70°C until analysis. Each treatment group was carried out in quadruplicate wells.

ELISA assays

Supernatants were than harvested and frozen at -70°C until ELISA analysis was preformed. The VEGF ELISA kit was purchased form R&D Biosystems (Minneapolis, MN). ELISAs were then preformed as per manufacture's instructions. ELISAs were developed with tetramethylbenzidine substrate (Sigma Chemical Co, St Louis, MO) and absorbance measured at 450 nm with an optical plate reader (Thermo Labsystems, Waltham, MA).

Proliferation assay

To ensure that LDC treatment was not toxic to the PECs, PEC survival was measure using a 3-(4,5-dimethylthylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma). Culture supernatants were removed and replaced with 100µl of 0.5 mg/ml MTT in PBS supplemented with 2% heat inactivated FBS, cultured at 37°C in 5% CO_2 for 3

hrs, lysed with 100 µl of 0.1N HCl in isopropanol, and absorbance measured at 570 nm with an optical plate reader (Thermo Labsystems).

Statistical analysis

In experiments with multiple groups of mice, statistical differences between treatment groups were compared using ANOVA and Tukey's multiple means comparisons test. For comparisons between two treatment groups, Student's t-test was used. Survival curves were generated using Kaplan-Meier analysis, and statistical significance was determined using Chi square analysis. Statistical analyses were done using GraphPad Prism software (San Diego, CA). A p-value < 0.05 was considered significant for these analyses.

RESULTS

Immunization with plasmid DNA or with LDC vaccines generates antigen-specific CD8+ T cell responses in spleen and tumor tissues

Previous studies have established that i.d. and i.m. administration of plasmid DNA is an effective means of generating $CD8^+$ T cell responses against tumor antigens (25, 26).

However, the effectiveness of LDC-based vaccines for eliciting $CD8^+$ T cell responses has not been previously reported. For these experiments, MHC class I tetramers were used to quantitate and compare $CD8^+$ T cell responses to DNA vaccination in tumor-bearing mice. The model antigen ovalbumin was used in these studies since the immunological reagents necessary for analysis of ova-specific T cells were readily available. We also used the B16 melanoma cell line transfected with the ovalbumin gene (B16-ova) to facilitate tracking of tumor-specific T cell responses *in vivo*.

In the first experiments, we compared the magnitude of $CD8^+$ T cell responses to vaccination with plasmid DNA when the DNA was administered by different routes and formulated either as "naked" plasmid DNA or complexed to cationic liposomes (LDC vaccines). Mice with established B16-ova tumors were immunized twice with DNA encoding ovalbumin and then spleen and tumor tissues were analyzed for presence of ova-specific $CD8^+$ T cells a week after the last immunization, using Kb-ova8 tetramers and flow cytometry. Following i.d. or i.m. immunization with 50 ug plasmid DNA, there was a significant expansion in the number of ova-specific $CD8^+$ T cells in spleen tissues and in tumor tissues **(Figure 1).** The increase in ova-specific $CD8^+$ T cells was particularly pronounced in tumor tissues of mice immunized i.m. with plasmid DNA, where approximately 20% of all tumor infiltrating $CD8^+$ T cells were ova specific **(Figure 1).** In mice immunized with 5 ug of plasmid DNA formulated as LDC and administered i.v., there was also a significant ($p < 0.05$) increase in ova-specific $CD8^+$ T cells in both spleen and tumor tissues **(Figure 1B).** Therefore, both conventional plasmid DNA immunization and immunization with LDC elicited significant increases in the

numbers of antigen-specific $CD8^+$ T cells in tumor-bearing mice. Notably, i.v. immunization with LDC was much more efficient than i.m. or i.d. immunization with plasmid DNA only, since only a tenth of the dose of plasmid DNA was utilized for LDC immunization. As a control, mice treated by i.v. administration of LDC containing non-coding plasmid DNA instead of ova DNA did not develop increased numbers of ova-specific $CD8^+$ T cells in spleen or tumor tissues.

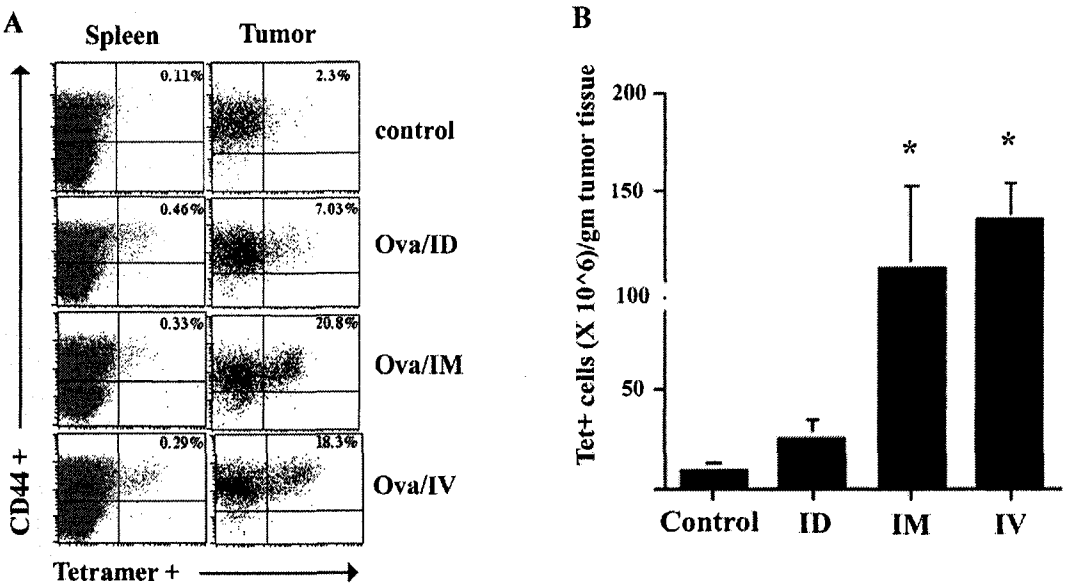

Figure 1: Intravenous and intramuscular DNA vaccination induces large increase in antigen-specific CD8+ T cells. The CD8+ T cell response to DNA vaccination was evaluated in mice with established cutaneous B16-Ova tumors, using K^b-ova8 tetramers and flow cytometry, as described in Methods. (A) Single cell suspensions were prepared from spleen and tumor tissues of mice 5 days after a booster DNA immunization (day 20 after tumor inoculation). Antigen specific CD8+ T cells were quantitated by staining with H-2K^b-ova8 tetramers, followed by flow cytometric analysis. The total CD8+ T cell population was analyzed, and the percentage of all CD8+ T cells that were positive for K^b-ova8 tetramer was determined. (**A**) Representative flow cytometric analyses of ova-specific $CD8^+$ T cells recovered from spleen and tumor tissues from tumor-bearing mice (4 per treatment group) that were untreated (control), vaccinated i.d. with Ova plasmid DNA (Ova/ID), i.m. with Ova plasmid DNA (Ova/IM), or i.v. with Ova LDC (Ova/IV). (**B**) The total number of K^b-$ova8^+$ tumor-infiltrating $CD8^+$ T cells for each mouse (4 per treatment group) was determined and then expressed based on the weight of the individual tumor. The mean (±SE) number of $tetramer^+$ cells per gm of tumor weight was plotted. Similar results were obtained in 2 additional experiments. * denotes significantly different ($p < 0.05$ compared to control mice), as assessed by ANOVA.

Mice were not treated with higher doses of LDC administered i.v. because excessive activation of innate immune responses induced toxicity, as reported previously (34). Immunization with LDC was not effective however when LDC were administered by the i.d. or i.m. routes. For example, the number of ova-specific CD8+ T cells was not

increased over background levels in mice immunized i.d. or i.m. with LDC, even when 50 ug DNA was delivered. Therefore, it appeared that LDC immunization was only effective in eliciting $CD8^+$ T cell responses when given by the i.v. route. The relative ineffectiveness of LDC immunization by the i.d. and i.m. routes may have stemmed from the fact that LDC injection induced local tissue damage in muscle and skin tissues and interfered with transgene expression. Intravenous administration of plasmid DNA alone also did not elicit detectable $CD8^+$ T cell responses. Therefore, for the remainder of the study the effects of LDC immunization were only evaluated by the i.v. route and plasmid DNA immunization was evaluated by the conventional i.d. and i.m. routes.

Humoral immune responses to DNA immunization were also assessed. In these experiments, antibody responses to β-gal immunization was evaluated in BALB/c mice, since C57Bl6 mice mounted very weak antibody responses to ovalbumin vaccination. Immunization with β-gal plasmid DNA by the i.d. or i.m. routes induced strong antibody responses (increased total IgG specific for β-gal), while i.v. immunization with LDC also elicited anti-β-gal antibody responses, though the magnitude of the response was weaker than for plasmid DNA immunization. In contrast, i.d. or i.m. immunization with β-gal DNA formulated as LDC encoding the β-gal gene did not elicit significant β-gal antibody responses, consistent with the previously observed lack of effectiveness for i.d. and i.m LDC immunization for eliciting $CD8^+$ T cell responses.

Immunization with LDC elicits CTL with greater functional activity than plasmid DNA immunization

The preceding experiments indicated that DNA immunization using either plasmid DNA or LDC could elicit significant increases in antigen-specific $CD8^+$ T cells. However, the functionality of T cells elicited by vaccination is also important in the generation of anti-tumor immunity. Therefore, we compared the cytotoxic activity of $CD8^+$ T cells elicited by the different DNA vaccines. Spleen cells were collected from tumor-bearing mice (4 C57Bl6 mice per group) immunized twice with Ova DNA by i.d. or i.m. administration of plasmid DNA or i.v administration of LDC. Control groups included untreated tumor-bearing mice and tumor-bearing mice treated by i.v. administration of LDC prepared with non-coding DNA. Spleen cells were re-stimulated *in vitro* for 5 days with 1 uM ova8 peptide and then assessed for their ability to lyse ^{15}Cr-labeled EL-4 target cells pulsed with the ova8 peptide. We found that spleen cells from mice immunized with LDC exhibited significantly greater specific cytotoxic activity than spleen cells from control mice ($p < 0.01$) **(Figure 2).** In addition, mice immunized with ova LDC also developed significantly greater cytolytic activity than mice immunized id. or i.m. with Ova plasmid DNA ($p < 0.01$). Mice treated by i.v. administration of LDC prepared with non-coding DNA however did not develop significantly greater CTL activity when compared to ova-LDC immunized mice ($p < 0.05$). In addition, spleen cells from mice immunized with ova LDC released greater amounts of IFN-γ in response to restimulation with ova8 peptide than spleen cells from mice immunized with ova plasmid DNA. These data indicated therefore that immunization with LDC not only elicited large numbers of

antigen-specific $CD8^+$ T cells, but that the T cells that were generated were also functionally more active.

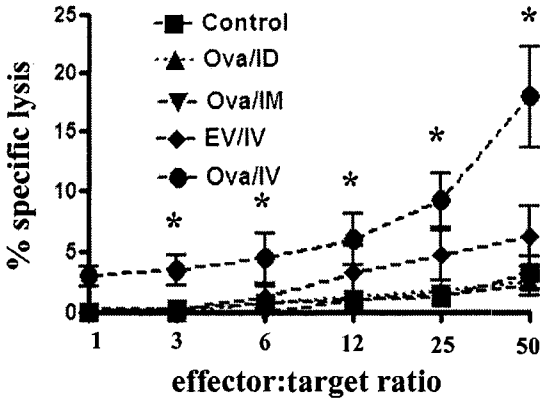

Figure 2: Comparison of CTL activity generated by DNA immunization. Tumor bearing mice (4 animals per treatment group) were immunized twice, one week apart, with Ova plasmid DNA by the i.d. (Ova/ID) or i.m (Ova/IM) routes, or i.v. with LDC prepared with Ova DNA (Ova/IV) or i.v. with empty vector LDC (EV/IV). Control mice were not vaccinated. After the second immunization, spleen cells were prepared from each mouse and restimulated *in vitro* with 1 uM ova8 peptide, as described in Methods. After restimulation, T cells were assayed for their ability to lyse ^{51}Cr-labeled EL-4 cells pulsed with 1 uM ova8 peptide, or unpulsed EL-4 control cells. The mean percentage specific lysis of target cells by each group of vaccinated mice, at the indicated effector:target cell ratios, was calculated and plotted. Significant differences (denoted by *) were determined by ANOVA. Similar results were obtained in 2 additional experiments.

Therapeutic immunization with LDC vaccines controls tumor growth in cutaneous and lung tumor models

Experiments were conducted next to assess the ability of DNA vaccines to elicit therapeutic anti-tumor immunity in mice with established cutaneous tumors, using two

well-studied tumor models. In the first experiments, B16-0va tumors were established in the skin of C57Bl6 mice and immunizations were not begun until day 7 after tumor inoculation, at which point the tumors were palpable (2-3 mm diameter). Treatment groups (5 mice per group) included untreated control mice, mice immunized i.d. with 50 ug Ova DNA, mice immunized i.m. with 50 ug ova DNA, mice immunized i.v. with 5 ug ova LDC, and mice immunized i.v. with 5 ug empty vector LDC. This last treatment group was included to control for the anti-tumor activity elicited by i.v. administration of empty vector LDC (34). Mice were each immunized twice, one week apart, and tumor size was determined every 2-3 days and mice were euthanized when tumor diameter exceeded 1.5 cm.

