DISSERTATION

EFFECTS OF CHEMOTHERAPY ON INNATE AND ADAPTIVE IMMUNE RESPONSES

Submitted by

Barbara J. Biller

Department of Microbiology, Immunology and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2007

COLORADO STATE UNIVERSITY

April 13, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BARBARA J. BILLER ENTITLED: "EFFECTS OF CHEMOTHERAPY ON INNATE AND ADAPTIVE IMMUNE RESPONSES" BE ACCEPTED AS FULFILLNG IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Committee on Graduate Work

Adviser

Department Head/Director

ABSTRACT OF DISSERTATION

EFFECTS OF CHEMOTHERAPY ON INNATE AND

ADAPTIVE IMMUNE RESPONSES

Chemotherapy has historically been viewed as immunosuppressive; however studies suggest that some chemotherapeutic agents possess immunostimulatory effects. Despite the promise offered by new treatment modalities such as immunotherapy, novel therapies appear most effective when used in combination with traditional treatments such as chemotherapy. Therefore, there is considerable interest in the identification of chemotherapeutics that can be synergistically combined with cancer immunotherapy. Little is known, however, concerning the immunomodulatory effects of chemotherapy on many aspects of immunity.

Therefore, we evaluated the effects of common chemotherapy drugs on dendritic cells and lymphocytes, two cell types critical to the generation of effective immune responses. In addition, because regulatory T cells are known to directly suppress antitumor immunity in human cancer patients, we developed an assay to identify canine regulatory T cells and initiated the study of this T-cell subset in dogs.

Studies of the effects of chemotherapy on lymphocytes were conducted in pet dogs with lymphoma or osteosarcoma. Through evaluation of lymphocyte subsets and humoral antibody responses before, during, and after chemotherapy, we found that

chemotherapy had little impact on adaptive immune responses. Compared to healthy dogs, however, tumor-bearing dogs had abnormally low T-cell numbers and increased numbers of regulatory T cells. Our findings demonstrate the minimal impact of chemotherapy on lymphocyte numbers, suggesting that chemotherapy itself is not likely to interfere with induction of cell-mediated antitumor responses. However, underlying malignancy could play a role in suppression of antitumor immunity.

The impact of chemotherapy on dendritic cell function was assessed in mice. These studies demonstrated that the drug doxorubicin stimulated multiple aspects of dendritic cell function, including costimulatory molecule expression and antigen presentation. In mice with melanoma, administration of doxorubicin led to increased numbers of mature dendritic cells within tumor tissues and tumor-draining lymph nodes. These experiments suggest that doxorubicin is a promising candidate for combination with tumor-specific immunotherapy.

The studies presented here provide important insight into the effects of chemotherapy on immunity in healthy and tumor-bearing animals. Most importantly, they set the stage for future work investigating combinations of chemotherapy and immunotherapy towards the development of more effective treatments for cancer.

Barbara J. Biller
Department of Microbiology,
Immunology and Pathology
Colorado State University
Fort Collins, CO. 80523
Summer 2007

ACKNOWLEDGMENTS

I would like to sincerely thank the following individuals who contributed in many important ways to my doctoral studies. First and foremost, I am deeply grateful to Dr. Steve Dow for his endless guidance, support and kindness throughout my graduate program. His mentorship has not only made completion of this work possible, but has also provided me with the skills I will need to build a productive career in science and academia. I also am very grateful for the friendship, support and assistance of the many members of the Dow lab, in particular Katy Bosio, Lance U'Ren, Amanda Guth, Debra Kamstock, Shayna Warner, Beth Stallman, Andrew Goodyear, Joe Sottnik and Abby Jones. These people, and many others in the Pathology building, have helped to make the lab a fun, productive and stimulating environment in which to work and learn. I also wish to acknowledge the members of my graduate committee - Paul Lunn, Jill Slansky and Anne Avery, each of whom always had time to help me no matter how busy they were and whose insight and guidance were invaluable. I also would like to acknowledge the special guidance of Dr. Stephen Withrow who has always managed to point me in the right direction at the right time and has been a source of inspiration throughout my career.

Finally, I am forever grateful for the incredible support and understanding of my husband, Rob, and my son, Matthew, whose patience, humor and counseling have made all the difference in the world.

TABLE OF CONTENTS

Chapter One

Literature Review and Project Rationale

CONVENTIONAL CHEMOTHERAPY

Current Uses of Chemotherapy in Human and Veterinary Oncology

For more than 50 years chemotherapy has been a central component of treating cancer, especially in the management of metastatic disease and hematopoietic malignancies. During this time our understanding of the mechanisms by which chemotherapy induces tumor cell death as well as its effects on normal tissues of the body have dramatically increased. This insight is enabling the development of new avenues of treatment in which chemotherapeutic agents can potentially be used to simultaneously kill tumor cells and stimulate host defenses against the tumor itself.

Chemotherapy can be used in a number of different settings. The drugs and dosages given are dependent on the tumor type, stage of disease and numerous other factors such as the age and overall health of the patient, cost, and goals of therapy. *Primary*, or *neoadjuvant*, chemotherapy refers to initial drug therapy (ideally for localized disease only) that is typically followed by a definitive local treatment such as surgery or radiotherapy. This approach is most often used to decrease the size of large tumors with the goal of generating smaller treatment fields for either surgery or radiation therapy. In both human and veterinary oncology, *adjuvant* chemotherapy is utilized more

commonly than neoadjuvant therapy. It is administered after surgery or radiation therapy in patients for which the risk of tumor recurrence and/or metastasis is high.

One of the most successful uses of chemotherapy is in patients with hematologic malignancies such as Non-Hodgkin's lymphoma (NHL) or multiple myeloma. The key to this success has been the development of multi-agent protocols that combine chemotherapy drugs with differing mechanisms of action and non-overlapping toxicities. Prior to the use of multiple drug therapy, for example, less than 20% of children with NHL were cured (1). Current treatment regimes for the treatment of children with early stage NHL have now extended the 5-year survival rate to higher than 90% (2, 3). Similar success has been achieved in veterinary oncology as well, where the treatment of dogs with lymphoma using combination drug protocols has improved median survival times from less than 3 months to slightly more than 12 months, with 25% of dogs experiencing long-term remissions of greater than 2 years (4, 5).

For many patients however, chemotherapy is far less effective, especially when used in the adjuvant setting. In people with melanoma, for example, there are a fairly large number of drugs with some degree of activity against melanoma. However, the overall response rate (< 25% complete or partial responses) in patients with metastatic disease is poor despite the use of high-dose combination protocols (6). Adjuvant chemotherapy of high-grade carcinomas, such as those of the breast and colon, is similarly discouraging; despite the use of dose-intense chemotherapy, response rates continue to hover in the 20 – 50% range with remission durations of 18 months or less (7, 8).

One of the more successful uses of adjuvant chemotherapy has been in the treatment of canine osteosarcoma (OSA). This commonly occurring bone tumor is highly metastatic; approximately 90% of dogs have pulmonary micrometastatic disease at the time of initial diagnosis. Without adjuvant chemotherapy the median survival time for dogs with appendicular OSA is less than 4 months, however administration of cisplatin and/or doxorubicin following surgery has extended the median survival to 12-14 months (9-11). For malignancies other than OSA or lymphoma, however, chemotherapy for dogs or cats with metastatic disease is often typified by low response rates (10-25%) and short survival times (typically less than 6 months) depending on the stage of disease and tumor type.

The Limitations of Conventional Chemotherapy

The inability of chemotherapy to cure most non-hematopoietic malignancies or even to limit tumor cell growth for long periods of time is an inherent property of the manner in which chemotherapeutic agents kill tumor cells. Cytotoxic chemotherapy drugs induce DNA damage that prevents cellular replication, interferes with critical parts of the cell cycle and/or induces apoptosis. Cells that are rapidly proliferating are more sensitive to the effects of DNA damage; therefore chemotherapy is most effective against rapidly growing tumors. Tumor growth, however, generally follows Gompertzian growth kinetics which is characterized by rapid proliferation of tumor cells in the latent period (prior to detection) followed by progressively slower growth as the tumor enlarges and becomes clinically detectable (12). Thus by the time most tumors are detected the majority of tumor cells are in the resting (G_0) phase of the cell cycle and are therefore

relatively resistant to the cytotoxic effects of chemotherapy agents. This is particularly true in veterinary oncology where tumors are typically detected much later than they are in people.

The other major limitation of chemotherapy is its toxicity to normal cells in the body. This limitation is also a consequence of mechanism of action since it is the rapidly proliferating stem cells of the bone marrow, hair follicles, gastrointestinal and reproductive tracts that are most susceptible to the non-specific effects of chemotherapy. Thus, although it might be theoretically possible to deliver a sufficient dose of drug to kill every tumor cell present, the toxicity induced with this approach would be devastating and likely result in the death of the patient. A number of other factors, such as the development of biochemical drug resistance, upregulation of drug-inactivating enzymes and dysregulation of apoptosis or cell proliferation in the tumor cell, may also be present and limit the efficacy of chemotherapy. The end result is that the use of chemotherapy is rarely associated with a cure in the treatment of non-hematopoietic malignancies and most patients ultimately succumb to drug-resistant metastatic disease.

TUMOR VACCINES

Introduction

One of the newer approaches to cancer treatment is the use of targeted forms of immunotherapy, such as vaccine strategies that actively elicit the development of tumor-specific immunity. The goal of cancer vaccine therapy is to generate an antitumor immune response that results in clinical regression of a tumor or its metastases. There are several important differences between the use of tumor vaccines and conventional

chemotherapy that should be kept in mind. Unlike cytotoxic chemotherapy, tumor vaccines can be targeted specifically to neoplastic cells, thus sparing normal tissues and potentially providing protection against relapsing disease (13, 14). Responses to vaccine therapy must be evaluated differently than those to chemotherapeutic agents. Instead of inducing rapid tumor cell death that is detectable within a few days, clinical responses to vaccine therapy depend on the development of an adaptive immune response that may take several months or more to appear. The observation of "mixed responses" is another frequent problem in assessing treatment outcome; this phenomenon is characterized by the differential response to therapy of metastases within different tissues of the same patient. Factors such as these pose significant challenges to the design and assessment of clinical trials, especially when cancer vaccines are combined with other treatment modalities.