We found that immunization with Ova LDC significantly inhibited tumor growth ($p < 0.001$) compared to untreated control mice or to mice immunized with Ova plasmid DNA **(Figure 3).** Immunization with Ova LDC also elicited significantly greater inhibition of tumor growth than i.v. administration of empty vector LDC ($p < 0.01$). We also observed that i.d. or i.m. immunization with ova LDC did not elicit detectable anti-tumor activity, nor did i.v. administration of ova plasmid DNA alone. These results indicated that i.v. immunization with LDC was uniquely effective in eliciting therapeutic anti-tumor activity, compared to conventional plasmid DNA immunization. Moreover, the effect was not simply due to activation of innate immunity, as i.v. administration of non-coding LDC was not effective in controlling tumor growth.

Another set of experiments was conducted to extend these findings to a different tumor model in a different strain of mice. For these studies, the CT26 colon carcinoma model was studied in BALB/c mice. A CT26 tumor line expressing the β–galactosidase (β-gal) gene (CL25) was utilized to facilitate the immunization experiments and mice were immunized with a plasmid expressing the β-gal antigen. The same treatment and control groups and treatment schedules were utilized as described above, except that mice were immunized with β-gal plasmid DNA. CL25 tumors were established in the skin and allowed to grow for 12 days (until tumors were palpable) before immunizations were initiated. Intravenous immunization with β-gal LDC significantly ($p < 0.001$) inhibited tumor growth compared to control mice or to mice immunized with β-gal DNA by the i.d. or i.m. routes **(Figure 3).** In addition, immunization with β-gal LDC was also significantly more effective ($p < 0.01$) than administration of empty vector LDC. These data therefore confirmed and extended the findings in the B16 tumor model system and underscored the potency of the LDC vaccine approach to eliciting therapeutic anti-tumor immunity.

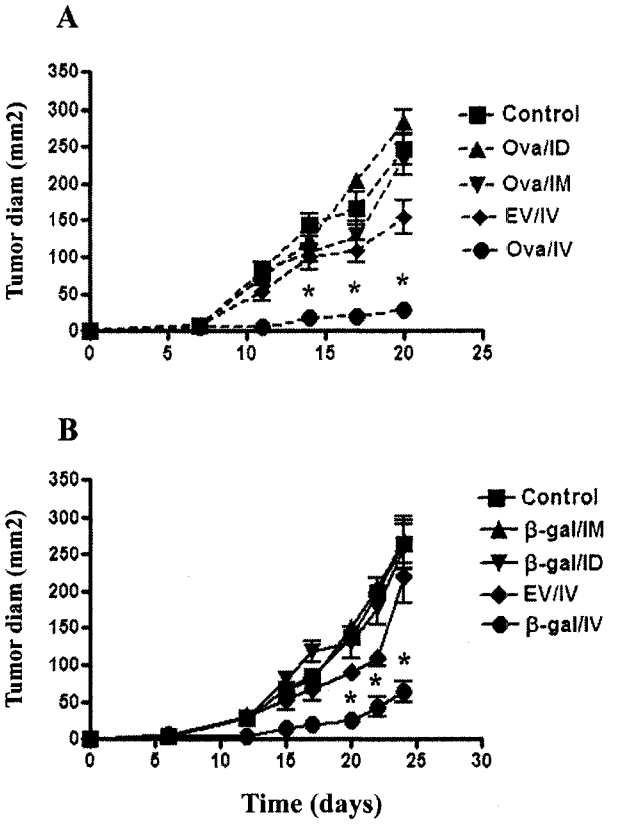

Figure 3: Effects of DNA vaccination on growth of established cutaneous B16 or CT26 tumors. (A) Cutaneous B16-ova tumors were established in C57Bl6 mice (5 per treatment group) 7 days prior to vaccination. Mice were immunized twice, one week apart, with DNA vaccines encoding Ova, using i.d. plasmid DNA (Ova/ID), i.m. plasmid DNA(Ova/IM), i.v. Ova LDC (Ova/IV), or i.v. empty vector LDC (EV/IV). Controls included mice that were not vaccinated. Tumor growth rates were assessed by measurement of tumor diameter every 2-3 days. The mean tumor diameter (± SE) was plotted for each time point. Statistical comparison was done by ANOVA and significant differences ($p < 0.01$) compared to control mice or EV/IV mice are represented by *. Similar results were obtained in 2 additional experiments. (B) Cutaneous CL25 tumors were established in BALB/c mice (5 per group) 12 days prior to vaccination. Mice were vaccinated twice, one week apart, with DNA vaccines encoding the β-galactosidase gene, using plasmid DNA by the i.d.(β-gal/ID) or i.m. (β-gal/IM) routes, or LDC by the i.v route (β-gal/IV). Controls included mice that were not vaccinated or mice injected i.v. with non-coding LDC (EV/IV). Tumor growth was monitored and tumor diameters plotted and analyzed as described above. (* denotes significant differences ($p < 0.01$) compared to control mice or EV/IV mice). This experiment was repeated once with similar results.

Immunization with LDC also increases survival in animals with established lung metastases

The preceding results were investigated further to determine whether reduction in tumor growth rate also correlated with increased survival times in mice with lung tumor metastases. For the lung tumor model, tumor metastases were established by i.v. injection of tumor cells prior to immunization. First, the effects of immunization on survival of mice with established cutaneous B16-ova tumors was assessed. Mice with cutaneous B16-ova tumors treated with ova LDC survived significantly longer ($p = .002$) than control mice **(Figure 4A).** In contrast, mice vaccinated with ova plasmid DNA by the i.d. or i.m. routes did not survive significantly longer than control mice ($p > 0.05$). Mice immunized with ova LDC also survived significantly longer ($p = .008$) than mice treated with empty vector LDC.

The effects of DNA immunization on survival times in mice with day 7 established B16-ova lung tumor metastases were assessed next in a more rigorous therapeutic vaccination model. Mice were immunized twice, one week apart, and euthanized when progressive tumor growth became apparent. Mice immunized with ova LDC survived significantly longer ($p = .002$) than untreated control mice **(Figure 4B).** Survival times in mice immunized with ova plasmid DNA by the i.d. or i.m. routes were not significantly different from control animals. Consistent with our previous findings in lung tumor studies with intravenously administered LDC, mice treated with LDC containing non-coding DNA survived significantly longer than control animals ($p = .005$) (34).

However, survival times in mice immunized with ova LDC were significantly longer than in mice treated with non-coding LDC ($p = .008$), indicative of an antigen-specific response in addition to non-specific activation of innate immunity. These results provided additional evidence of the therapeutic anti-tumor efficacy elicited when DNA vaccines were formulated as LDC.

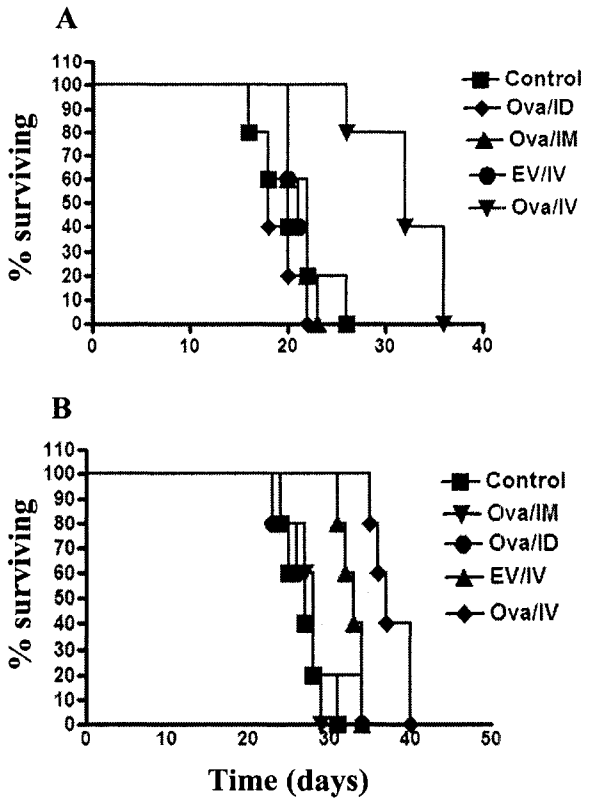

Figure 4: Effects of LDC vaccination on survival in mice with established B16-ova tumors in the skin or lungs. (A) Cutaneous tumors were established in C57Bl6 mice (5 per group) and the mice were vaccinated 7 days later, (twice one week apart) with plasmid DNA encoding the ova gene by the i.d or i.m. routes, or with LDC by the i.v. routes, as described in Figure 3. Controls included mice that were not vaccinated (control) or mice immunized i.v. with LDC containing non-coding plasmid DNA. Mice were euthanized when tumor diameter exceeded 1.5 cm and the survival times were plotted. Statistical differences in survival times were determined using Kaplan-Meier survival curves and Chi square analysis. Mice in the Ova/IV group had a significant increase in survival times ($p < 0.005$) compared to all 4 other groups of mice. Similar results were obtained in one additional experiment. (B) Lung tumors were established in C57Bl6 mice (5 per group) by i.v. injection of B16-ova cells and vaccination was initiated 7 days later, as described in (A) above. Overall survival times were calculated and compared by Kaplan-Meier survival curves. Mice in the Ova/IV group had statistically increased survival times ($p < 0.01$) compared to control, EV/IV, Ova/ID, and Ova/IM groups. Similar results were obtained in one additional experiment.

Anti-tumor activity elicited by immunization with LDC vaccines is mediated by $CD8^+$ T cells

DNA immunization has been shown to elicit both $CD4^+$ and $CD8^+$ T cell responses, and both effector cells may help control tumor growth. In addition, we have found that NK cells are one of the major effectors of anti-tumor activity elicited by i.v. injection of non-coding LDC (34). Therefore, cell subset depletion experiments were used to assess the relative roles of T cells and NK cells in mediating the anti-tumor activity elicited by immunization with LDC. B16-ova tumors were established in the skin of C57Bl6 mice (5 mice per group) for 7 days, then the mice were depleted of $CD4^+$ or $CD8^+$ T cells or NK cells and then immunized with ova-LDC. Controls included non-immunized mice, mice immunized with LDC but not depleted, and mice treated with an irrelevant (non-depleting) rat IgG and then immunized. The effects of cell depletion were assessed by measuring tumor growth kinetics **(Figure 5).** We found that depletion of $CD8^+$ T cells, but not $CD4^+$ T cells or NK cells, significantly inhibited the anti-tumor activity elicited by immunization with ova-LDC. For example, tumor growth was still slowed significantly ($p < 0.001$) following immunization of mice depleted of $CD4^+$ T cells or NK cells. In contrast, tumor size was not significantly different ($p > 0.05$) from tumors in control mice on days 12 and 18 in mice depleted of $CD8^+$ T cells. These results suggested therefore that the majority of the anti-tumor effect elicited by immunization with LDC were mediated by $CD8^+$ T cells.

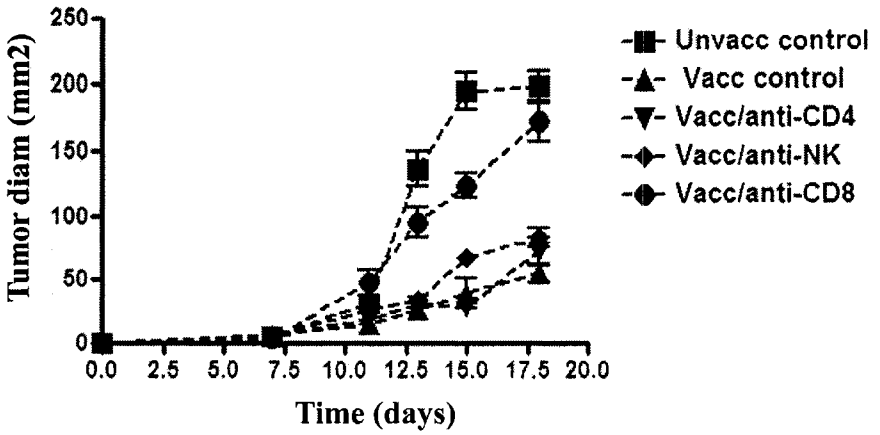

Figure 5: Effects of T cell or NK cell depletion on tumor response to immunization with LDC. Cutaneous B16-ova tumors were established in C57Bl/6 mice (5 per group). One day before vaccination (day 6 after tumor inoculation), $CD4^+$ and $CD8^+$ T cells and NK cells were depleted by injection of depleting antibodies, as described in Methods. Mice were vaccinated i.v. with LDC containing 5 ug Ova DNA one day later. The antibody depletion was repeated 7 days later and LDC vaccination was administered again 24 hours later. Treatment groups included untreated tumor-bearing mice (Unvacc control), tumor-bearing vaccinated mice (Vacc control), mice depleted of $CD4^+$ T cells and vaccinated (Vacc/anti-CD4), mice depleted of NK cells and vaccinated (Vacc/anti-NK), and mice depleted of $CD8^+$ T cells and vaccinated (Vacc/anti-CD8). An additional group of mice was treated with an irrelevant rat IgG and vaccinated (not shown). Tumor growth rates were measured every 2-3 days and the mean tumor diameter (± SE) for each treatment group was plotted. Statistical comparison of tumor diameters was done using ANOVA. The mean tumor diameter for all groups of vaccinated mice except the CD8-depleted mice were significantly different ($p < 0.05$) than control mice for days 12, 15 and 18. In the case of CD8-depleted mice, tumor diameters were not significantly different on days 12 and 18 when compared to control mice. Similar results were obtained in one additional experiment.