To overcome some of these obstacles, a new set of criteria was recently proposed by the National Cancer Institute. Termed RECIST (Response Evaluation Criteria in Solid Tumors), an objective clinical response is defined as a 30% reduction in the sum of the maximum diameters of lesions as well as the appearance of no new or progressive lesions (15). The RECIST response criteria greatly facilitate comparisons between trials as well as standardize the description of tumor responses.

Determination of appropriate immunological endpoints, in addition to tumor response is crucial to cancer vaccine and immunotherapy trial design. Methods to assess humoral and cellular immune responses, such as measurement of antibody titers and delayed-type hypersensitivity reactions, are critical in correlating immune response with clinical outcome. In addition, measuring the success or failure of a trial with more

realistic clinical endpoints, for example long-term stabilization of disease rather than induction of complete remission, will also facilitate the transition between basic research and the conduction of meaningful clinical trials.

The Use of Liposome-DNA Complexes in Tumor Vaccines

There are numerous tumor vaccine strategies under investigation in veterinary and human clinical trials including immunization with whole tumor cell or tumor lysate vaccines, peptide vaccines, virally-delivered vaccines, dendritic cell vaccines and plasmid DNA vaccines (14-19). Our laboratory has been evaluating the use of liposome-DNA complexes (LDCs) as a means of increasing the efficacy of plasmid DNA and peptide vaccines in the development of antitumor immunity.

LDCs were initially investigated as an efficient means of systemic gene delivery, especially for targeting pulmonary tissue. During these studies, Dow et al. observed that i.v. injection of "empty" LDCs, those with non-coding plasmid-DNA vectors, dramatically reduced the number of established lung tumor metastases and extended survival in mice with B16 (melanoma), CT-26 (colon carcinoma) or MCA-205 (fibrosarcoma) metastases (20, 21). The antitumor effect was shown to be mediated by increased NK cell cytotoxicity as well as induction of IL-12, IFN-γ and Th1 cell-mediated immunity.

Recent studies in our laboratory and by other investigators have extended these results to show that LDCs induce potent dendritic cell (DC) activation with marked increase in costimulatory molecule expression, pro-inflammatory cytokine production and tumor growth inhibition (22-24). In a murine model of atopic dermatitis,

immunization with LDC-based vaccine induced a strongly T helper type 1 (Th1) biased immune response while preventing development of a T helper type 2 (Th2) response (25). When compared to the efficacy of a "naked" plasmid DNA vaccine, addition of LDCs significantly increased the antitumor activity of antigen-specific $CD8^+$ T cells and led to slower tumor growth in mice bearing either CT-26 carcinoma or B16 melanoma tumors (24). An additional advantage of using LDCs as an adjuvant is that they can be readily combined with defined tumor antigens and administered as a tumor-specific vaccine. We recently evaluated the antitumor effects of a tyrosinase-related-protein-2 (trp2)-LDC vaccine in mice with established B16 melanoma. The vaccine elicited strong trp2-specific cytotoxic T-lymphocyte (CTL) responses, greatly facilitated the study of CTL function, and significantly slowed tumor progression (26).

The use of LDCs has also been evaluated in dogs. We investigated the potential therapeutic role of an allogeneic tumor cell lysate vaccine combined with LDCs in a non-randomized phase I/II trial in dogs with either melanoma or hemangiosarcoma (27, 28) Client-owned dogs received the tumor vaccine once every 2 weeks for 5 treatments then once monthly for at least 3 months. Toxicity was minimal in this study with 6 of 75 dogs experiencing nausea, transient fever, or mild to moderate abdominal pain. Determination of overall efficacy for the trials was complicated by the use of multiple treatment protocols in addition to the tumor vaccine, but median survival of vaccinated dogs with hemangiosarcoma was significantly longer than a control population. Together, the studies with LDCs demonstrate its potential use as an immunotherapeutic agent that can readily be adapted to translational studies in companion animals with cancer.

The Limitations of Tumor Vaccines

Despite the potential of cancer vaccines to control tumor growth and prevent the development of metastatic disease, the overall success rate for human and veterinary clinical trials has been discouragingly low (27-32). Plasmid DNA vaccines, for example, have been evaluated in human patients with melanoma, lymphoma, prostate cancer, and renal cell carcinoma. In many of these trials a high percentage of patients (from 80 – 100%) developed large numbers of tumor-specific CTLs in peripheral circulation following vaccination. However, only 10 - 20% of these patients experienced significant tumor regression and even fewer had significantly longer survival times (33-35).

A somewhat more promising clinical trial was recently completed in dogs with malignant melanoma (36). The investigators immunized dogs with human DNA encoding tyrosinase, a melanoma differentiation antigen essential in melanin synthesis. The nine dogs of this study received a xenogeneic DNA vaccine biweekly for 2 weeks and did not receive any other treatments during the course of vaccination. All of the dogs had advanced disease (WHO stage II, III, or IV) at the start of the study; one dog with multiple pulmonary metastases experienced complete remission lasting 329 days and two other dogs with stage IV disease had long-term survivals of greater than 420 days. The overall response rate was 44% with a 389 day median survival; toxicity was limited to mild pain at the vaccine injection site.

The majority of clinical trials have shown that the best use of tumor vaccination is in the setting of minimal disease and as an adjuvant to more traditional methods of tumor reduction (15, 34, 37). These trials have also illustrated the magnitude and breadth of mechanisms of immune evasion set in place by growing tumors which include faulty DC

activation and function, production of potent immunosuppressive cytokines and induction of regulatory T cells (Treg). Efforts to surmount these problems are the focus of new approaches to tumor vaccine therapy.

THE EFFECTS OF CHEMOTHERAPY ON THE IMMUNE SYSTEM

Introduction

The limitations of chemotherapy and tumor vaccine therapy as single therapeutic modalities have generated considerable interest in their combination. Indeed, there is mounting evidence that certain chemotherapy agents possess immunomodulatory properties and that the combination of cytotoxic tumor reduction with stimulation of antitumor immunity may provide an ideal opportunity to maximize the benefits and minimize the disadvantages of each treatment approach.

Because of the non-specific cytotoxic effects by which chemotherapeutics kill tumor cells, chemotherapy has historically been viewed as being immunosuppressive. Although several early studies described apparent synergy between select chemotherapy drugs and host immunity in experimental murine tumor as early as 40 years ago, clear evidence for the immunostimulatory properties of chemotherapy agents, as well as potential mechanisms for these effects, are just beginning to be described (38-40). These advances have occurred because of the recent development of techniques such as tetramer analysis of tumor-specific T cells, intracellular cytokine staining and the use of transgenic mice, all of which have greatly facilitated analysis of the complex interactions between chemotherapy and multiple components of the immune response.

As recently reviewed by several investigators, there are multiple ways in which chemotherapy can potentially augment antitumor immunity (41-44). These effects include both direct interactions of chemotherapy agents with immune cells such as lymphocytes and antigen presenting cells as well as indirect immunomodulatory effects secondary to chemotherapy-induced tumor cell death. In the following section, we will discuss our current understanding of these interactions, focusing on the potential of commonly used chemotherapeutics to augment responses to cancer vaccine therapy.

Immunomodulatory Properties of Key Chemotherapy Drugs

a) Alkylating agents. Drugs such as cyclophosphamide (CYC), chlorambucil and melphalan, contain active alkylating moieties that irreversibly bind and damage DNA, thus inhibiting cell division and protein synthesis in rapidly proliferating cells. One well-characterized example of chemotherapy-induced immunomodulation in this class of drugs is the ability of CYC to enhance cellular immunity in combination with tumor vaccination. In a HER-2/*neu* mouse model of spontaneous breast cancer, treatment with low-dose CYC 24 hours prior to vaccination with a GM-CSF secreting whole cell vaccine significantly slowed mammary tumor growth and increased the number of tumor-specific CTL. In this study the importance of giving CYC prior to antigen exposure was underscored by the development of tumor tolerance when the drug was administered simultaneously with or subsequent to vaccination (45). The immunostimulatory effects of CYC largely stem from the drug's ability to inhibit the suppressive influence of CD4+CD25+ regulatory T cells (Tregs) via a number of mechanisms, including down-regulation of the FoxP3 transcription factor and interruption of proliferation. CYC has

also been shown to induce Type I IFN production as well as support the development of a Th1 immune response (46-48).

Besides direct effects of drugs such as CYC on lymphocytes, several investigators have shown that the type of tumor cell death elicited by chemotherapy can greatly influence the nature of the antitumor immune response (44, 49-52). For example, the ability of alkylating agents to stimulate antigen cross-presentation by DCs has been described. In a murine leukemia tumor model co-culture of immature DCs with melphalan or chlorambucil-treated tumor cells triggered DC maturation and enhanced antigen presentation (53). This effect was not as dramatic when leukemia cells were treated with drugs such as mitomycin C, suggesting that the way in which alkylating agents damage tumor cell DNA impacts whether DCs will respond to or ignore tumor antigens.

b) Paclitaxel. Paclitaxel (PTX), a taxane originally derived from the Pacific yew tree, is a relatively new drug with broad spectrum antitumor activity and multiple mechanisms of action. Like CYC, PTX has been shown to enhance vaccine efficacy when administered prior to antigen exposure (41, 54). As for the alkylating agents, part of this effect is secondary to enhanced antigen presentation through PTX-mediated induction of immunostimulatory tumor cell apoptosis. In a human clinical trial for advanced breast cancer, PTX given before tumor vaccination induced an apoptotic response within tumors that resulted in dramatically increased numbers of tumor-infiltrating lymphocytes and correlated with induction of complete clinical responses (55, 56). PTX has also been shown to directly impact the immune system by enhancing priming of antigen-specific CD4+ T cells, increasing T cell blastogenesis, and augmenting NK cell function (57, 58).

The best characterized immunomodulatory activity of PTX, however, is its ability to mimic lipopolysaccharide (LPS). PTX binds to Toll-like receptor (TLR)-4 on macrophages, leading to increased tumoricidal properties through enhanced production of nitric oxide activity and pro-inflammatory cytokines (59, 60). The effect of PTX on DCs is less clear but is thought to be similar; PTX appears to activate DC through TLR-4 and MyD88 signaling pathways (61, 62). Interestingly, however, although MHC Class II expression on DCs was increased by paclitaxel exposure in a recent in vitro study, antigen presentation was markedly impaired (60-63).

c) Gemcitabine. Gemcitabine is an analog of deoxycytidine that undergoes intracellular activation and results in inhibition of DNA synthesis. As recently observed by Nowak, et al., gemcitabine can induce extensive tumor-cell apoptosis both in vitro and in vivo (64). More importantly, however, these investigators found that gemcitabine-induced apoptosis enhanced DC cross-presentation of a model antigen to CD8+ T cells in tumor-draining lymph nodes, significantly priming effective cell-mediated antitumor immunity that resulted in slower tumor growth. Interestingly, gemcitabine has also been found to selectively deplete B cells, leading to suppression of antibody responses to tumor antigens (65). Although B cell proliferation was markedly inhibited in this study, gemcitabine did not inhibit T cell proliferation or interfere with the development of effective cell-mediated antitumor immunity. These studies suggest that combination of gemcitabine with tumor vaccines might be beneficial in generation of CTLs but detrimental to the development of a humoral immune response.

Gemcitabine-induced loss of myeloid suppressor cells has also been recently described (66). In this study the investigators found that treatment of mice bearing large

tumors led to dramatic reduction in the numbers of Gr-1+/CD11b+ cells in the spleens of treated mice. This loss was accompanied by an increase in CD8+ T cells and activated NK cells. The mechanism(s) for these effects have not been described.

d) Platinum drugs. The platinum containing drugs, such as cisplatin (CIS) and carboplatin, are heavy metal compounds that intercalate between DNA strands leading to inhibition of transcription. Although there is little information available describing the direct effects of these drugs on immune cells, the ability of cisplatin to increase tumor cell Fas receptor expression and apoptosis is well described (67). Importantly, induction of tumor cell death with cisplatin increases the susceptibility of tumors to CTL-mediated killing, an effect which has been demonstrated in several tumor models (67-69). When combined with either intratumoral CD40-ligand gene therapy or with a papillomavirus subunit vaccine, treatment of mice with cisplatin increased CTL-mediated tumor cell killing and led to increased cure rates (68, 69). In a study of human patients with advanced pancreatic cancer, chemotherapy with a combination of gemcitabine and cisplatin restored abnormally low levels of T lymphocytes, DCs and NK cells that were present prior to treatment (70). However, separation of cisplatin's effects from those of gemcitabine was not attempted.

e) Vinblastine, vincristine. Vinblastine and vincristine are alkaloid compounds derived from the flowering herb periwinkle (also known as the vinca alkaloids). Both drugs interfere with mitotic spindle formation through their effects on microtubular proteins, thereby disrupting cell division. The primary effects of vinblastine and vincristine on the immune response appear to be immunosuppressive rather than immunostimulatory in nature. In an early study on the effects of antineoplastic drugs on the function of tumor-

specific CTLs, Nigam et al., found that treatment of mice with either drug following administration of a GM-CSF cancer vaccine led to the significantly decreased lytic ability of CD8+ CTLs (45). More recently, vincristine has been shown to suppress the allostimulatory potential of human monocyte-derived DCs in vitro (71). Interestingly, these investigators also found that incubation of DCs with vincristine interfered with production of IL-12 but stimulated production of IL-10, an effect similar to that described for the glucocorticoids (72).

g) Glucocorticoids. The glucocorticoids or corticosteroids are a large group of natural and synthetic hormones with a wide range of functions including potent anti-inflammatory and immunosuppressive effects. The cytotoxic uses of the corticosteroids include chemotherapy of hematopoietic malignancies such as lymphoma and myeloma as well as some cutaneous tumors such as mast cell tumors. As for the vinca alkaloids, the primary effects of the glucocorticoids are immunosuppressive. The ability of the drug prednisone to induce apoptosis in activated lymphocytes is well-described (73, 74). This effect occurs primarily through down-regulation of CD3 expression on activated T cells and negatively impacts CD4 and CD8 gene expression as well (74). Glucocorticoids are also well known for their ability to inhibit inflammatory cytokine production by antigen presenting cells and Th1 T cells, selectively suppressing Th1 cell-mediated immunity in favor of a Th2 humoral immune response (75, 76).

Multiple direct inhibitory effects of naturally occurring and synthetic glucocorticoids on DCs have also been described. As reviewed recently by Hackstein et al., corticosteroids block differentiation and maturation of DCs both in vitro and in vivo. This occurs through multiple mechanisms including increased apoptosis, inhibition of

costimulatory molecule expression, interference of LPS or CD40-ligand-induced inflammatory cytokine production and inhibition of signals that enable DC migration to lymphoid tissues (77-79). Exposure of DCs to corticosteroids also induces IL-10 production in CD40-triggered DCs and promotes the generation of IL-10 regulatory T cells (72, 80).

f) Doxorubicin. Doxorubicin (DOX) is an anthracycline antibiotic (also known as Adriamycin), originally isolated from *Streptomyces sp.,* that induces cytotoxicity in rapidly dividing cells through several mechanisms including inhibition of topoisomerases I and II, inhibition of free radical formation and direct interference with DNA and RNA synthesis. The anthracyclines, which also include drugs such as mitoxantrone and idarubicin, are some of the most potent and broad-spectrum chemotherapeutic agents currently available and are very widely used in clinical practice.

DOX-induced stimulation of macrophages has long been appreciated. In 1977, for example, Orsini et al. found that whole spleen cell populations from DOX-treated mice demonstrated an increased ability to lyse tumor target cells in a ^{51}Cr-release cytotoxicity assay (81). Crude separation of monocytic versus lymphocytic cell populations revealed that this ability was a property of an immature monocytic/macrophage cell type. This finding was both confirmed and extended over the next several years by others to show that treatment of mice with DOX or culture of spleen cells harvested from DOX-treated mice increased the phagocytic and tumoricidal properties of macrophages and improved T cell-mediated tumor cytotoxicity (40, 82, 83). Interestingly, Mihich and Ehrke also found that DOX treatment induced the expansion of an immature population of cells within the monocyte/macrophage lineage while

simultaneously suppressing the activity of a T cell with a "regulatory" phenotype (40). A more recent study by Ujhazy, et al. described the increased tumoricidal potential of macrophages isolated from the peritoneal cavity of DOX-treated mice as well as increased production of TNF-α, and IL-1 (84).

In contrast, however, DOX has also been shown to impair macrophage function and slow wound healing in vivo when given twice weekly at the same relatively low doses used in the preceding studies. Asmis et al. found that LPS-stimulated production of TNF-α and IL-1 were decreased by macrophages both in vivo and in vitro and that wound healing was significantly impaired (85). These conflicting data suggest that evaluation of mice receiving repeated treatments with DOX should be undertaken; especially since multiple dosing strategies would more closely mirror the treatment of human and veterinary oncology patients.

The impact of DOX on NK cell function has also been assessed, although the drug's immunomodulatory effects are less well-understood than those described for macrophages. Ujhazy et al. found that treatment of mice with DOX augmented NK cell activity in peritoneal NK cells but inhibited this activity in the spleen (84). More recently, Markasz et al. observed that in vitro exposure of human NK cells to DOX increased their ability to kill tumor target cells (86). Interestingly, DOX augmented NK cell function and did not elicit cytotoxicity even when used at concentrations higher than therapeutic levels typically achieved in vivo in humans.

The ability of DOX to stimulate CTL responses in vivo has been described in multiple clinical studies, although separation of the drug's direct effects on T cells from those mediated by macrophage and NK cell activation remains unclear. When combined

with a GM-CSF whole cell vaccine in mice with CT-26 colon carcinoma, DOX given at the time of or 1 week after vaccination resulted in enhancement of tumor-specific CTL function. In contrast to CYC, this effect was abrogated when DOX was given prior to the vaccine (45). In a HER-2/*neu* model of tolerogenic breast cancer, DOX administered 1 week after GM-CSF vaccination slowed tumor growth but did not change the number or function of neu-specific T cells (58). Using this tumor model, Eralp and co-workers found that administration of DOX prior to giving an RNA plasmid vaccine synergistically increased numbers of tumor-specific CTLs and slowed tumor growth (87). Potential differences in sequencing of DOX administration with vaccination were not evaluated. In the murine E0771 breast tumor model treatment with a single dose of DOX followed by moderate doses of IL-2 resulted in significant increases in survival as well as protection from subsequent tumor challenge. These therapeutic effects were not seen with either DOX or IL-2 therapy alone and were dependent on CD8+ T cells but independent of NK cell function (88).

Induction of immunogenic tumor cell apoptosis is another important aspect of DOX's immunostimulatory activity. Although apoptosis has been traditionally regarded as being immunosuppressive, Casares et al. demonstrated that DOX treatment of CT-26 colon carcinoma cells induces caspase 3-dependent apoptosis that activates DCs and elicits a therapeutically effective cytotoxic T cell response (50). DOX-treated apoptotic tumor cells could also be given as an intratumoral vaccine in this model, eliciting protection against the development of pulmonary metastases.

Using the intratumoral vaccine approach, Obeid et al. recently extended these findings to show that the anthracycline drugs induce translocation of calreticulin, a

chaperone and calcium regulating protein, from its normal intracellular location within the endoplasmic reticulum to the surface of injured cells before apoptosis occurs (89). Tumor cells expressing calreticulin were readily phagocytosed by DCs and these DCs were critical in generation of effective antitumor CD4+ and CD8+ T-cell responses. Therefore, these studies suggest that it might be possible to increase the immunogenicity of chemotherapeutic agents in general by "tagging" tumor cells with molecules that augment DC-mediated cross presentation to T cells.