Uptake of LDC by macrophages and DCs in the spleen

Experiments were done to investigate the interaction of LDC with antigen presenting cells (APC) to better understand how LDC interacts with different components of the immune system. Liposomes labeled with a fluorescent dye (BODIPY) were used to track

uptake of LDC by splenic APC. At various time points after i.v. administration of labeled LDC, spleen cells were collected from C57Bl6 mice (4 mice per group), immunostained with antibodies to relevant cell surface determinants on APCs, then analyzed by multi-color flow cytometry. Panels of antibodies were selected to identify macrophages ($CD11b^+$, $Gr\text{-}1^-$), neutrophils ($CD11b^+$, $Gr\text{-}1^+$), dendritic cells (DC) ($CD11c^+$, NK 1.1^-), NK cells ($NK1.1^+$), and $CD4^+$ and $CD8^+$ T cells.

One hour after i.v. injection of BODIPY-labeled LDC, the majority of labeled LDC in the spleen were found in macrophages, neutrophils and DCs **(Figure 6).** Significantly fewer NK cells **(Figure 6B)** contained labeled LDC. When the kinetics of uptake of LDC were examined, high levels of uptake of labeled LDC were detectable at one and 4 hours after injection, but by 24 hours post-injection the number of labeled LDC in cells had declined significantly. The disappearance of the complexes most likely reflected processing and degradation of the complexes within the APC, though it could not be excluded that the BODIPY label itself was being selectively degraded following binding and internalization of the LDC. Thus, uptake of the LDC appeared to be specifically targeted to key APC in the spleen, including macrophages and DC.

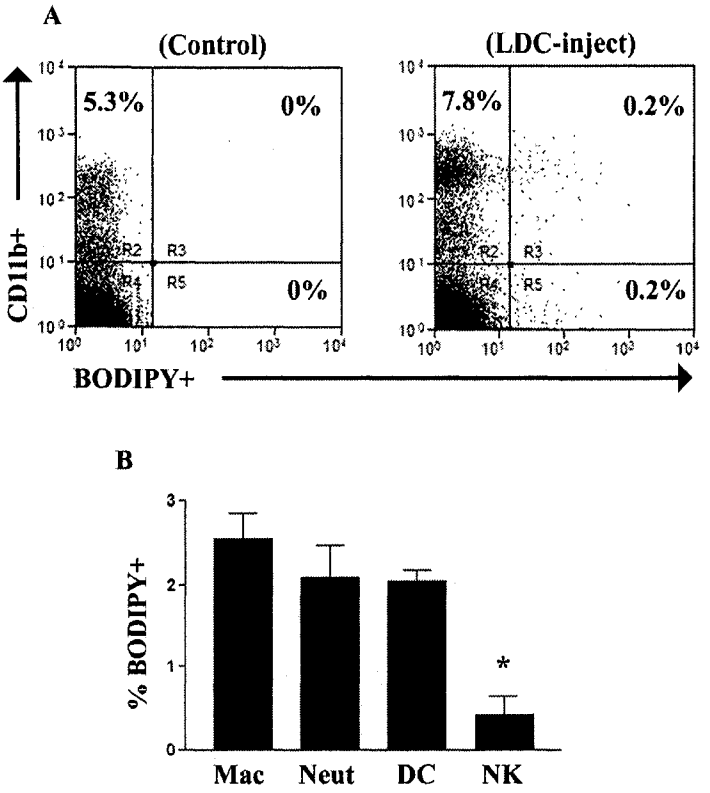

Figure 6: Uptake of labeled LDC by antigen presenting cells in the spleen. LDC were prepared using fluorescent BODIPY$^+$ liposomes (see Methods) to track their distribution and uptake by cells in the spleen following i.v. administration. Mice (4 per group) were injected i.v. with labeled LDC and spleens were collected 1 hour later; control mice were un-injected. Spleen cells were immunostained for flow cytometric analysis of antigen presenting cells (APC), as described in Methods. (**A**) Labeled liposomes were detected in CD11b+ spleen cells following i.v. injection, as depicted in this representative facs plot. (**B**) The mean percentage of cells containing labeled LDC (± SE) was determined for several different relevant spleen APC populations. The indicated populations were first gated and the percent of BODIPY positive cells calculated from that gate. The majority of labeled liposomes were detected in CD11b$^+$/Gr-1$^-$ cells (macrophages), CD11b$^+$/Gr-1$^+$ cells (neutrophils), and CD11c$^+$ cells (dendritic cells). In contrast, labeled liposomes were much rarer in NK cells. Similar results were obtained in 3 additional experiments.

LDC elicit NK cell production of IFN-γ and DC production of IL-12

We next assessed the functional effects of LDC on splenic APCs. Intravenous administration of LDC has been shown previously to elicit systemic production of pro-inflammatory cytokines, yet the cellular origin of these cytokines has not been previously identified *in vivo* (34). Therefore, intracellular cytokine staining was used to identify the cells producing two key cytokines (IL-12 and IFN-γ) that are largely responsible for generating Th1 polarized T cell responses.

Intravenous administration of LDC was found to elicit production of very high levels of IFN-γ by splenic NK cells **(Figure 7A and 7B).** For example, nearly 45% of all NK cells in the spleen were positive for intracellular IFN-γ at the peak of the immune response following LDC injection. Production of IFN-γ peaked between 4 hours and 24 hours after administration of LDC. Moreover, it appeared that NK cells were the major source of IFN-γ production in the spleen, as very little intracellular IFN-γ was detected in T cells, DC, or macrophages following injection of LDC.

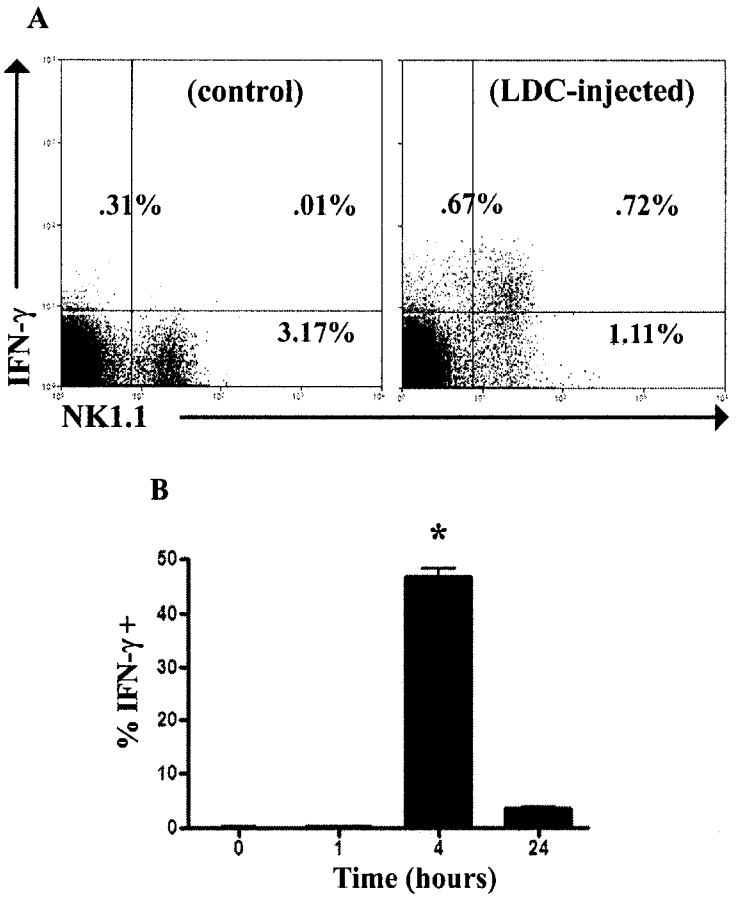

Figure 7: NK cell production of IFN gamma following i.v. injection of LDC. C57Bl6 mice (4 per group) were injected i.v. with LDC and spleen cells were collected at the indicated time points post-injection. Single cell suspension of spleen cells were prepared, then cultured for 4 hours *in vitro* in the presence of 10 ug/ml Brefeldin A. Cells were then immunostained for detection of intracellular IFN-γ as described in Methods. (**A**)Intracellular production of IFN-γ by splenic $NK1.1^+$ cells in control mice or in mice injected 4 hours prior with LDC. (**B**) Percentage of splenic NK1.1+ cells producing IFN-γ over time following i.v. injection of LDC. Only NK cells were found to express significant amounts of intracellular IFN-γ at any of the time points examined. * denotes significant differences ($p < 0.001$) compared to control mice, as determined by ANOVA. Data are representative of 3 independent experiments.

Intracellular cytokine staining for IL-12 production revealed that splenic DCs were the major cell type responsible for producing IL-12 in the spleen following injection of LDC

(Figure 8). Production of IL-12 was detectable as soon as one hour after injection of LDC, then peaked at 4 hours and declined thereafter (though not to baseline) by 24 hours after injection. At the peak of IL-12 production, approximately 3% of all splenic DC were producing IL-12. In addition, it appeared that DC were the only cells in the spleen capable of producing IL-12 in response to LDC, as intracellular IL-12 was not detected in other cell types. These data indicate therefore that injection of LDC elicited selective production of IL-12 and IFN-γ *in vivo* and that the cytokine response on a per cell basis was quite robust.

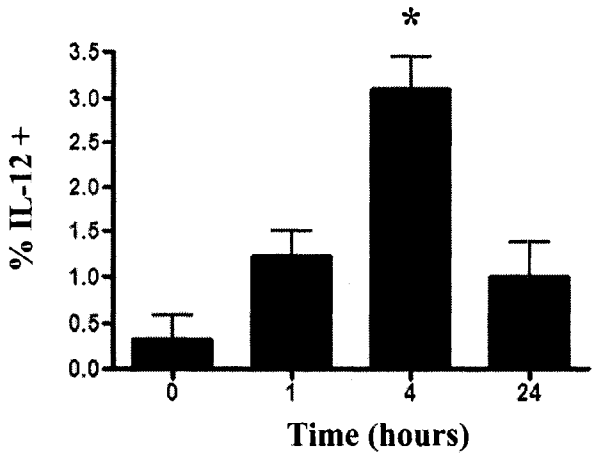

Figure 8: Production of IL-12 by splenic DCs following i.v. injection of LDC. C57Bl6 mice (4 per group) were injected i.v. with LDC and spleen cells were collected at the indicated time points post-injection. Single cell suspension of spleen cells were prepared, then cultured for 4 hours *in vitro* in the presence of 10 ug/ml Brefeldin A. Cells were then immunostained for detection of intracellular IL-12 by flow cytometry as described in methods. Data is depicing the percentage of splenic CD11C+ cells that are producing IL-12. * denotes significant differences ($p < 0.001$) compared to control mice, as determined by ANOVA. Data are representative of 2 independent experiments.

LDC injection triggers upregulation of co-stimulatory molecules on antigen presenting cells

The effects of LDC on expression of the key co-stimulatory molecule CD86 by APC in the spleen was assessed. Twenty-four hours after injection of LDC, significant upregulation of CD86 expression on both macrophages and DC in the spleen was noted **(Figure 9).** This response was maximal by 24 hours after injection of LDC, and also occurred on other cell types including B cells. Taken together, these data indicate that LDC have a marked stimulatory effect on APCs, triggering not only cytokine release but also upregulation of co-stimulatory molecules. In contrast, we did not observe evidence of systemic immune activation in mice injected with plasmid DNA only by the i.d. or i.m. routes. Thus, it is likely that strong activation of APCs and robust cytokine release accounts for the efficient induction of functionally active T cells by immunization with LDC.

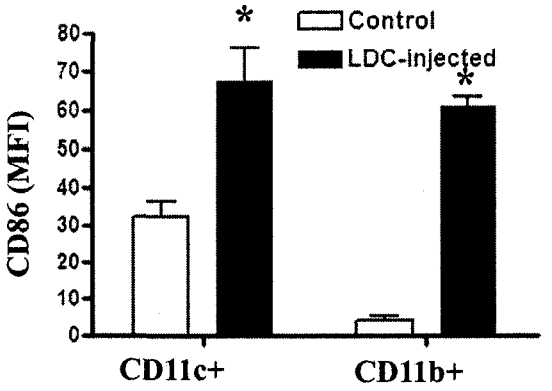

Figure 9: Activation of antigen presenting cells by i.v. injection of LDC. The response of splenic APC to injection of LDC was determined by flow cytometric assessment of co-stimulatory molecules. C57Bl6 mice (4 per group) were injected i.v. with LDC and spleen cells were collected and immunostained for detection of cell surface expression of co-stimulatory molecules. Upregulation of CD86 expression on CD11c+ and CD11b+ spleen cells was assessed by flow cytometry, 24 hours after i.v. injection of LDC. In both cell types, there was a significant increase ($p < 0.05$) in expression of CD86 following treatment with LDC.

LDC treatment can inhibit macrophage VEGF production in response to apoptotic tumor cell clearance

Finally, we assessed if LDC treatment could inhibit the ability of macrophage to produce VEGF in response to apoptotic tumor cell clearance. PECs were pretreated with LDC for 1 hr, washed, and than treated with apoptotic jurkat cells. LDC was able to inhibit macrophage production of VEGF in a dose dependant manner **(Figure 10)**. A MTT assay was also preformed to assure that the decrease in VEGF production was not simply the result of cytotoxicity to the LDC. This suggests that in addition to activating

macrophages, LDC can inhibit their ability to produce VEGF in response to apoptotic cell stimuli.

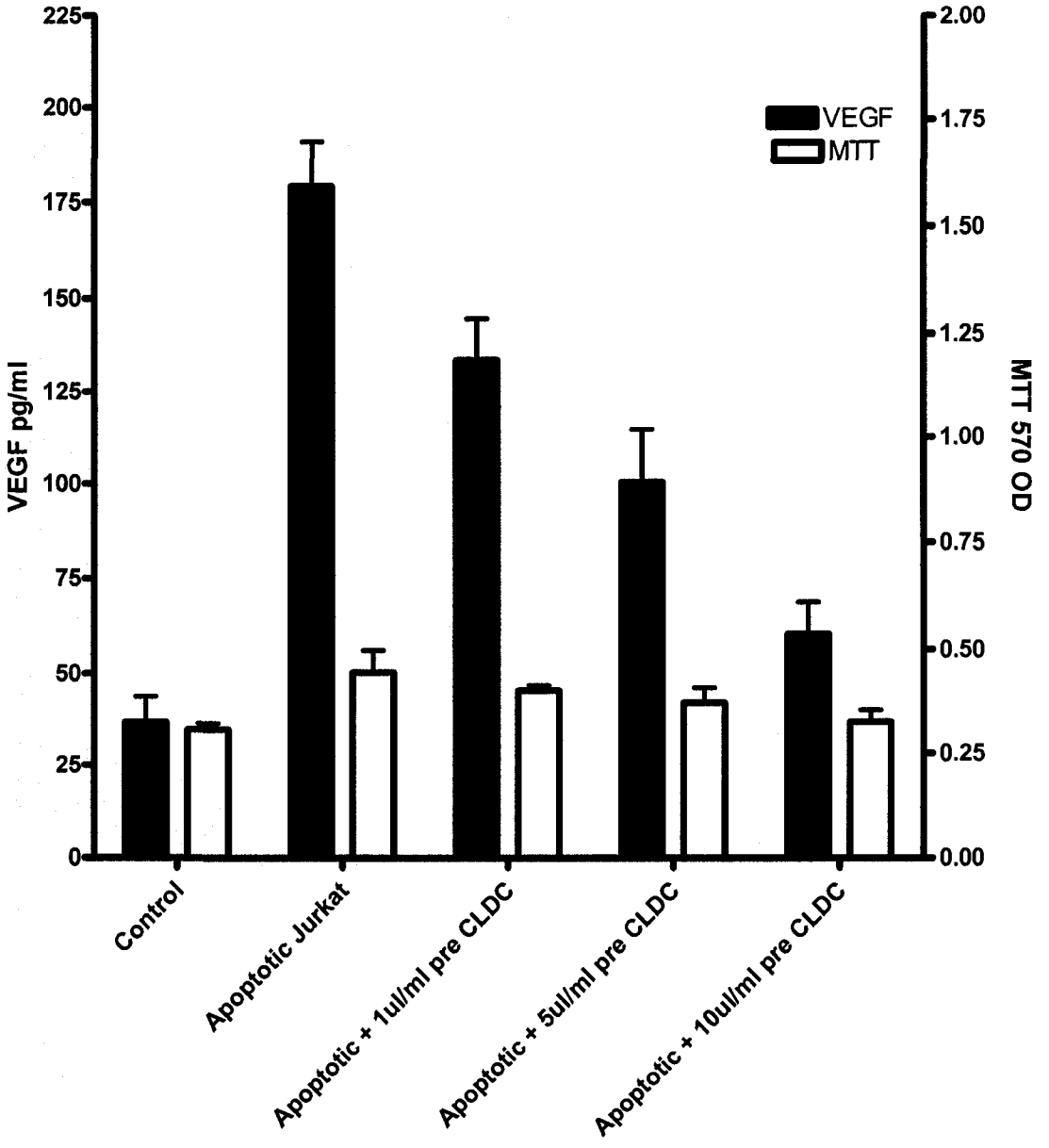

Figure 10: LDC abrogates the macrophage VEGF response to apoptotic tumor cells. ICR thioglycollate elcited PECs pretreated with varying concentrations of LDC for 1 hr prior to the addition of apoptotic jurkat targets. Follwing a 36 hr incubation VEGF was measured in culture supernatants by ELISA (Black Bars). MTT was also preformed to insure that the results were not a artifact of cell viability (White Bars). Four Replicates per group. Experiment repeated 3 times. Mean +/- SEM.

DISCUSSION

The results of the studies reported here indicate that immunization with LDC is an effective means of generating therapeutic anti-tumor immunity with plasmid DNA. The enhanced anti-tumor activity elicited by LDC immunization relative to immunization with conventional plasmid DNA vaccines was mediated in part by generation of $CD8^+$ T cells with increased functional activity. The additional non-specific anti-tumor effects elicited by activation of innate immunity with LDC probably also contributed to the overall anti-tumor activity observed. Studies of LDC targeting indicated that LDC were selectively taken up by splenic macrophages and DCs and initiate a TH1 biased cytokine profile, which may explain in part the unique ability of LDC to generate $CD8^+$ T cell responses.

The efficacy of DNA vaccines depends in part on the inherent immunogenicity of bacterial DNA and its ability to trigger elaboration of local Th1 cytokine responses (33, 45, 46). Therefore, increasing the inherent immunogenicity of plasmid DNA by complexing to cationic liposomes would be expected to potentially increase the overall effectiveness of a DNA vaccine. Therefore, we reasoned that LDC might serve as a more efficient means of delivering DNA vaccines for cancer. There is precedent for the improved efficacy of LDC-based vaccines. For example, liposomes have been shown previously to increase the effectiveness of DNA vaccines for mucosal immunization (47-

51). In our case, the i.v route of immunization with LDC may be particularly effective because of the strong induction of systemic innate immunity that occurs following i.v. delivery of LDC (Figure 7) (34). Intravenous administration of LDC probably also results in more efficient uptake of DNA by DCs in the spleen (Figure 6). In addition, cationic liposomes are known to promote endosomal targeting of plasmid DNA, thereby increasing the efficiency of activation of TLR9 and promoting MHC class I-restricted antigen presentation (52-54).

The systemic immunological responses triggered by intravenous administration of LDC may also synergize with T cell responses to augment anti-tumor activity. For example, the strong release of type I and II interferons triggered by LDC may have direct anti-tumor effects (35). Release of interferons may therefore render tumor cells more susceptible to attack by $CD8^+$ T cells. It is likely that the generation of Type I interferons plays a role in the generation of DC derived IL-12 and NK-cell derived IFN-γ described here (Figures 7 and 8). We also show that LDC therapy can activate DCs and macrophages as assessed by the up regulation of the co-stimulatory molecule CD86 (Figure 9).

We were also able to show that LDC could inhibit the ability of macrophages to produce VEGF in response to apoptotic cell clearance (Figure 10). It is possible that this is the result of LDC activating the macrophages and not allowing them to produce growth factors, such as VEGF, that are typical of a M2 phenotype or LDC could be inhibiting the ability of macrophages to physically engulf apoptotic cells. This is an important finding

because stimulation of tumor angiogenesis is one of the major means by which TAMs are thought to promote tumor growth(55, 56). Tumors with higher TAM density also produce higher levels of VEGF, a major stimulus for blood vessel growth(57-59). It has been shown that LDC are capable of inhibiting tumor angiogenesis *in vivo* in a large animal spontaneous tumor model (60). Our data suggests that a potential mechanism for this inhibition of tumor angiogenesis is that LDC may be targeting TAMs and inhibiting their VEGF production.

In summary, our findings indicate that LDC administered i.v. may be a particularly efficient means of generating therapeutic anti-tumor immunity. Such an approach could be considered for patients whose tumors express tumor antigens that are known $CD8^+$ T cell targets, including melanoma, renal cell carcinoma, prostate cancer, and colon cancer. Our finding show that LDC administration primarily target macrophages and DCs. This stimulates DCs to produce IL-12 and NK cells to produce IFN-γ. LDC therapy ultimately leads to the activation and upregulation of co-simulatory molecules on macrophages and DCs. Additionally, LDC can inhibit the ability of macrophages to produce VEGF, which could play an important role in the regulation of tumor angiogenesis.

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Chapter Five

Evaluation of a Liposome-DNA Complex Vaccine in Dogs With Hemangiosarcoma

ABSTRACT

Hemangiosarcoma (HSA) is a highly metastatic and often rapidly fatal tumor of dogs. At present, adjuvant chemotherapy is the only proven effective treatment for dogs with HSA, though the benefits from chemotherapy are modest. Administration of immunotherapy together with chemotherapy has also been reported to improve survival in dogs with HSA. Therefore, we evaluated safety and immunologic responses to a novel tumor vaccine administered together with doxorubicin chemotherapy in dogs with different stages of HSA. We hypothesized that tumor vaccination could be safely and effectively combined with doxorubicin chemotherapy for treatment of dogs with HSA.

Twenty-eight dogs with different stages of HSA were enrolled in the study. The HSA vaccine was prepared using lysates of allogeneic canine HSA cell lines mixed with an adjuvant comprised of liposome-DNA complexes (LDC). Dogs received a series of 8 immunizations administered over a 22-week period and most also received

chemotherapy. Clinical side-effects were noted, immune responses were measured by elisa and flow cytometry, and survival times were calculated.

The most common side-effects observed in vaccinated dogs also treated with doxorubicin chemotherapy were diarrhea and anorexia. Vaccinated dogs were found to mount strong humoral immune responses against a control antigen and most dogs also mounted antibody responses against canine HSA cells. Thirteen dogs with stage II splenic HSA that received the tumor vaccine plus doxorubicin chemotherapy had an overall median survival time of 182 days. We conclude that an allogeneic tumor lysate vaccine is safe in dogs with HSA and can elicit humoral immune responses in dogs that are receiving concurrent doxorubicin chemotherapy.

INTRODUCTION

Hemangiosarcoma is an aggressive and highly metastatic tumor of dogs. The tumor is thought to arise from transformed endothelial cells and typically occurs in the spleen, right heart, or skin(1-5). The most common cause of death in dogs with HSA is tumor rupture and hemorrhage, which is often complicated by coagulation abnormalities (6-8).

Survival times for dogs with the most common form of HSA (splenic HSA) are typically less than 2-3 months following splenectomy alone(4, 9-13). Several studies report a modest survival benefit from the addition of adjuvant doxorubicin (DOX)-based chemotherapy of HSA following surgical splenectomy(10, 12-14). A trial of combination therapy with minocycline, a putative angiogenesis inhibitor, and DOX chemotherapy failed to show a survival benefit over treatment with DOX alone(10). However, administration of the immune stimulant liposomal muramyl tripeptide phosphatidylethanolamine in combination with DOX-based chemotherapy did show a significant survival advantage compared to dogs treated with chemotherapy alone(15), suggesting that immunotherapy combined with chemotherapy may have utility in the treatment of HSA in dogs.

Targeted immunotherapy is promising as new treatment option for cancer in humans and companion animals. Unfortunately, there are currently few effective immunotherapeutics available for use in dogs with cancer. We have investigated a novel immune stimulant,

comprised of cationic liposome and DNA complexes (LDC), for use in cancer immunotherapy. We previously reported that LDC were very potent activators of innate immunity and anti-tumor activity in mice (16-19). More recently we also reported that intravenous administration of LDC with the IL-2 gene elicited immune activation and significantly prolonged survival times in dogs with metastatic osteosarcoma (20). Moreover, we also recently reported that infusion of LDC could inhibit tumor angiogenesis and cause tumor regression in some dogs with soft tissue sarcoma (21).