In summary, the studies reviewed in this section demonstrate the potential for many chemotherapeutic agents to augment multiple aspects of antitumor immunity and illustrate the close link to DC function. Much of the work to date has focused on the effects of various drugs on the development of adaptive immune responses or on the immunomodulatory effects of tumor cell death. Although there are a few studies of chemotherapy's effects on macrophage and NK cell function, there is very little information regarding direct effects on DCs. These studies also reveal the complexities of drug interactions between tumor cells and immunity, emphasizing the need to perform careful analyses of in vitro and in vivo effects in animal models using both healthy and tumor-bearing animals. Finally, successful clinical trials exploring combinatorial therapies will need to consider multiple factors such as proper sequencing of chemotherapy with vaccination, dosages of drugs, nature of vaccine adjuvants, possible competing effects of multiple chemotherapeutics and definition of clinical endpoints of therapy.

THE EFFECTS OF CANCER ON DENDRITIC CELLS AND REGULATORY T CELLS

Introduction

Research efforts directed at effective combination of chemotherapy and tumor vaccines have yielded not only a better understanding of how each treatment modality impacts the immune response, but also a growing appreciation for the breadth and magnitude of mechanisms that tumors evolve to escape immune control. These mechanisms can be inherent properties of tumor cells themselves, such as decreased expression of MHC molecules or down regulation of genes associated with antigen presentation, or can involve the dysfunction of components of the host's innate and adaptive immune responses. Two critical cell populations that are markedly impacted during tumor growth are DCs and Treg. Tumor-induced defects in DC differentiation and maturation have far-reaching effects on the ability of the host to mount an effective antitumor immune response. Indeed, one of the major reasons for the failure of cancer vaccines to control tumor growth is likely a consequence of impaired DC development and function (90-93).

Another reason for the limited success of tumor immunotherapy is the immunosuppressive activities of Treg. Treg are increased in the large majority of cancer patients and have been shown to directly interfere with the development of antitumor immunity. Therefore, in this section we will focus on current understanding of the impact of cancer on these two cell types and how these effects interfere with antitumor immunity.

Impaired DC Differentiation and Activation

Given the critical role of DC function in regulating T cell responses to tumor antigens it is not surprising that one of the major reasons for the failure of cancer vaccines to control tumors may be mediated by tumor-induced defects in DC differentiation and activation. In both mice and humans with cancer, defective DC function is thought to arise from impaired DC differentiation from myeloid and lymphoid progenitor cell populations (90). Thus, the defects induced in DCs occur systemically and are not restricted to DCs in the tumor microenvironment.

a) The role of tumor-derived factors. A number of tumor-derived factors are now known to contribute to impaired DC differentiation including vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF), GM-CSF, IL-6 and IL-10 (93). Of these factors, VEGF was the first to be shown to inhibit DC differentiation. This was demonstrated by in vitro experiments in which neutralizing VEGF-specific antibodies abolished the negative effects of tumor cell conditioned tissue culture medium on DC differentiation (94). Inhibition was also confirmed in vivo by showing that administration of recombinant VEGF to normal mice resulted in abnormal DC development, leading to increased production of immature myeloid cells (95).

The clinical relevance of VEGF has been documented by studies in human cancer patients that show that mature DC numbers can be negatively correlated with VEGF levels in blood and tumor tissue for some types of cancer such as gastric adenocarcinoma and metastatic carcinomas of various locations (96-98). In breast cancer patients the intratumoral ratio of soluble VEGF-receptor-1 levels (sVEGFR1) to VEGF levels is also prognostic; tumors in which the sVEGFR1 level exceeded VEGF levels by more than 10-

fold were associated with a markedly better prognosis. Analysis of this ratio was also helpful in prediction of sentinel lymph node metastases (99). The effects of other cytokines such as IL-6 and IL-10 on DC development are similar to VEGF; both IL-6 and IL-10 have inhibitory effects on DC maturation that can be demonstrated in vitro and in vivo (100-103).

b) STAT3 signaling in tumor cells. Most of the tumor-derived factors implicated in aberrant DC maturation exert their effects through the STAT3 (signal transducer and activator of transcription 3) pathway. The STAT3 pathway is constitutively activated in most tumors and is crucial for tumor cell proliferation and survival (104). Activation of this pathway in tumor cells inhibits production of multiple inflammatory cytokines while simultaneously producing factors such as VEGF and IL-10. In turn, the immunosuppressive cytokines inhibit functional DC maturation through disruption of STAT3 and NF-κB signaling pathways in DC progenitor cells (105, 106). Thus, the tumor's "two-pronged" approach facilitates evasion of an immune response by preventing DC activation and by fostering the accumulation of immature DCs and myeloid cells.

Using an inducible STAT3 knock-out approach, Kortylewski et al. recently investigated the impact of STAT3 signaling on the development of antitumor immunity in several mouse tumor models (105). These investigators found that STAT3 was constitutively activated in tumor-infiltrating cells. Comparison of the phenotype of tumor-infiltrating DCs isolated from $STAT3^{-/-}$ or $STAT3^{+/+}$ mice showed that expression of MHC Class II and costimulatory molecules such as CD80 and CD86 were considerably increased in the $STAT3^{-/-}$ mice. They also found that ablation of the STAT3

pathway using small molecule inhibitors restored normal DC maturation in vivo and significantly inhibited tumor growth.

c) Immunosuppressive effects of iMCs. Immature myeloid cells (also termed myeloid suppressor cells) are a heterogeneous population that comprises immature macrophages, granulocytes, DCs and other myeloid cells at early stages of differentiation. In mice, iMCs can be identified as $Gr1^{+}CD11b^{+}TCR^{-}$ cells but may also express markers reflecting their mix of partially differentiated cell types (107, 108). Under normal homeostatic conditions, iMCs differentiate in the bone marrow and spleen into mature granulocytes, macrophages and DCs. However in the cancer patient, precise definition or isolation of iMCs can be challenging and additionally complicated by aberrant development under the influence of various cytokines and factors produced by the tumor (107, 109).

As discussed above, iMCs arise primarily through production of tumor-derived factors such as VEGF, IL-10 and IL-6 which disrupt normal DC differentiation from progenitor cells or through production of GM-CSF which mobilizes DC precursor release from the bone marrow. Regardless of the source, iMCs play a key role in active suppression of antitumor immunity. The ability of iMCs to inhibit both MHC class I and class II-restricted T cell responses in a cell-to-cell contact dependant manner has been recognized for some time (91, 110). This contact mediated inhibition is thought to be mediated by reactive oxygen species such as H_20_2, although the precise mechanism is not clear (109).

Recent work by Gallina et al. has helped to clarify this mechanism by showing that contact with CD8+ T cells elicits IL-13 and IFN-γ production from the iMC (111).

IL-13 and IFN-γ then act in an autocrine fashion to increase production of the enzymes iNOS and Arginase I, both of which are important to normal T cell function. Increased enzyme production interferes with IL-2-receptor signaling on CD8+ T cells and leads to T cell inactivation through depletion of local L-arginase levels.

d) Clinical consequences of impaired DC activation. The clinical relevance of aberrant DC development is supported by numerous studies that show that decreased numbers of mature DCs and/or increased numbers of iMCs are commonly found in cancer patients and that these changes often correlate with extent of disease or prognosis (112). In people with squamous cell carcinoma of the head and neck or with breast cancer for example, DC number correlates inversely with stage of disease (96, 113). In the study of people with squamous cell carcinoma, patients with early stages of the disease had two-fold fewer DCs in their peripheral circulation while those with end stage disease had more than four-fold fewer DCs than healthy donors (113). Other studies have reported similar findings for other types of cancer and several have shown that the number of mature DCs in peripheral blood increases towards normal following surgical removal of the primary tumor (90).

Another abnormality in the immune system of cancer patients is that normal populations of mature DCs are replaced with increased numbers of immature DCs which can be identified in addition to iMCs. Although immature DCs process and present antigen in the context of MHC class I molecules, their expression of very low levels of costimulatory molecules and lack of pro-inflammatory cytokine production induces antigen-specific T-cell tolerance rather than an effective adaptive immune response. People with breast and prostate cancer, for example, have accumulation of HLA-DR^+

DCs which display multiple features of immature DCs including poor responsiveness to maturation stimuli, decreased ability to present antigen and preferential induction of a Th_2 rather than a Th_1 immune response (93).

A subset of plasmacytoid DCs that express the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO) has also been shown to directly interfere with the development of antitumor immunity (114). The immunosuppressive effects of IDO have been previously described; expression of IDO by macrophages and DCs inhibits T cell proliferation and is an important component of maternal tolerance towards the allogeneic fetus (115-117). Munn hypothesized, therefore, that IDO production might be induced in DCs from cancer patients and contribute to immune evasion by the tumor (114). Indeed, these investigators identified a subset of plasmacytoid DCs, expressing B220 and CD19, within tumor-draining lymph nodes that constitutively expressed immunosuppressive levels of IDO. Using the B16 melanoma model, they demonstrated that the IDO-expressing DCs potently suppressed antigen-specific T cell responses to both IDO^+ DCs as well as to non-IDO^+ DCs. When plasmacytoid DCs were isolated from the tumor-draining lymph nodes of IDO knock-out mice, the DCs did not demonstrate any suppressive effects. The investigators also retrospectively identified large numbers of IDO^+ DCs in the tumor-draining lymph nodes of human cancer patients with melanoma, breast carcinoma and a variety of solid tumor types. In these cancer patients the presence IDO^+ DCs in sentinel lymph nodes at the time of initial diagnosis correlated with a significantly worse prognosis.

Another consequence of impaired DC activation is associated with the induction of regulatory T cells (Treg). DCs exposed to myeloma tumor cells, for example, produce

large amounts of the immunosuppressive cytokine IL-10 and very little IL-12. These DCs were found to support generation of T cells with a suppressive phenotype rather than an effector phenotype (118). Immature DCs may also promote the proliferation of Tregs through tumor-induced production of TGF-β (119). In this study Ghiringhelli and co-workers identified accumulation of immature DCs (defined as a population of $CD11c^{+}CD11b^{+}$ cells with low levels of MHC Class II, CD80, and CD86) in the spleen and draining lymph nodes of tumor-bearing mice and rats. When purified and cultured with Treg, the immature DCs supported proliferation of Treg in a TGF-β dependent manner. Interestingly, while the tumor cells themselves did not produce biologically active TGF-β, incubation of the immature DCs with tumor culture supernatants induced TGF-β production by the DCs within 24 hours.