We hypothesized therefore that LDC could also be used as an effective cancer vaccine adjuvant in dogs. This hypothesis was based in part on a recent report where we found that LDC had potent adjuvant properties in mice (43). In addition, we also reported that LDC could be used as a vaccine adjuvant for immunization of dogs with refractory atopic dermatitis and that immunization with the LDC-based vaccine could reduce clinical signs and suppress the Th2 polarization of the T cells from atopic dogs (22). In addition, we showed recently that in cancer-bearing dogs the LDC adjuvant could be used recently to elicit humoral immune responses even in dogs receiving chemotherapy (28). Others have also reported that liposomes complexed with DNA can be used to produce effective vaccine adjuvants (23-25). However, LDC-based tumor vaccines have not been previously evaluated in dogs. Therefore, we conducted an open, phase I study of an allogeneic tumor vaccine based on the LDC adjuvant in 28 dogs with HSA. The purposes of the study were to assess safety and immunological responses in dogs receiving concurrent doxorubicin chemotherapy and also survival times in a subset of dogs with Stage II HSA.

The tumor vaccine was found to be safe in dogs with HSA, including many dogs treated concurrently with chemotherapy, with the major side-effects being transient diarrhea and vomiting. The vaccine was found to elicit humoral immune responses against control antigens and HSA tumor cells in most dogs that were vaccinated with while receiving concurrent DOX chemotherapy. The humoral immune responses that were elicited appeared to be largely tumor-specific inasmuch as humoral responses were not observed against irrelevant tumor cell lines. We conclude therefore that additional studies of tumor vaccination as adjuvant therapy for dogs with HSA are warranted

MATERIALS AND METHODS

Patients

Twenty-eight dogs with HSA (2 with stage I, 18 with stage II and 8 with stage III disease; Table 1) were recruited by participating oncologists the United States to participate in this open-label phase I study (oncologists other than those at Colorado State University and the Veterinary Cancer Specialists included: Drs. Steve Atwater, Michele Cohen, Steve Crow, Juliana Cyman, Ravi Dhaliwal, Sue Downing, Kim Freeman, Lisa Fulton, Joe Impellizeri, Liz Korgosien, Cheryl London, Susan Plaza, Gerry Post, Philip Reis, Carlos Rodriguez, Mona Rosenberg, Cecile Siedlecki, Avanelle Turner, and Courtney

Zwhalen). The protocol for these studies was approved by the Animal Care and Use Committee and the Clinical Review Board at Colorado State University. A histologic diagnosis of HSA was required for entry into the study. Patients were excluded from participation if they had serious concurrent medical diseases, including renal failure or hepatic disease, or had been treated in the preceding two weeks with systemic corticosteroids. Staging and administration of chemotherapy was performed according to standard of practice at each participating institution. Dogs were staged as follows: Stage I, tumor < 5 cm diameter and confined to the primary tumor site, Stage II, tumor > 5 cm diameter, with SC invasion, tumor rupture (eg, splenic HSA), and/or regional lymph node spread; Stage III, tumor with measurable metastases, or multicentric disease. Follow-up assessment for side-effects and treatment responses was done by telephone survey of participating veterinarians and/or veterinary technicians at each practice.

Survival times and disease-free intervals were calculated for the subset of 13 study dogs with Stage II splenic HSA that received the tumor vaccine plus DOX chemotherapy. In addition, we compiled historical data (cases evaluated from 1996 to 2006) for 24 dogs with Stage II splenic HSA that were treated only with DOX chemotherapy from two participating institutions (Colorado State University and Veterinary Referral Center of Colorado). The survival times and disease-free interval data for these 24 historical control dogs were calculated and compared to survival times and disease-free interval times for study dogs.

Vaccine Preparation

The HSA vaccine was prepared using lysates from two canine HSA cell lines (DEN-HSA and Fitz-HSA). The endothelial derivation of one of these cell lines (DEN-HSA) was described recently(26). Tumor lysates were prepared from equal numbers of pooled HSA cells, which were washed extensively in PBS to remove any residual FBS, then resuspended in distilled water and subjected to 4 freeze-thaw cycles, followed by sonication (Microson XL, Misonix Inc, Framingham, NY). The cell lysate solution was then centrifuged at 1,500 X G and filtered through a 0.22 um filter, adjusted to a final protein concentration of 5 mg/ml, and stored at -80°C until used.

The LDC vaccine adjuvant was prepared as described previously (16). Briefly, equimolar amounts of the cationic lipid 1,2-diacyl-3-trimethylammonium propane (DOTAP) and cholesterol (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, then dried down to a thin film in round-bottomed tubes in a vacuum desiccator (Virtis Benchtop lyophilizer, Virtis Co, Gardiner, NY). Liposomes were prepared by rehydration in a 10% sucrose solution, followed by filtration, as described previously(27). Liposome-DNA complexes were prepared by adding plasmid DNA (non-coding plasmid DNA with < 0.05 EU/ml endotoxin content; Althea Technologies, San Diego, CA) to liposomes in 10% sucrose to form LDC, as described previously(16). The vaccine was then prepared by adding tumor lysate to the liposome-DNA complexes at a concentration of 5 mg tumor lysate antigen per 4 ml LDC, followed by gentle mixing. In addition, 50 ug keyhole limpet hemocyanin (KLH; Sigma-Aldrich Chemical

Company, St Louis, MO) was added to each vial of vaccine as a control antigen. The vaccine was aliquotted to vials (4 ml per vial) and frozen to -80C, after which the vials were lyophilized. Following lyophilization, the vials were capped and stored at -20°C until used.

Vaccine administration schedule

Patients enrolled in the study were scheduled to receive a series of 8 tumor vaccines, administered over a 22-week period. The first 5 vaccines were administered once every other week, while the remaining 3 vaccines were administered once monthly. The vaccine was administered intraperitoneally, based on data in mice which indicated superior immune responses following vaccination by that route (43). The vaccine was rehydrated at room temperature to a 10 ml final volume and administered in a single intraperitoneal injection site. Dogs weighing >10 kg received 10 ml of vaccine, while dogs of <10 kg body weight received 5 ml of vaccine. Treated dogs were monitored for signs of gastrointestinal disturbances (nausea, vomiting, diarrhea), abdominal pain (vocalization, licking injection site; looking at abdomen) and fever (body temperature) in the hospital for side-effects for the first 6-8 hours following administration of the first vaccine.

Determination of KLH antibody responses

Humoral immune responses to vaccination were assessed using serum samples obtained pre-vaccination and on week 8 of the study (after the third vaccine) from 6 patients with stage II splenic HSA. Within this group of 6 patients, 5 were treated concurrently with DOX and vaccine, while one dog (Dog #2, Figure 2) was treated with DOX prior to vaccination. Serum was stored frozen at -20°C prior to immunological evaluation. Anti-KLH antibody responses were assessed using an ELISA assay, as described previously (28). Briefly, KLH-specific antibody titers were determined using ELISA plates coated with KLH at a concentration of 5 μg/ml. Plates were pre-blocked with nonfat dried milk prior to incubating serum samples. Serum samples were diluted 1:8,000 in PBS and 1% bovine serum albumin prior to addition to the elisa assay. Plates were washed and incubated with goat-anti dog IgG conjugated to HRP, then washed again and incubated with substrate solution. The optical density of the wells in the plate was determined using an automated optical density reader (Multiskan Ascent, Thermo Lab Systems, Waltham, MA).

Flow Cytometric assessment of Anti-HSA antibody responses

The effects of vaccination on development of antibody responses against canine HSA cell lines was assessed using flow cytometry to detect cell surface antibody responses. The

two canine HSA cell lines (DEN-HSA and Fitz-HSA) used to prepare the vaccine were also used for the serological studies. For flow cytometric analysis, serum samples were diluted 1:100 in FACS buffer (PBS with 2% fetal bovine serum and 0.1% sodium azide; Sigma-Aldrich Chemical Company, St Louis, MO) (dilution based on prior assay optimization experiments; data not shown) and then incubated with viable HSA cells. The HSA cells were detached by brief typsinization and then added at a concentration of 1 X 10^5 cells per well in a 96-well round bottomed plate to wells containing pre-diluted test serum and incubated for 30 minutes at 4°C. The cells were then washed twice in FACS buffer, incubated with pre-diluted PE-conjugated goat anti-dog IgG antibody (Jackson ImmunoResearch, West Grove, PA) for 20 minutes at 4°C, then washed and then fixed in 1% paraformaldehyde (Sigma-Aldrich Chemical Company, St Louis, MO) prior to analysis. Controls included HSA cells incubated with secondary antibody only and cells incubated with serum from control dogs without cancer.

Additional controls for tumor specificity of humoral responses included assessment of antibody binding to three different irrelevant canine tumor cell lines. These cell lines included one canine melanoma cell line (developed in our laboratory) and two canine osteosarcoma cell lines (D-17 and Abrams, kindly provided by Dr. G. MacEwen, University of Wisconsin-Madison). These cell lines were incubated with patient pre- and post-vaccination serum diluted to the same concentrations as used for analysis of HSA cell lines.

Cell surface binding of dog antibodies was assessed using a Cyan flow cytometer (DakoCytomation, Ft Collins, CO) and data analysis was done using Summit software (DakoCytomation, Ft Collins, CO). Gates for determining the degree of antibody binding were set on live cells based on forward and side-scatter characteristics. Analysis gates for each sample were adjusted to allow approximately 1-2% positive events, based on the degree of autofluorescence observed in each of the unstained cell lines. Positive responses to vaccination were defined as at least a doubling of the percentage of antibody-positive cells when pre- and post-vaccination serum samples were compared between the same dogs using the same cell line.

Statistical analyses

Disease-free intervals and overall survival times were calculated for 13 dogs with stage II splenic HSA that received treatment with tumor vaccine plus DOX chemotherapy. Four of the 17 Stage II HSA dogs in the study population (see Table 1) were excluded from this analysis because although they were vaccinated with the tumor vaccine, they were not treated with DOX chemotherapy. In addition, survival times and disease-free intervals were also calculated for 24 historical control dogs with Stage II splenic HSA that were treated with DOX only. Comparison of survival and disease-free interval times between study dogs and historical control dogs was done using Kaplan-Meier curves generated using GraphPad software (San Diego, CA). Comparison of pre- and post-

vaccination antibody titers was done using the Wilcoxon signed rank test. A p value of < 0.05.was considered significant for all statistical analyses performed in this study.

RESULTS

Patient Population Characteristics

Twenty-eight dogs were enrolled in the study and complete follow-up was obtained for all of the animals (**Table 1**). Of these 28 dogs, 21 dogs had primary splenic HSA (one stage I, 17 stage II, 3 stage III), 4 dogs had primary cutaneous HSA (one stage I, one stage II, 2 stage III), 2 dogs had primary renal HSA (stage III), and one dog had primary cardiac HSA (stage III). Five dogs (one with stage I disease, three with stage II disease, and one with stage III disease) received the vaccine but did not receive chemotherapy. Of the 17 dogs with stage II splenic HSA that received the HSA vaccine, three did not receive any chemotherapy, one was treated with low-dose cyclophosphamide therapy and 13 dogs received DOX chemotherapy. Two of the stage II dogs treated with DOX chemotherapy received the VAC protocol (DOX plus cyclophosphamide and vincristine). The HSA vaccine was administered concurrently with chemotherapy protocols, generally on alternating weeks.

Tumor Stage	# of Dogs
Stage I	
Splenic	1
Skin	1
Stage II	
Splenic	17
Skin	1
Stage III	
Splenic	3
Skin	2
Cardiac	1
Renal	2

Table 1: Characteristics and staging of patients enrolled in the HSA vaccine trial.

Toxicity and side effects

The vaccine was well tolerated by most dogs enrolled in the study. The most common side-effect observed (see **Table 2**) was mild to moderate diarrhea that occurred after at least one treatment in 5 of 28 treated dogs (2 dogs grade I toxicity, 3 dogs grade II), as determined by the VCOG toxicity scale (44). Other side-effects included mild to moderate anorexia in 3 treated dogs (one grade I, two grade II toxicity). Other side-effects noted included mild to moderate vomiting in 2 dogs (one grade I and one grade II toxicity), mild fever in 2 treated dogs (two grade I toxicity), a mild vaccine site reaction in one dog (grade I toxicity), abdominal pain in one dog (grade I toxicity), and one dog exhibited weakness and muscle tremors (grade I toxicity). None of the side-effects

necessitated in-hospital treatment or discontinuation of the vaccine. It should be noted that many of these same-side effects (especially diarrhea) also occur in dogs treated with DOX chemotherapy alone (29-31). Therefore, it was not possible from the design of this study to ascertain whether the toxicity observed was due to the effects of vaccination alone or to chemotherapy alone. Nevertheless, the observed incidence of GI side-effects in this study (17%) is consistent with the incidence noted with DOX alone, suggesting that the combination of vaccination with DOX chemotherapy did not increase the overall incidence of side-effects typically observed with DOX chemotherapy alone(30).