Identification and Function of Regulatory T Cells

The key role played by a subset of T cells called regulatory T cells, or Treg, in hindering the development of antitumor immunity is now well-supported by numerous studies in tumor-bearing mice and human cancer patients that document both the presence and immunosuppressive effects of Treg. However even the existence of a distinct subset of T cells with inhibitory properties, let alone their contribution to regulation of immunity, was a highly controversial topic for more than 25 years. North and colleagues were among the first to propose that a group of "suppressor" T cells with a CD4+ phenotype could mediate suppression of antitumor immunity in a mouse tumor model (120). Although other investigators working during this time also detected similar CD4+ T cells within human melanoma tumors, the entire field of suppressor T cell

biology was subsequently lost in a confusing disarray of circuitous regulatory loops and poorly-defined factors and was largely discredited (121-123).

Renewed interest in the Treg field was stimulated nearly 20 years later in 2000 by the observations of Sakaguchi et al. who found that a subset of T cells, identified based on expression of CD4+ and CD25+, were crucial to the prevention of autoimmune disorders following thymectomy of neonatal mice (124). In a series of pioneering experiments, Sakaguchi demonstrated that depletion of CD25+ T-cells or adoptive transfer of CD4+CD25- T cells to lymphopenic mice resulted in the development of a slowly progressive, inflammatory multi-organ autoimmune disorder. Shortly thereafter these investigators demonstrated that depletion of CD4+CD25+ T cells or injections of anti-CD25 antibody could induce T-cell mediated antitumor immune responses, making a connection for the first time between tumor immunity and autoimmunity (125, 126). Since this time there has been a tremendous interest in the role of Treg in regulating many aspects of immune responses including those to self-antigens, infectious agents, tumor antigens and transplantation antigens.

Treg are now accepted as a distinct type of T-lymphocytes that comprise about 5 – 8% of the total population of T cells in the lymph node, or 10 – 12% of the total $CD4^+$ population (127, 128). Several subsets of Treg have been described, including naturally-occurring, thymic-derived Treg and those induced by exposure to antigen and activated in the periphery (129, 130). When stimulated through their TCR, endogenous CD4+ Treg function to directly suppress the proliferation of harmful self-reactive T cells in a cell-contact dependent, cytokine-independent manner (124, 128). In contrast, antigen-

induced Treg typically regulate immune homeostasis via production of cytokines such as IL-10 and TGF-β (131, 132).

In mice, cats and humans Treg can be identified based on surface expression of high levels of the IL-2-receptor-α chain (CD25). Although other cell surface molecules such as glucocorticoid-induced TNF-receptor (GITR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and CD103 aid in Treg identification, definitive delineation from activated $CD4^+$ non-regulatory T cells using cell surface marker expression alone is not possible. Recently, however, intracellular detection of the transcription factor FoxP3 has been shown to uniquely identify the rodent Treg population (133, 134). In mice FoxP3 is a "master regulator" of the Treg phenotype; constitutive FoxP3 expression is essential both for initial Treg development as well as maintenance of their suppressive function (135).

The role of FoxP3 expression in human Treg is not as well understood. Although FoxP3 expression is highly enriched within the human $CD4^+CD25^{high}$ population as it is in mice, several investigators have demonstrated induction of FoxP3 expression in activated CD4+ T cells that lack suppressive function (136-138). Nevertheless, identification of CD4+FoxP3+ T cells is currently accepted as the most specific method of Treg identification.

The Role of Regulatory T Cells in Cancer

Over the past 6 to 7 years there have been numerous clinical studies that have demonstrated increased levels of Treg in people with a variety of malignancies such as melanoma, Hodgkin lymphoma, or ovarian, gastric, pancreatic, breast and colorectal

cancer (139-145). Although a number of studies implicated Treg in suppression of antitumor immunity, the first evidence for a direct role in human cancer was provided by Curiel et al. (139). In a large study of women with various stages of ovarian carcinoma, these investigators demonstrated Treg-mediated suppression of antitumor immune responses both in vitro and in vivo. They found that women with more advanced stages of the disease had higher numbers of Treg within tumor tissue and ascitic fluid than women with less advanced cancer. Tumor Treg numbers were also correlated with increased death rates and decreased survival times. Interestingly, Treg numbers were decreased in tumor-draining lymph nodes in patients with more advanced disease; an effect that was found to be mediated by production of the chemokine CCL22 which induced migration of Treg from the lymph node to the tumor.

A direct role for Treg-mediated suppression of antitumor immunity within tumor-draining lymph nodes has also been reported. In contrast to the Curiel study, Viguier et al. found that Treg preferentially accumulated in tumor-draining lymph nodes of people with metastatic melanoma compared to tumor-free lymph nodes (142). These Treg, identified based on $CD4^+CD25^+FoxP3^+$ expression, inhibited proliferation and Th_1 cytokine production of $CD4^+CD25^-$ and $CD8^+$ T cells through a cell-contact dependent mechanism. High levels of IL-10 and TGF-β were also present in metastatic lymph nodes, providing a possible mechanism for recruitment and/or expansion of Treg.

Because of their central role in the pathology of cancer, there has been considerable interest in Treg depletion to improve the efficacy of tumor immunotherapy. Several studies have examined the feasibility of enhancing antitumor responses using antibodies directed against the CD25 surface marker. Although administration of an anti-

CD25 antibody to mice can uncover effective T-cell mediated immune responses, this approach has also been shown to hinder the adoptive transfer of activated CD8+CD25+ CTLs and tumor-reactive CD4+ T cells (146). In human cancer patients Treg can be depleted from the peripheral circulation using a recombinant IL-2 diphtheria toxin fusion protein (also known as ONTAK) (147). Although this approach is selective for Treg expressing high levels of CD25, some toxicity to activated CD4+ non-regulatory T-cells can also occur.

More selective Treg depletion is possible with the chemotherapy agent cyclophosphamide. The ability of CYC to improve the efficacy of various types of immunotherapy, in particular adoptive transfer therapy, has been recognized for some time; an effect that has been associated with decreased Treg numbers. Recently, Lutsiak et al. provided direct evidence for CYC's ability to suppress Treg function (47). These investigators found that intraperitoneal administration of CYC to healthy mice significantly inhibited the suppressive effects of Treg as well as diminished their overall numbers. The mechanism of this effect occurred at several levels including down-regulation of FoxP3 expression, increased sensitivity of Treg to apoptosis and a transient decrease in Treg proliferation (47).

The administration of low, frequent doses of CYC, an approach also known as metronomic chemotherapy, is now commonly used in the treatment of both people and companion animals with cancer. Metronomic chemotherapy has been shown to be anti-angiogenic, leading to slowed tumor growth in a number of tumor models. But in addition to effects on tumor angiogenesis, CYC can clinically decrease Treg levels and improve antitumor immune responses. In a study of people with a variety of advanced

cancers, for example, oral administration of low-dose CYC was found to significantly decrease circulating Treg numbers, restoring peripheral T cell proliferation and NK cell function (148). Interestingly, another chemotherapy agent, fludarabine, has also recently been reported to selectively decrease Treg numbers. In people with chronic lymphocytic leukemia, administration of fludarabine led to decreased peripheral Treg numbers as well as decreased suppressive function (149). The mechanisms for fludarabine's suppressive effects on Treg are unknown but may include down-regulation of FoxP3 expression and inhibition of Treg proliferation. Combination of CYC and fludarabine chemotherapy may be particularly effective in Treg depletion; this approach is presently under investigation to efforts to increase the success of adoptive transfer therapy in people with advanced malignancies (150).

SUMMARY AND PROJECT RATIONALE

Despite progress in treating some types of cancer, the ability of conventional cytotoxic chemotherapy to completely eradicate malignant cells is limited by non-specific toxicity to normal tissues and by the development of drug resistance in the tumor itself. Although efforts to protect normal cells and to sensitize tumor cells to chemotherapy are under investigation, it is unlikely that chemotherapy as a single therapeutic modality will be able to elicit cures for the majority of human and companion animal cancer patients.

A tremendous amount of research over the past decade, therefore, has focused on new approaches to cancer therapy. One such approach is the administration of tumor vaccines; a strategy that takes advantage of the potential for the immune system to recognize and attack cancerous cells. Tumor vaccines can be designed to be tumor-

specific and even patient-specific and can deliver immunostimulatory cytokines and costimulatory molecules as well as tumor antigens. However, although tumor vaccine therapy has shown significant promise in mouse models and in a few clinical trials in people, the overall success rate of tumor vaccination in cancer patients has been disappointingly low (15, 37, 151). To be effective then, tumor vaccination and most other types of immunotherapy will need to be combined with standard treatment modalities such as chemotherapy or radiation therapy.

The overall objective of this dissertation research was to develop a better understanding of the impact of conventional chemotherapy on innate and adaptive immune responses in animals with cancer as a foundation for the effective combination of chemotherapy and tumor vaccination. As a first step in this process we evaluated the effects of two commonly used chemotherapy protocols on adaptive immune responses in dogs with cancer (**Chapter 2: Effects of Chemotherapy on Adaptive Immunity and Regulatory T Cells in Dogs with Cancer**). Although chemotherapy has historically been viewed as immunosuppressive, there are now numerous studies in mice and humans demonstrating the immunostimulatory potential of certain chemotherapy drugs. However, there are a very limited number of studies examining the impact of chemotherapy on immune response in dogs and thus the potential for chemotherapy to modulate immune function is unknown. Prior studies have focused primarily on dogs with lymphoma and were retrospective in nature (152-154). Therefore, we designed a study to prospectively assess the short and intermediate-term effects of two common chemotherapy protocols on T-cell and B cell numbers and humoral immune responses in dogs with either osteosarcoma or lymphoma. We hypothesized that chemotherapy might actually

increase, or at least not suppress, numbers of T and B lymphocytes and antibody titers during the course of therapy. Flow cytometry was used to determine changes in CD4+ and CD8+-T cell and B cell numbers before, during and after chemotherapy; these parameters were also compared to those of healthy control dogs. In addition, dogs were vaccinated with a novel antigen during chemotherapy to permit functional evaluation of the humoral immune response to vaccination with a novel antigen.