Side Effect	No. Affected	% Affected
Diarrhea	5/28	17.8
Anorexia	3/28	10.7
Vomiting	2/28	7.1
Fever	2/28	7.1
Abdominal Pain	1/28	3.5
Vaccine Site reaction	1/28	3.5
Weakness, tremors	1/28	3.5

Table 2: Side-effects associated with immunization with a HSA tumor lysate vaccine combined with DOX chemotherapy in dogs with HSA.

Vaccine-induced antibody responses against a control antigen

A control antigen (KLH) was incorporated into the vaccine to allow assessment of humoral immune responses elicited by the vaccine. Serum samples obtained from 6 dogs prior to vaccination and after the third vaccination were assessed for anti-KLH antibody

titers. All 6 vaccinated dogs evaluated mounted strong antibody responses against the KLH antigen incorporated into the vaccine and there was a significant difference ($p = 0.03$) when pre- and post-vaccination antibody titers were compared (**Figure 1**). These results indicated therefore that the LDC vaccine adjuvant was capable of eliciting strong humoral immune responses against a novel antigen (KLH) in dogs with cancer treated concurrently with DOX chemotherapy.

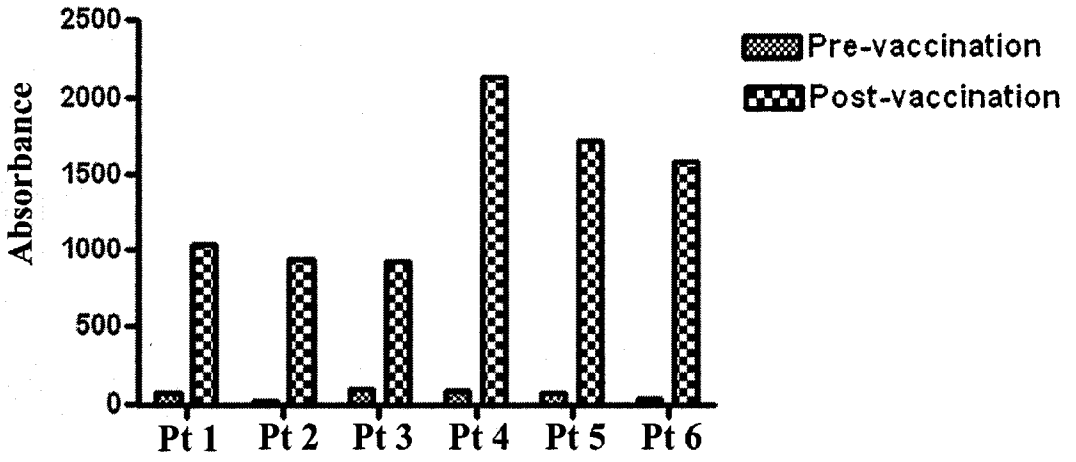


Figure 1: Development of antibody responses to a control antigen in 6 dogs following immunization with tumor vaccine and chemotherapy. Serum was obtained from 6 dogs with Stage II splenic HSA that were vaccinated and treated with DOX chemotherapy. Serum was obtained prior to immunization and after immunization with the HSA vaccine, which contained 50 ug KLH in addition to HSA lysate antigens. Serum was assessed for anti-KLH antibodies by elisa, as described in Methods. When pre- and post-vaccine anti-KLH titers were compared, vaccinated dogs developed significant ($p < 0.03$) antibody responses.

Generation of immune responses against HSA cells

We next assessed the ability of the HSA lysate vaccine to elicit antibody responses against canine HSA cells. This was done using flow cytometry to assess pre- and post-vaccination serum samples from for the presence of antibodies against cell surface determinants expressed on two different canine HSA cell lines (**Figure 2**).

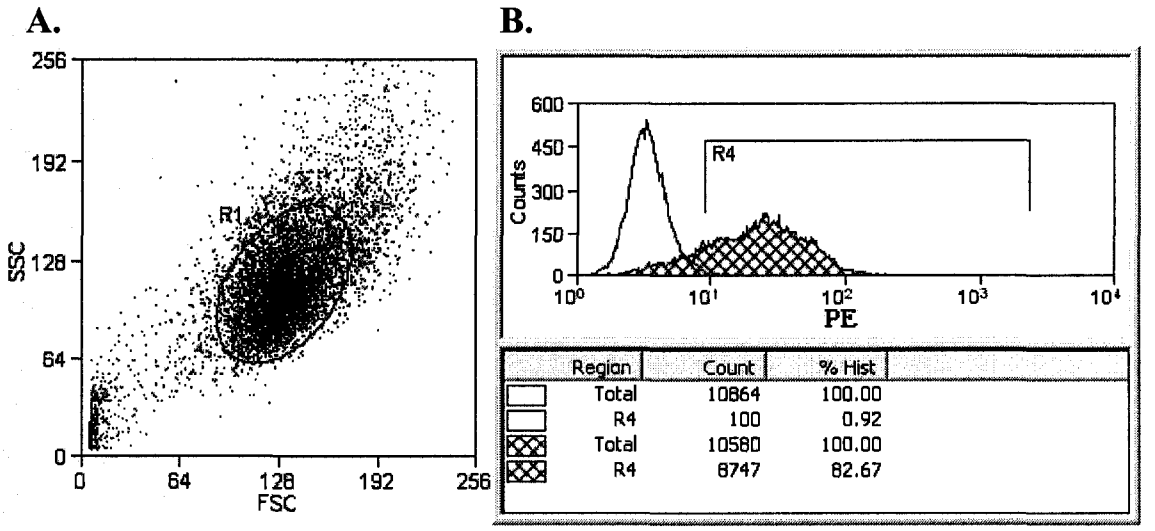
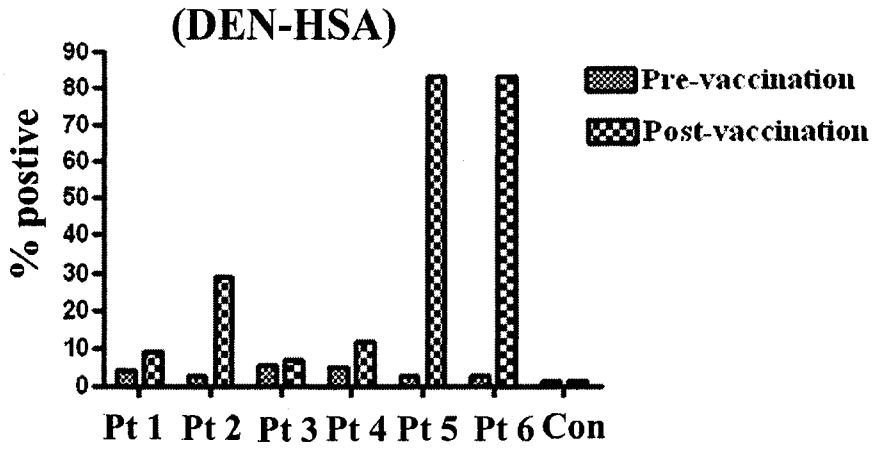


Figure 2: Example of how antibody responses were assessed against HSA cells following vaccination with tumor vaccine. HSA cells were incubated with test serum for 30 min and than labeled with PE-conjugated goat anti-dog IgG antibody. With flow cytometry live cells were gated by SSC x FSC characteristics (A) and the number of PE positive events quantified in pre-vaccine serum samples (open plot) and post-vaccine serum samples (hashed plot) (B).

Positive responses were considered to be those where the percentage of positive staining cells was at least two times greater in post-vaccination samples than in pre-vaccination serum samples from the same dog. Based on this analysis, we found that 5 of 6 dogs had

an increase in antibody responses against the DEN-HSA cell line, while 4 of 6 dogs had an increase in antibody responses against the Fitz-HSA cell line (**Figure 3**). There was a significant difference ($p = 0.05$) when pre- and post-vaccination antibody responses against the DEN-HSA cell line were assessed, but the differences in pre- and post-vaccination responses were not significantly different ($p = 0.12$) against the Fitz-HSA cell line. Due to the relatively small number of samples evaluated, we were unable to correlate antibody responses with survival times (see below). The fact that dogs mounted stronger and more consistent antibody responses against the foreign antigen KLH than against HSA tumor cells most likely reflects the greater degree of immune tolerance elicited against tumor antigens than against foreign antigens such as KLH.



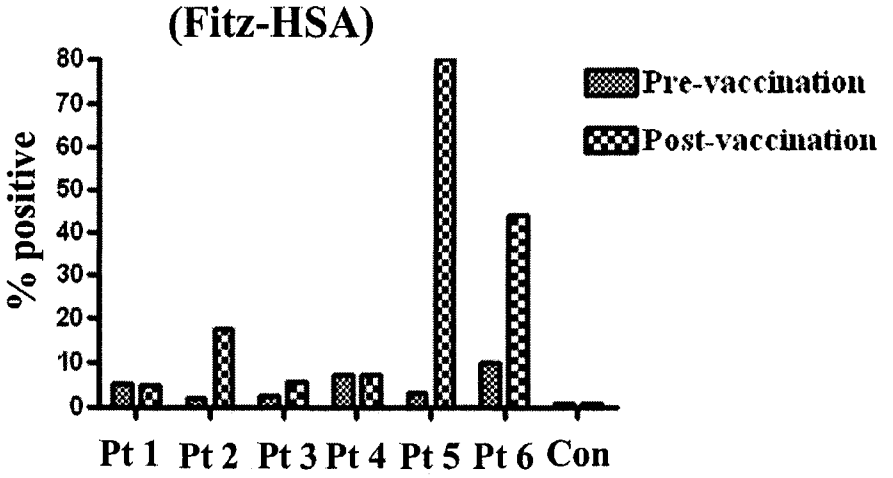


Figure 3: Development of antibody responses against HSA cells following vaccination with tumor vaccine. Serum was obtained prior to immunization and following vaccination with the HSA tumor lysate vaccine and development of antibody responses against cell surface determinants on two different canine HSA cell lines (DEN-HSA and Fitz-HSA) was assessed using flow cytometry, as described in Methods. There was a significant difference ($p = 0.05$) in pre- and post-vaccine titers against DEN-HSA cells, while the difference in pre- and post-vaccine titers against Fitz-HSA cells did not reach the level of significance ($p = 0.12$).

To control for induction of non-specific antibodies by the tumor lysate vaccine, we also assessed binding of pre- and post-vaccination serum samples to 3 non-HSA canine tumor cell lines (one canine melanoma cell line, two canine osteosarcoma cell lines). These cells were prepared and incubated with serum for flow cytometry exactly as described for the HSA cell lines. We found that there was no reactivity against either of the two canine osteosarcoma cell lines when pre- and post-vaccination serum samples were evaluated by flow cytometry (**Figure 4A and 4B**). Against the canine melanoma cell line, there was increased binding of post-vaccination serum from patients #1 and #3, while there was no increased binding by post-vaccination serum from the other 4 patient serum samples (**Figure 4C**). These data suggest therefore that the majority of the increase in antibody binding to HSA cell lines was due to induction of tumor-specific humoral immune responses. However, since there was reactivity of two patient serum samples against a canine melanoma cell line, we cannot completely exclude the possibility that some of the reactivity observed against HSA cell lines may have been due to induction of humoral immune responses against alloantigens. Alternatively, the melanoma cells may have expressed tumor antigens also expressed by the HSA cells.