Another objective of this chapter was to compare the effects of single agent doxorubicin chemotherapy with those of a multi-drug protocol. Given the greater degree of myelosuppression associated with the use of combination protocols, we hypothesized that dogs treated with multi-agent chemotherapy might exhibit greater decreases in lymphocyte numbers or antibody titers than dogs treated with doxorubicin alone and thus might be less suitable candidates for therapies combining chemotherapy and tumor vaccination.

During the course of the preceding studies we developed an interest in the effects of chemotherapy on regulatory T cells in cancer-bearing dogs. This interest arose from the growing body of literature that describes the ability of Treg to actively participate in the evasion of antitumor immune responses in both mice and humans with cancer. Although the role of Treg in cancer patients is becoming better understood, the effects of chemotherapy on this important T cell subset are largely unknown. Therefore, in the second section of Chapter Two, we took advantage of the wealth of information provided by the samples collected for the prospective study to initiate work directed towards identification of the canine Treg population and evaluation of the effects of chemotherapy on Treg numbers. We hypothesized that Treg would be present in dogs as they are in

other species and that the type of chemotherapy given might differentially impact Treg levels.

Our initial approach to Treg identification was to determine whether antibodies used to detect various cell-surface markers associated with murine, human or feline Treg could be used to identify the canine Treg population. Using flow cytometry, lymphocytes collected from peripheral blood or lymph node aspirates were screened for the presence of Treg-associated markers such as Lag-3, CD103, CD25, GITR and CTLA-4 using antibodies designed to recognize these markers in other species. Unfortunately none of the reagents used in this project were useful in the detection of these cell surface markers on CD4+ T cells in either normal dogs or those with cancer.

We met with somewhat better success, however, using a more indirect approach to Treg identification. Thus, we developed a flow cytometry-based assay to tentatively identify canine Treg levels by evaluating cell-surface expression of the IL-2 receptor (IL-2R) in CD4+ lymphocytes. Although this approach did not permit definitive differentiation of Treg from activated CD4+ T-cells, which can also express the IL-2R on their cell surface, the assay was used to determine whether the number of CD4+IL-2R+ T-cells in the peripheral circulation of dogs with cancer differed than those of normal dogs and to follow changes in CD4+IL-2R+ T-cells during the course of chemotherapy. Because Treg numbers are known to be high in the peripheral circulation of human cancer patients, we surmised that numbers of CD4+IL-2R+ T-cells would be higher in dogs with cancer than in normal dogs.

Frustrated with the relatively low sensitivity and poor specificity of this approach, however, we continued to optimize the canine Treg assay. Fortunately, at this point in

our research, the role of the FoxP3 transcription factor in the development and maintenance of the human and murine Treg population was described (155, 156). Given the high degree of conservation between FoxP3 transcription factors across species, we hypothesized that the use of a mouse antibody to FoxP3 might aid in identification of canine Treg. As described in **Chapter 3 (Use of FoxP3 Expression to Identify Regulatory T Cells in Healthy Dogs and Dogs with Cancer)**, this approach was much more successful. Using this new tool we then performed a preliminary study of Treg numbers in dogs with different types of cancer with the goal of determining whether dogs have increased Treg in their peripheral circulation and tumor-draining lymph nodes as is the case in many human cancer patients.

Finally, in the last chapter of this dissertation (**Chapter 4: The Immunomodulatory Effects of Doxorubicin on Dendritic Cells**), we focused on the effects of common chemotherapeutic agents on DCs in healthy and tumor-bearing mice with the objective of identifying drugs that might ultimately enhance antitumor immunity through augmentation of dendritic cell function. As for the work in Chapter 2, the impetus for these studies arose from the need to better understand the impact of chemotherapy on the immune system so that multi-modality approaches to cancer therapy might become more effective. Although a few chemotherapy drugs, such as doxorubicin and cyclophosphamide are known to enhance DC function secondary to their apoptotic effects on tumor cells, very little is known about the effects of chemotherapy on DCs themselves. Therefore we hypothesized that a drug (or drugs) could be identified that would enhance DC function and potentially could be combined with tumor vaccination to generate a more effective antitumor immune response.

To this end, we analyzed the effects of a panel of commonly used chemotherapy drugs on bone-marrow derived DCs (BMDCs) in vitro; a setting in which the potential interactions of chemotherapy drugs with cells other than DCs could be kept to a minimum. We found that, among a panel of 6 chemotherapeutic agents, the anthracycline derivative doxorubicin was uniquely able to stimulate multiple aspects of DC function including antigen presentation, increased production of the common p40 subunit of IL-12 and IL-23, and increased expression of the costimulatory molecule CD40. Further work was then focused more closely on the effects of DOX on DCs in vivo in healthy and tumor-bearing mice and confirmed the potential for DOX to activate several important components of DC function. In conclusion, the work of this chapter has set the stage for future investigations into the use of DOX chemotherapy in combination with tumor vaccines in both mice and companion animals with cancer. It is hoped that the research described in this dissertation as a whole will help to guide the development of increasingly more effective approaches to the treatment of people and companion animals with cancer.

REFERENCES

1. Murphy SB, Fairclough DL, Hutchison RE, Berard CW. Non-Hodgkin's lymphomas of childhood: an analysis of the histology, staging, and response to treatment of 338 cases at a single institution. J Clin Oncol 1989;7(2):186-93.

2. Reiter A, Schrappe M, Parwaresch R, *et al.* Non-Hodgkin's lymphomas of childhood and adolescence: results of a treatment stratified for biologic subtypes and stage--a report of the Berlin-Frankfurt-Munster Group. J Clin Oncol 1995;13(2):359-72.

3. Sun XF, Zhen ZJ, Lui DG, *et al.* Improved treatment outcome in Chinese children and adolescents with Burkitt's lymphoma and large cell lymphoma by using the modified B-non-Hodgkin's lymphoma-Berlin-Frankfurt-Munster-90 protocol. Eur J Haematol 2006;77(5):365-71.

4. MacDonald VS, Thamm DH, Kurzman ID, Turek MM, Vail DM. Does L-asparaginase influence efficacy or toxicity when added to a standard CHOP protocol for dogs with lymphoma? J Vet Intern Med 2005;19(5):732-6.

5. MacEwen EG, Hayes AA, Matus RE, Kurzman I. Evaluation of some prognostic factors for advanced multicentric lymphosarcoma in the dog: 147 cases (1978-1981). J Am Vet Med Assoc 1987;190(5):564-8.

6. Khan MA, Andrews S, Ismail-Khan R, *et al.* Overall and progression-free survival in metastatic melanoma: analysis of a single-institution database. Cancer Control 2006;13(3):211-7.

7. Natarajan N, Shuster TD. New agents, combinations, and opportunities in the treatment of advanced and early-stage colon cancer. Surg Clin North Am 2006;86(4):1023-43.

8. Seidman AD. Systemic treatment of breast cancer. Two decades of progress. Oncology (Williston Park) 2006;20(9):983-90; discussion 91-2, 97-8.

9. Chun R, Kurzman ID, Couto CG, Klausner J, Henry C, MacEwen EG. Cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma: a pilot study. J Vet Intern Med 2000;14(5):495-8.

10. Shapiro W, Fossum TW, Kitchell BE, Couto CG, Theilen GH. Use of cisplatin for treatment of appendicular osteosarcoma in dogs. J Am Vet Med Assoc 1988;192(4):507-11.

11. Thompson JP, Fugent MJ. Evaluation of survival times after limb amputation, with and without subsequent administration of cisplatin, for treatment of appendicular osteosarcoma in dogs: 30 cases (1979-1990). J Am Vet Med Assoc 1992;200(4):531-3.

12. Norton L. A Gompertzian model of human breast cancer growth. Cancer Res 1988;48(24 Pt 1):7067-71.

13. Lysaght J, Todryk S. Developments in cancer vaccination. Curr Opin Investig Drugs 2003;4(6):716-21.

14. Pardoll DM. Cancer vaccines. Nat Med 1998;4(5 Suppl):525-31.

15. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10(9):909-15.

16. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10(5):475-80.

17. Mach N, Dranoff G. Cytokine-secreting tumor cell vaccines. Curr Opin Immunol 2000;12(5):571-5.

18. Pardoll DM. Therapeutic vaccination for cancer. Clin Immunol 2000;95(1 Pt 2):S44-62.

19. Prud'homme GJ. DNA vaccination against tumors. J Gene Med 2005;7(1):3-17.

20. Dow SW, Elmslie RE, Fradkin LG, *et al.* Intravenous cytokine gene delivery by lipid-DNA complexes controls the growth of established lung metastases. Hum Gene Ther 1999;10(18):2961-72.

21. Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J Immunol 1999;163(3):1552-61.

22. Higgins RJ, McKisic M, Dickinson PJ, *et al.* Growth inhibition of an orthotopic glioblastoma in immunocompetent mice by cationic lipid-DNA complexes. Cancer Immunol Immunother 2004;53(4):338-44.

23. Sellins K, Fradkin L, Liggitt D, Dow S. Type I interferons potently suppress gene expression following gene delivery using liposome(-)DNA complexes. Mol Ther 2005;12(3):451-9.

24. U'Ren L, Kedl R, Dow S. Vaccination with liposome-DNA complexes elicits enhanced antitumor immunity. Cancer Gene Ther 2006;13(11):1033-44.

25. Mueller RS, Veir J, Fieseler KV, Dow SW. Use of immunostimulatory liposome-nucleic acid complexes in allergen-specific immunotherapy of dogs with refractory atopic dermatitis - a pilot study. Vet Dermatol 2005;16(1):61-8.

26. Zaks K, Jordan M, Guth A, *et al.* Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 2006;176(12):7335-45.

27. U'Ren LB, B. Elmslie, R. Rose, B. Dow, S. Evaluation of an allogeneic tumor vaccine in dogs with oral melanoma. Veterinary Cancer Society Annual Conference Proceedings; 2004; Kansas City, MO.; 2004. p. 104.

28. U'Ren LW, Biller BJ, Elmslie RE, Thamm DH, Dow SW. Evaluation of a novel tumor vaccine in dogs with hemangiosarcoma. J Vet Intern Med 2007;21(1):113-20.