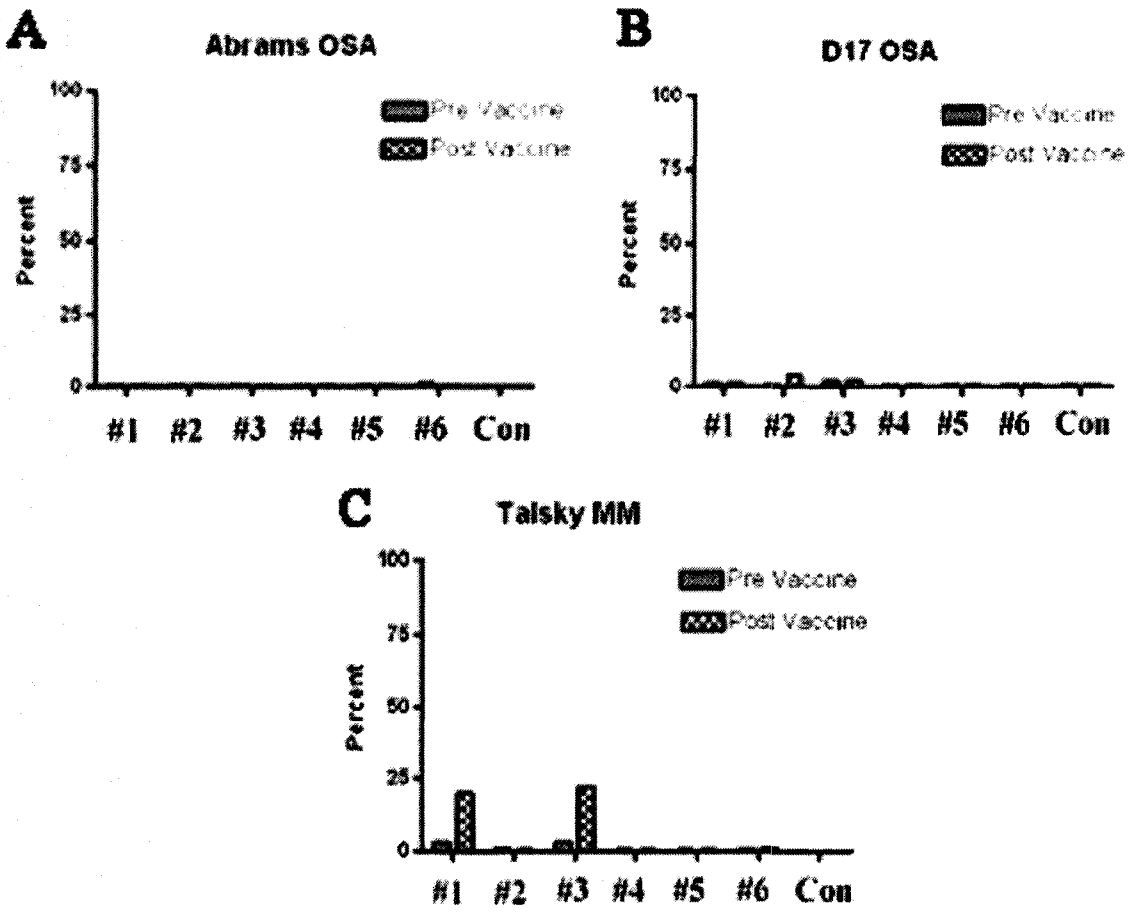


Figure 4: Assessment of antibody responses against irrelevant tumor cell lines. Serum from six dogs vaccinated with the HSA vaccine and reactivity against three non-HSA cell lines was assessed, using flow cytometry as described in Methods. Immune reactivity against two canine osteosarcoma cell lines (Abrams OSA and D17 OSA) and one canine melanoma cell lines (Talsky MM) was assessed. Antibody binding to the osteosarcoma cell lines was not observed (data not shown), while 2 of 6 vaccinated dogs (patients #1 and #3) had reactivity against the Talsky melanoma cell line.

The median disease-free interval for 13 study dogs with stage II splenic HSA treated with vaccine and DOX chemotherapy was 146 days and their median overall survival time was 182 days. The median disease-free interval for the 24 historical control dogs with stage II splenic HSA treated with DOX chemotherapy only was 126 days and their median overall survival time was 133 days (**Figures 5 and 6**). Comparison of overall survival times revealed a statistically significant increase in survival times in study dogs compared to historical control dogs, whereas disease-free intervals were not significantly different between the two groups (**Figures 5 and 6**). .

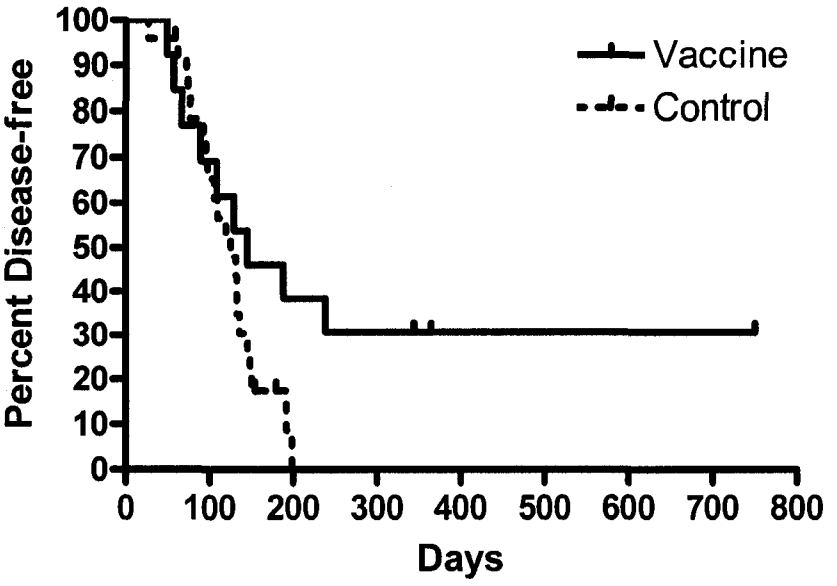


Figure 5: Assessment of disease-free interval for vaccine and DOX treated dogs versus historical DOX treated dogs . The median disease-free interval for 13 study dogs with stage II splenic HSA treated with vaccine and DOX chemotherapy was 146 days while that of historical control dogs treated with DOX chemotherapy was 126 days. Kaplan-Meier p value = 0.08.

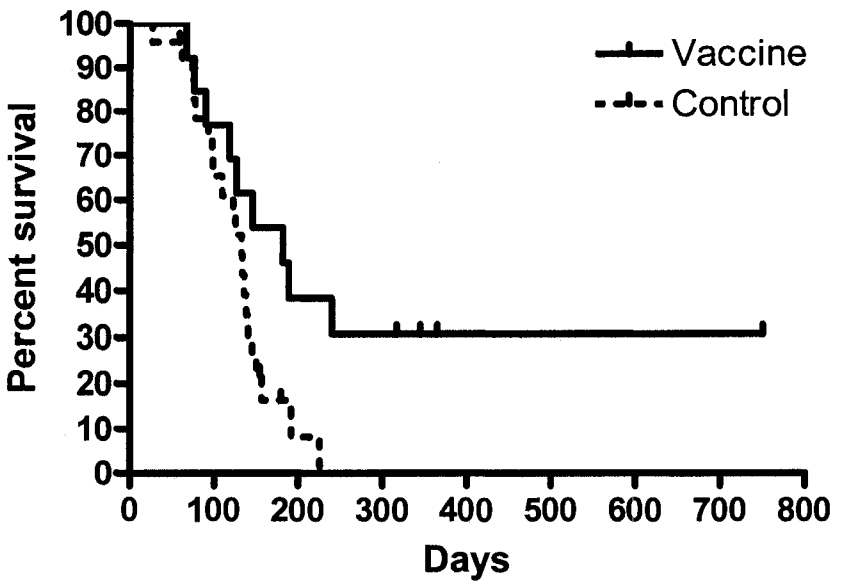


Figure 6: Assessment of survival curves for vaccine and DOX treated dogs versus historical DOX treated dogs . The median survival times for 13 study dogs with stage II splenic HSA treated with vaccine and DOX chemotherapy was 182 days while that of historical control dogs treated with DOX chemotherapy was 133 days. Kaplan-Meier p value = 0.03

DISCUSSION

Several important findings emerged from the studies reported here. For one, we found that a novel tumor lysate vaccine utilizing a potent vaccine adjuvant (liposome-DNA complexes) was well-tolerated in dogs with a variety of different stages of HSA. The most common side-effects were transient diarrhea and anorexia. These side-effects have also been reported previously in studies where the LDC were administered intravenously

to dogs for gene delivery or non-specific tumor immunotherapy (20, 21). However, these side-effects are often noted in dogs treated with DOX chemotherapy alone(30). Therefore, it is not clear from this study which side-effects were elicited by the vaccine and which by DOX chemotherapy. However, it is apparent that an overall increase in the expected frequency of possible DOX-related toxicity was not observed. Another important finding was the fact that the HSA tumor lysate vaccine was capable of eliciting humoral immune responses against canine HSA cell lines and against a novel antigen incorporated into the vaccine in dogs that were receiving concurrent DOX chemotherapy. Finally, administration of the vaccine together with DOX chemotherapy may have increased survival times over treatment with chemotherapy only, though these last results will require confirmation in a prospective, randomized trial.

Tumor vaccines can be prepared using several different technologies, and each has advantages and disadvantages when used for designing vaccines for use in canine cancer patients. Autologous whole tumor cell vaccines have been evaluated extensively in humans, but with the exception of GM-CSF transfected tumor cell vaccines, this approach has not been extensively adopted (32-34). Autologous tumor cell vaccines have major drawbacks related to difficulty in establishing cell lines and preparing vaccines in a timely and efficient fashion. Since few true tumor antigens have been identified in tumors of dogs and none in the case of HSA, recombinant tumor antigen vaccines are unlikely to be of much use in the immunotherapy of HSA in dogs. Moreover, clinical experience in human tumor vaccine trials suggests that whole tumor cell vaccines are

likely to be more effective than single tumor antigen vaccines, despite the greater difficulty associated with the preparation of whole cell vaccines (32).

Dogs with melanoma have been successfully immunized using DNA vaccination against the human melanoma antigen tyrosinase (35). In addition, an allogeneic dog melanoma cell line transfected with human gp100 has also been used to vaccinate dogs with melanoma (35, 36). However, similar human tumor antigens are not available for use against other common tumors of dogs, such as lymphoma, mast cell tumor, or HSA. Dendritic cell (DC) vaccines have received a great deal of attention over the past several years and there is one report of the use of DC vaccination against cancer in dogs (37). However, the preparation of DC vaccines is quite labor intensive and costly and requires use of autologous DC, along with autologous tumor cell lysates, thereby severely limiting their applicability in veterinary medicine.

Allogeneic tumor vaccines prepared from canine tumor cell lines are an attractive alternative to autologous tumor vaccines. This approach holds promise because many tumors of the same histiotype may share common antigens, so that vaccination against one tumor may elicit cross-reactive immune responses against other tumors of the same type (32, 38, 39). An allogeneic tumor lysate vaccine for melanoma has been evaluated in a number of clinical trials in humans and has shown clinical benefit in an adjuvant setting (40, 41). Vaccines prepared using tumor cell lysates are particularly promising because of the ease of preparation, storage and shipping of the vaccine. An allogeneic whole melanoma cell vaccine in dogs has shown promise in an early clinical trial (36).

Vaccines prepared using tumor lysates typically require use of strong vaccine adjuvants to elicit both cell-mediated and humoral immune responses against protein antigens. Prior studies in our lab have shown that LDCs can function as strong vaccine adjuvants in mice and also in dogs with atopy(22,43). Therefore, we designed a tumor lysate HSA vaccine for dogs that utilized pooled tumor lysates prepared from two canine HSA cell lines, in conjunction with the LDC adjuvant platform. The vaccine was administered by the intraperitoneal route because vaccine studies in mice demonstrated that this was the most effective route for eliciting T cell and antibody responses (43).

The HSA vaccine was evaluated in dogs with a variety of different stages of HSA to determine safety and immunological effectiveness. Some of side-effects that developed in dogs in this study were those that we have previously associated with activation of innate immunity by the LDC component of the vaccine (20). In addition, a number of side-effects observed, especially gastrointestinal side-effects such as diarrhea, are also commonly associated with DOX chemotherapy(29, 30). Therefore, it was impossible from the design of this study to fully distinguish vaccine-induced side-effects from those elicited by treatment with DOX alone.

While development of anti-HSA antibodies following vaccination was observed in most dogs in this study, the specificity of the immune response was not determined. For example, it is possible that the antibody response was directed against allogeneic MHC antigens on the HSA cells, rather than against novel HSA tumor antigens. Arguing

against this possibility is the fact that the vaccine was prepared using cell lysates, which are unlikely to contain MHC molecules that are typically associated with the cell membrane. Moreover, the tumor lysates were extensively washed to remove proteins that might have been present in the fetal bovine serum used to culture the cells. In addition, we did not observe antibody recognition of two unrelated canine osteosarcoma cell lines by pre- or post-treatment serum from HSA-vaccinated dogs, again arguing against recognition of allogeneic antigens (Figure 4). However, when a canine melanoma cell line was evaluated as a target for antibody binding, an increase in immunoglobulin binding by post-vaccination serum was observed in 2 of the 6 vaccinated dogs (Figure 4). This response against the melanoma cell line could represent an immune response against alloantigens or the presence of cross-reactive antigens shared between the HSA and melanoma cell lines. Assessment of humoral immune responses against autologous HSA cell lines, which was not possible in this study, would be necessary to help further resolve this issue.

The development of immune responses against normal endothelial cells is a theoretical concern with the use of vaccines prepared using HSA cell lines. This is because the HSA tumor arises from transformed endothelial cells and canine HSA cell lines have been shown to express normal endothelial cell antigens (3, 26). However, we did not observe side-effects that one might expect with the development of anti-endothelial immune responses, such as coagulopathies or thrombotic events.

Survival times in dogs treated with tumor vaccine plus DOX chemotherapy were also compared to those of disease-matched historical control dogs with stage II HSA. The median survival time for 13 dogs treated with vaccine plus DOX was 182 days, whereas the median survival time for the 24 historical control dogs treated with DOX only was 133 days. The survival time of 182 days found in the current study is longer than survival times reported with DOX only therapy in some studies (15), but is shorter than reported in other studies for dogs with stage II HSA treated with DOX or other chemotherapies (10, 13, 14). The reasons for these discrepancies are not fully apparent, but may be related to differences in staging of the tumors or to differences in case selection. The increase in survival times observed in vaccine plus DOX treated dogs suggests a positive interaction between the tumor vaccine and DOX chemotherapy. However, it is also important to note that statistical comparison of unrelated data sets such as these may introduce unintended bias and error into study results. Therefore, before these survival and disease-free interval results can be accepted, a randomized prospective trial comparing dogs treated with DOX alone to dogs treated with DOX plus vaccine would be necessary.