29. Alexander AN, Huelsmeyer MK, Mitzey A, *et al.* Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II clinical trial in canine patients with malignant melanoma. Cancer Immunol Immunother 2006;55(4):433-42.

30. Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? Cancer Res 2004;64(18):6827-30.

31. Bodey B, Bodey B, Jr., Siegel SE, Kaiser HE. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. Anticancer Res 2000;20(4):2665-76.

32. Gilboa E. The promise of cancer vaccines. Nat Rev Cancer 2004;4(5):401-11.

33. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P. Human T cell responses against melanoma. Annu Rev Immunol 2006;24:175-208.

34. Ferrantini M, Capone I, Marincola FM, Parmiani G, Belardelli F. International meeting "immunotherapy of cancer: challenges and needs". Cancer Immunol Immunother 2007;56(4):581-5.

35. Mocellin S, Mandruzzato S, Bronte V, Marincola FM. Cancer vaccines: pessimism in check. Nat Med 2004;10(12):1278-9; author reply 9-80.

36. Bergman PJ, McKnight J, Novosad A, *et al.* Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 2003;9(4):1284-90.

37. Yu Z, Restifo NP. Cancer vaccines: progress reveals new complexities. J Clin Invest 2002;110(3):289-94.

38. Braun DP, Harris JE. Modulation of the immune response by chemotherapy. Pharmacol Ther 1981;14(1):89-122.

39. Kempf RA, Mitchell MS. Effects of chemotherapeutic agents on the immune response. I. Cancer Invest 1984;2(6):459-66.

40. Mihich E, Ehrke MJ. Immunomodulating effects of anticancer drugs: the example of adriamycin. Transplant Proc 1984;16(2):499-501.

41. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 2005;65(18):8059-64.

42. Emens LA, Reilly RT, Jaffee EM. Cancer vaccines in combination with multimodality therapy. Cancer Treat Res 2005;123:227-45.

43. Lake RA, Robinson BW. Immunotherapy and chemotherapy--a practical partnership. Nat Rev Cancer 2005;5(5):397-405.

44. van der Most RG, Currie A, Robinson BW, Lake RA. Cranking the immunologic engine with chemotherapy: using context to drive tumor antigen cross-presentation towards useful antitumor immunity. Cancer Res 2006;66(2):601-4.

45. Nigam A, Yacavone RF, Zahurak ML, *et al.* Immunomodulatory properties of antineoplastic drugs administered in conjunction with GM-CSF-secreting cancer cell vaccines. International journal of oncology 1998;12(1):161-70.

46. Ghiringhelli F, Larmonier N, Schmitt E, *et al.* CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34(2):336-44.

47. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;105(7):2862-8.

48. Schiavoni G, Mattei F, Di Pucchio T, *et al.* Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. Blood 2000;95(6):2024-30.

49. Buttiglieri S, Galetto A, Forno S, De Andrea M, Matera L. Influence of drug-induced apoptotic death on processing and presentation of tumor antigens by dendritic cells. Int J Cancer 2003;106(4):516-20.

50. Casares N, Pequignot MO, Tesniere A, *et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med 2005;202(12):1691-701.

51. Casati A, Zimmermann VS, Benigni F, Bertilaccio MT, Bellone M, Mondino A. The immunogenicity of dendritic cell-based vaccines is not hampered by doxorubicin and melphalan administration. J Immunol 2005;174(6):3317-25.

52. Lake RA, van der Most RG. A better way for a cancer cell to die. N Engl J Med 2006;354(23):2503-4.

53. Rad AN, Pollara G, Sohaib SM, Chiang C, Chain BM, Katz DR. The differential influence of allogeneic tumor cell death via DNA damage on dendritic cell maturation and antigen presentation. Cancer Res 2003;63(16):5143-50.

54. Emens LA, Machiels JP, Reilly RT, Jaffee EM. Chemotherapy: friend or foe to cancer vaccines? Curr Opin Mol Ther 2001;3(1):77-84.

55. Demaria S, Volm MD, Shapiro RL, *et al.* Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. Clin Cancer Res 2001;7(10):3025-30.

56. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 1999;59(3):734-41.

57. Carson WE, 3rd, Shapiro CL, Crespin TR, Thornton LM, Andersen BL. Cellular immunity in breast cancer patients completing taxane treatment. Clin Cancer Res 2004;10(10):3401-9.

58. Machiels JP, Reilly RT, Emens LA, *et al.* Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001;61(9):3689-97.

59. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Involvement of TLR4/MD-2 complex in species-specific lipopolysaccharide-mimetic signal transduction by Taxol. J Endotoxin Res 2001;7(3):232-6.

60. Wang J, Kobayashi M, Han M, *et al.* MyD88 is involved in the signalling pathway for Taxol-induced apoptosis and TNF-alpha expression in human myelomonocytic cells. Br J Haematol 2002;118(2):638-45.

61. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. Eur J Immunol 2001;31(8):2448-57.

62. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem 2000;275(4):2251-4.

63. Joo HG. Altered maturation of dendritic cells by taxol, an anticancer drug. J Vet Sci 2003;4(3):229-34.

64. Nowak AK, Lake RA, Marzo AL, *et al.* Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J Immunol 2003;170(10):4905-13.

65. Nowak AK, Robinson BW, Lake RA. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. Cancer Res 2002;62(8):2353-8.

66. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 2005;11(18):6713-21.

67. Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel MT. Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. J Natl Cancer Inst 1997;89(11):783-9.

68. Bae SH, Park YJ, Park JB, Choi YS, Kim MS, Sin JI. Therapeutic synergy of human papillomavirus E7 subunit vaccines plus cisplatin in an animal tumor model: causal involvement of increased sensitivity of cisplatin-treated tumors to CTL-mediated killing in therapeutic synergy. Clin Cancer Res 2007;13(1):341-9.

69. Merritt RE, Mahtabifard A, Yamada RE, Crystal RG, Korst RJ. Cisplatin augments cytotoxic T-lymphocyte-mediated antitumor immunity in poorly immunogenic murine lung cancer. J Thorac Cardiovasc Surg 2003;126(5):1609-17.

70. Bang S, Kim HS, Choo YS, Park SW, Chung JB, Song SY. Differences in immune cells engaged in cell-mediated immunity after chemotherapy for far advanced pancreatic cancer. Pancreas 2006;32(1):29-36.

71. Rubio MT, Ittelet D, Raymond E, Blay JY, Bernard J, Chouaib S. The immunosuppressive effect of vincristine on allostimulatory potential of human dendritic cells interferes with their function and survival. International journal of oncology 2004;25(2):407-12.

72. Rea D, van Kooten C, van Meijgaarden KE, Ottenhoff TH, Melief CJ, Offringa R. Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. Blood 2000;95(10):3162-7.

73. Lanza L, Scudeletti M, Puppo F, *et al.* Prednisone increases apoptosis in in vitro activated human peripheral blood T lymphocytes. Clin Exp Immunol 1996;103(3):482-90.

74. Scudeletti M, Lanza L, Monaco E, *et al.* Immune regulatory properties of corticosteroids: prednisone induces apoptosis of human T lymphocytes following the CD3 down-regulation. Ann N Y Acad Sci 1999;876:164-79.

75. Almawi WY, Saouda MS, Stevens AC, Lipman ML, Barth CM, Strom TB. Partial mediation of glucocorticoid antiproliferative effects by lipocortins. J Immunol 1996;157(12):5231-9.

76. Elenkov IJ. Glucocorticoids and the Th1/Th2 balance. Ann N Y Acad Sci 2004;1024:138-46.

77. Cumberbatch M, Dearman RJ, Kimber I. Inhibition by dexamethasone of Langerhans cell migration: influence of epidermal cytokine signals. Immunopharmacology 1999;41(3):235-43.

78. Hackstein H, Morelli AE, Thomson AW. Designer dendritic cells for tolerance induction: guided not misguided missiles. Trends Immunol 2001;22(8):437-42.

79. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nat Rev Immunol 2004;4(1):24-34.

80. Matyszak MK, Citterio S, Rescigno M, Ricciardi-Castagnoli P. Differential effects of corticosteroids during different stages of dendritic cell maturation. Eur J Immunol 2000;30(4):1233-42.

81. Orsini F, Pavelic Z, Mihich E. Increased primary cell-mediated immunity in culture subsequent to adriamycin or daunorubicin treatment of spleen donor mice. Cancer Res 1977;37(6):1719-26.

82. Haskill JS. Adriamycin-activated macrophages as tumor growth inhibitors. Cancer Res 1981;41(10):3852-6.

83. Salazar D, Cohen SA. Multiple tumoricidal effector mechanisms induced by adriamycin. Cancer Res 1984;44(6):2561-6.

84. Ujhazy P, Zaleskis G, Mihich E, Ehrke MJ, Berleth ES. Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells. Cancer Immunol Immunother 2003;52(7):463-72.

85. Asmis R, Qiao M, Rossi RR, Cholewa J, Xu L, Asmis LM. Adriamycin promotes macrophage dysfunction in mice. Free Radic Biol Med 2006;41(1):165-74.

86. Markasz L, Stuber G, Vanherberghen B, *et al.* Effect of frequently used chemotherapeutic drugs on the cytotoxic activity of human natural killer cells. Mol Cancer Ther 2007;6(2):644-54.

87. Eralp Y, Wang X, Wang JP, Maughan MF, Polo JM, Lachman LB. Doxorubicin and paclitaxel enhance the antitumor efficacy of vaccines directed against HER 2/neu in a murine mammary carcinoma model. Breast Cancer Res 2004;6(4):R275-83.

88. Ewens A, Luo L, Berleth E, *et al.* Doxorubicin plus interleukin-2 chemoimmunotherapy against breast cancer in mice. Cancer Res 2006;66(10):5419-26.

89. Obeid M, Tesniere A, Ghiringhelli F, *et al.* Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007;13(1):54-61.

90. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4(12):941-52.

91. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 2001;166(9):5398-406.

92. Jonuleit H, Schmitt E, Steinbrink K, Enk AH. Dendritic cells as a tool to induce anergic and regulatory T cells. Trends Immunol 2001;22(7):394-400.