This study did not fully address the effect of timing of administration of the vaccine relative to timing of administration of DOX chemotherapy. Studies in mouse tumor models have shown that timing of administration of chemotherapy can have a significant impact on the efficacy of tumor vaccination (42). For example, DOX administration on the same day as or within 7 days after administration of tumor vaccine was optimal for enhancing anti-tumor immunity, whereas administration of DOX 7 days prior to

vaccination significantly inhibited tumor immunity. In our case, since DOX chemotherapy was given in repeated cycles, it was impossible to achieve the exact optimal timing of administration of DOX suggested in mouse studies. Additional studies in dogs would be required to more completely address these timing issues.

In summary, the results presented here, combined with results from a recent study by our group examining the effects of chemotherapy on immune responses in dogs with cancer, suggest that administration of DOX chemotherapy does not significantly impact generation of humoral immune responses to vaccination in dogs with cancer (28). The data also suggest that vaccination combined with chemotherapy may improve overall survival times. A previous study of the immunotherapeutic L-MTP-PE also found an additive effect of immunotherapy when combined with chemotherapy for HSA in dogs (15). Therefore, additional studies investigating the use of combined immunotherapy and chemotherapy for treatment of particularly aggressive tumors of dogs such as HSA and osteosarcoma are warranted. Critical questions remaining to be addressed include determining which chemotherapeutic drugs can be most effectively combined with immunotherapy, the optimal timing of chemotherapy with immunotherapy, and whether tumor specific immunotherapy (ie, tumor vaccines) are more effective than non-specific immunotherapy when combined with chemotherapy.

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Chapter Six

General Conclusions and Future Directions

The researched presented in this dissertation provides important insight into the biology of tumor-associated macrophages (TAMs). This research will help clarify the mechanisms which allow for TAM accumulation and induction of a pro-angiogenic phenotype. We also provide support that immunotherapy may be a viable treatment option in trying to limit the detrimental effects of TAMs. We specifically identify cationic lipid-DNA complexes (LDC) as a novel immunotherapy that elicits a strong antitumor T cell response, targets macrophages and inhibits their ability to produce VEGF, and is an effective and safe immunotherapy in canine cancer patients. We are hopeful that this research will lay the groundwork for further study into treatments for human cancers which can limit the impact of TAMs and potentially augment the effectiveness of current cancer therapies.

It is apparent from the work described here that type I IFNs play a critical role in tumor progression. The endogenous production of type I IFNs inhibit tumor growth and reduce tumor vascularity. We show that tumors grown in an IFNα/βR-/- mouse have an increase in TAM accumulation. It is likely that the increase in TAMs results in a better vascularized, faster growing tumor. Using *in vitro* experimentation, we were able to

show that type I IFNs inhibit the ability of macrophage precursor cells to respond to CSF-1 and differentiate into macrophages. Therefore, we conclude that the endogenous production of type I IFNs can limit the accumulation of TAMs which results in a decrease of TAM stimulated angiogenesis and subsequent slower tumor growth **(Figure 1).**

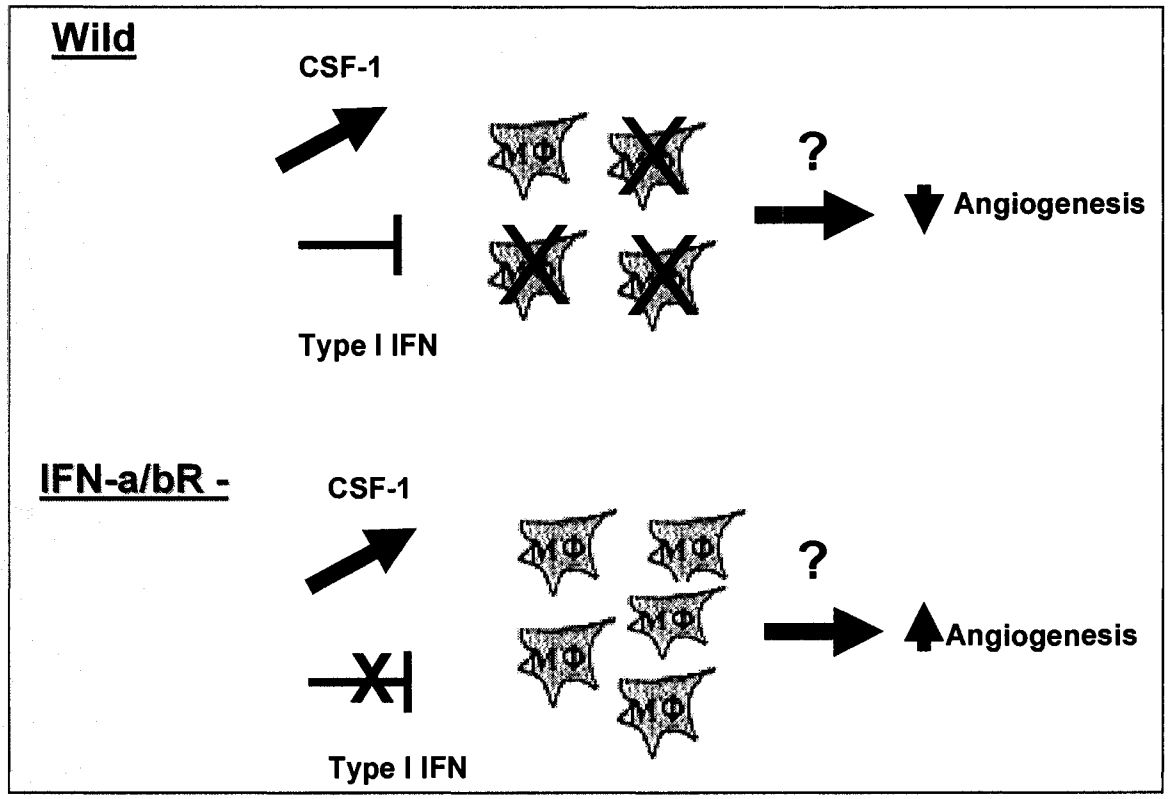

Figure 1: Schematic describing how type I IFNs inhibit macrophage (Mϕ) differentiation, resulting in an increase in tumor angiogenesis.

It is possible that the increase in TAM numbers in the IFNα/βR-/- mouse is simply the result of there being more access to tumor vasculature allowing for more monocyte migration into the tumor. We think that this unlikely since other model systems using the IFNα/βR-/- mice have also noted increases in macrophage numbers independent of vascularization. When IFNα/βR-/- mice are infected with listeria there is an increase in splenic and hepatic macrophage numbers which end up playing a protective role for these mice(1). Additionally, IFNα/βR-/- mice are described as being osteopenic when they become older as a result of increased osteoclast differentiation(2). This provides further evidence that the differentiation of a macrophage-like cell can be inhibited by type I IFNs. However, even with all the precedence in the literature the best way to refute the possibility of TAM accumulation being simply the result of increased vascular access would be to provide a direct link between the increase in TAM numbers and enhanced angiogenesis.

We were unable to identify a specific mechanism by which the TAMs in the IFNα/βR-/- mice enhanced angiogenesis in our system. Although there is ample literature that establishes a direct link between TAM accumulation and angiogenesis(3, 4), it would be important to understand the relationship with endogenous type I IFNs. This could be most completely studied by utilizing microarray technology to compare mRNA expression between tumor tissue of the WT or IFNα/βR-/- mice. Additionally, TAMs could be sorted from individual tumors and mRNA expression of TAMs directly could be compared. To exemplify the importance of TAMs in driving tumor angiogenesis and tumor growth within our model system, macrophages could be depleted with liposomal

clodronate to determine if tumor growth and angiogenesis decreased to a level that is comparable with that of the wild type mouse.

It would also be interesting to assess if type I IFNs play any role in the accumulation of myeloid derived suppressor cells (MDSCs). MDSCs are under intense investigation because of their propensity to enhance tumor progression by inhibiting CTL responses and promoting angiogenesis(5, 6).Type I IFNs may play a role in their differentiation since they are monocyte derived and closely related to the macrophage. Recently a more specific phenotype of the MDSC has been identified that would make it possible to quantify their numbers within the tumor, blood, and spleen of tumor bearing mice(5). This involves the use of IL4Rα in addition to GR-1 has a more specific phenotype for MDSCs.

Within chapter 3 we describe how the engulfment of apoptotic tumor cells induces macrophage VEGF production. A similar observation was first described by Golpin et al(7). We further show that this response is PS dependant and requires actual engulfment of the apoptotic cells. This response could have a profound impact on tumor biology for several reasons. First, the field currently believes that TAMs are induced to produce VEGF due to the influence of hypoxia and the transcription of HIF inducible genes. Our results could provide an alternative or complimentary explanation for the induction of VEGF by TAMs. Secondly, cancer therapies can induce massive tumor apoptosis while not effecting the TAM population. This would allow for large scale clearance of apoptotic cells by macrophages and result in the reciprocal production of growth and

angiogenic factors that could allow for tumor reoccurrence. Finally, with the identification of some tumors that constitutively externalize PS, it is possible that TAM engulfment of shed PS expressing tumor cell vesicles could induce the production of growth and angiogenic factors.

It is still necessary to establish a strong *in vivo* link between the clearance of apoptotic cells and TAM VEGF production. This can be achieved by examining TAM VEFG production following treatment with chemotherapy. Alternatively, TAM clearance of apoptotic cells could be blocked by using broad spectrum caspase inhibitors in combination with chemotherapy to inhibit the process of apoptosis and allow progression of cellular necrosis. The inhibitory effects that TAMs may be having on chemotherapy treatment could also be assessed through the depletion of TAMs using reagents like liposomal clodronate. It would be anticipated that TAM depletion would augment the effect of chemotherapy.

Since, TAMs have often lost their ability to be effective antitumor immune cells and are beneficial to tumor progression, it is important to explore immunotherapies that can target TAMs. By targeting TAMs it may be possible to "rescue" them from a pro-tumor phenotype and turn them into an antitumor effecter cells. The purpose of chapter 4 was to determine if LDC immunotherapy could be a platform to enhance the generation of therapeutic anti-tumor immunity through the combined activation of systemic innate and adaptive immune responses. In addition to the generation of a T cell mediated response we wanted to study if LDC could target macrophages, activate them, and inhibit their

ability to produce VEGF in response to apoptotic cells. We were able to determine that LDC therapy did target macrophages and activate them as assessed by up regulation of co-stimulatory markers. In addition, pretreatment of macrophages with LDC did inhibit their ability to produce VEGF in response to apoptotic cell engulfment.

Although looking at splenic macrophages is an important starting point in understanding the response of macrophages to LDC therapy, it needs to be determined how LDC affects TAMs specifically. This would include trying to assess if LDC can target to TAM populations and change them into an activated phenotype. It is known that LDC has potent antitumor properties but it is not known what contribution, if any, TAM activation plays(8-11). Since LDC can induce the production of type I IFNs, it would be interesting to determine if this can influence TAM accumulation(12). As we showed with the use of type I IFN receptor KO mice in chapter 2, type I IFNs can influence the accumulation of TAMs. Additionally, to help understand the role that type I IFNs are playing in the antitumor activity of LDC in general, the efficacy of LDC could be assessed in the IFNα/βR-/- mice and compared against the antitumor response in wt mice.

In addition to evaluating LDC in mouse models of cancer, we evaluated the safety and efficacy of LDC therapy in canine hemangiosarcoma (HSA) patients. We concluded that an allogeneic tumor lysate LDC vaccine is safe in dogs with HSA and can elicit humoral immune responses will receiving concurrent doxorubicin chemotherapy. There are still critical questions which need to be answered in the evaluation of LDC as a cancer immunotherapy such as determining which chemotherapeutic drugs can be most

effectively combined with immunotherapy, the optimal timing of chemotherapy with immunotherapy, and whether tumor specific vaccines are more effective than non-specific immunotherapy when combined with chemotherapy. However, this study was an important first step in the evaluation of LDC outside of mouse models and will set the stage for a prospective, placebo driven clinical trail.

The research described herein expands our understanding of the biology of TAMs. This work outlines mechanisms that regulate the accumulation of TAMs and their ability to promote angiogenesis. I believe that the identification and understanding of these mechanisms could open the door to new anti-cancer therapies. This work shows that not only does LDC immunotherapy induce a strong antitumor T cell response but it also may be able to target TAMs and inhibit their pro-tumor functions. Additionally, LDC immunotherapy not only induces a potent antitumor immunity in mouse models but is all safe and effective when combined with chemotherapy for the treatment of canine HSA. Taken together, these findings could uncover new avenues in which TAMs can be targeted and identified a novel immunotherapy as a potential candidate.

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