93. Pinzon-Charry A, Maxwell T, Lopez JA. Dendritic cell dysfunction in cancer: a mechanism for immunosuppression. Immunol Cell Biol 2005;83(5):451-61.

94. Gabrilovich DI, Patterson S, Timofeev AV, Harvey JJ, Knight SC. Mechanism for dendritic cell dysfunction in retroviral infection of mice. Clin Immunol Immunopathol 1996;80(2):139-46.

95. Gabrilovich D, Ishida T, Oyama T, *et al.* Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. Blood 1998;92(11):4150-66.

96. Almand B, Resser JR, Lindman B, *et al.* Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 2000;6(5):1755-66.

97. Saito H, Tsujitani S, Ikeguchi M, Maeta M, Kaibara N. Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. Br J Cancer 1998;78(12):1573-7.

98. Toi M, Taniguchi T, Yamamoto Y, Kurisaki T, Suzuki H, Tominaga T. Clinical significance of the determination of angiogenic factors. Eur J Cancer 1996;32A(14):2513-9.

99. Toi M, Bando H, Ogawa T, Muta M, Hornig C, Weich HA. Significance of vascular endothelial growth factor (VEGF)/soluble VEGF receptor-1 relationship in breast cancer. Int J Cancer 2002;98(1):14-8.

100. Menetrier-Caux C, Montmain G, Dieu MC, *et al.* Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. Blood 1998;92(12):4778-91.

101. Park SJ, Nakagawa T, Kitamura H, *et al.* IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. J Immunol 2004;173(6):3844-54.

102. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. Blood 2002;99(7):2468-76.

103. Yang AS, Lattime EC. Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. Cancer Res 2003;63(9):2150-7.

104. Yu Y, Zeng H, Vijay-Kumar M, *et al.* STAT signaling underlies difference between flagellin-induced and tumor necrosis factor-alpha-induced epithelial gene expression. J Biol Chem 2004;279(34):35210-8.

105. Kortylewski M, Kujawski M, Wang T, *et al.* Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 2005;11(12):1314-21.

106. Wang T, Niu G, Kortylewski M, *et al.* Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. Nat Med 2004;10(1):48-54.

107. Frey AB. Myeloid suppressor cells regulate the adaptive immune response to cancer. J Clin Invest 2006;116(10):2587-90.

108. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 2002;51(6):293-8.

109. Kusmartsev S, Gabrilovich DI. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. J Leukoc Biol 2003;74(2):186-96.

110. Almand B, Clark JI, Nikitina E, *et al.* Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001;166(1):678-89.

111. Gallina G, Dolcetti L, Serafini P, *et al.* Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. J Clin Invest 2006;116(10):2777-90.

112. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5(4):263-74.

113. Hoffmann TK, Muller-Berghaus J, Ferris RL, Johnson JT, Storkus WJ, Whiteside TL. Alterations in the frequency of dendritic cell subsets in the peripheral circulation of patients with squamous cell carcinomas of the head and neck. Clin Cancer Res 2002;8(6):1787-93.

114. Munn DH, Sharma MD, Hou D, *et al.* Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004;114(2):280-90.

115. Hwu P, Du MX, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol 2000;164(7):3596-9.

116. Mellor AL, Keskin DB, Johnson T, Chandler P, Munn DH. Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses. J Immunol 2002;168(8):3771-6.

117. Munn DH, Sharma MD, Lee JR, *et al.* Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002;297(5588):1867-70.

118. Fiore F, Nuschak B, Peola S, *et al.* Exposure to myeloma cell lysates affects the immune competence of dendritic cells and favors the induction of Tr1-like regulatory T cells. Eur J Immunol 2005;35(4):1155-63.

119. Ghiringhelli F, Puig PE, Roux S, *et al.* Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med 2005;202(7):919-29.

120. Berendt MJ, North RJ. T-cell-mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor. J Exp Med 1980;151(1):69-80.

121. Mukherji B, Chakraborty NG, Sivanandham M. T-cell clones that react against autologous human tumors. Immunol Rev 1990;116:33-62.

122. Mukherji B, Nashed AL, Guha A, Ergin MT. Regulation of cellular immune response against autologous human melanoma. II. Mechanism of induction and specificity of suppression. J Immunol 1986;136(5):1893-8.

123. Mukherji B, Wilhelm SA, Guha A, Ergin MT. Regulation of cellular immune response against autologous human melanoma. I. Evidence for cell-mediated suppression of in vitro cytotoxic immune response. J Immunol 1986;136(5):1888-92.

124. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101(5):455-8.

125. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 1999;59(13):3128-33.

126. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 1999;163(10):5211-8.

127. Itoh M, Takahashi T, Sakaguchi N, *et al.* Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 1999;162(9):5317-26.

128. Shevach EM. Regulatory T cells in autoimmmunity*. Annu Rev Immunol 2000;18:423-49.

129. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006;108(3):804-11.

130. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 2001;193(11):1295-302.

131. Hara M, Kingsley CI, Niimi M, *et al.* IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol 2001;166(6):3789-96.

132. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;194(5):629-44.

133. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4(4):330-6.

134. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol 2005;6(4):331-7.

135. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 2007;8(3):277-84.

136. Allan SE, Passerini L, Bacchetta R, *et al.* The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. J Clin Invest 2005;115(11):3276-84.

137. Morgan ME, van Bilsen JH, Bakker AM, *et al.* Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum Immunol 2005;66(1):13-20.

138. Walker MR, Kasprowicz DJ, Gersuk VH, *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003;112(9):1437-43.

139. Curiel TJ, Coukos G, Zou L, *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942-9.

140. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 2005;65(6):2457-64.

141. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN, Whiteside TL. Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 2005;92(5):913-20.

142. Viguier M, Lemaitre F, Verola O, *et al.* Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004;173(2):1444-53.

143. Alvaro T, Lejeune M, Salvado MT, *et al.* Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 2005;11(4):1467-73.

144. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003;9(12):4404-8.

145. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003;9(2):606-12.

146. Sutmuller RP, van Duivenvoorde LM, van Elsas A, *et al.* Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 2001;194(6):823-32.

147. Dannull J, Su Z, Rizzieri D, *et al.* Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005;115(12):3623-33.

148. Ghiringhelli F, Menard C, Puig PE, *et al.* Metronomic cyclophosphamide regimen selectively depletes CD4(+)CD25 (+) regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56(5):641-8.

149. Beyer M, Kochanek M, Darabi K, *et al.* Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood 2005;106(6):2018-25.

150. Dudley ME, Wunderlich JR, Yang JC, *et al.* Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005;23(10):2346-57.

151. Dalgleish AG, Whelan MA. Cancer vaccines as a therapeutic modality: The long trek. Cancer Immunol Immunother 2006;55(8):1025-32.

152. Gauthier MJ, Aubert I, Abrams-Ogg A, Woods JP, Bienzle D. The immunophenotype of peripheral blood lymphocytes in clinically healthy dogs and dogs with lymphoma in remission. J Vet Intern Med 2005;19(2):193-9.

153. Henry CJ, McCaw DL, Brock KV, *et al.* Association between cancer chemotherapy and canine distemper virus, canine parvovirus, and rabies virus antibody titers in tumor-bearing dogs. J Am Vet Med Assoc 2001;219(9):1238-41.

154. Winnicka A, Jagielski D, Hoffmann-Jagielska M, Lechowski R. Cytometric evaluation of peripheral blood lymphocytes in dogs with lymphoma during chemotherapy. J Vet Med A Physiol Pathol Clin Med 2002;49(6):303-6.

155. Yagi H, Nomura T, Nakamura K, *et al.* Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004;16(11):1643-56.

156. Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209-26.

Chapter Two

Effects of Chemotherapy on Adaptive Immunity and Regulatory T Cells in Dogs with Cancer

ABSTRACT

Although certain chemotherapeutic agents demonstrate immunostimulatory effects and may therefore be logical candidates for combination with immunotherapy, the immunomodulatory effects of chemotherapy on adaptive immune responses in dogs have not been previously evaluated. Furthermore, the impact of chemotherapy on regulatory T cells (Treg), a subset of T lymphocytes often detrimental to effective combinatorial therapy, is unknown. Therefore, a study was conducted to evaluate the effects of two common chemotherapy protocols on adaptive immunity and Treg numbers in dogs with cancer. Twenty-one dogs with cancer (12 dogs with lymphoma, 9 dogs with osteosarcoma) were enrolled in a prospective study to assess the effects of doxorubicin versus multi-drug chemotherapy on adaptive immunity and Treg levels (characterized as CD4+IL-2R+ T-cells). Numbers of T cells, B cells, and CD4+IL-2R+ T-cells were assessed by flow cytometry and antibody responses to *de novo* vaccination were measured by ELISA before, during, and after chemotherapy. Pre-treatment CD4+IL-2R+ T-cells, CD4+ and CD8+ T-cells and B cell numbers were also compared to those of healthy, age-matched control dogs.

We found that dogs with cancer had significantly fewer CD4+ and CD8+ T cells than healthy dogs prior to administration of chemotherapy. In addition, dogs with lymphoma had significantly more CD4+IL-2R+ T-cells than healthy dogs, which persisted despite induction of clinical remission. Treatment with doxorubicin did not result in significant changes in CD4+IL-2R+ cells or lymphocyte numbers compared to pre-treatment values. Similarly, treatment with multi-agent chemotherapy did not elicit persistent changes in CD4+IL-2R+, CD4+ or CD8+ T-cell numbers, but did cause a significant and persistent decline in B cell numbers. Despite the decline in the number of B cells, antibody titers were similar between control and chemotherapy-treated dogs following vaccination with a novel antigen, suggesting that chemotherapy does not suppress antibody production. As a whole, our findings point towards a minimal impact of chemotherapy on adaptive immune responses but also emphasize the need to better assess the role of Treg in dogs with cancer.

INTRODUCTION

Although chemotherapy has typically been viewed as immunosuppressive, there is a growing body of evidence that demonstrates the immunostimulatory effects of a number of commonly used chemotherapy drugs. Studies in mice and humans show that administration of chemotherapeutic agents has variable and sometimes dose-dependent effects on different components of the immune system. For example, administration of high doses of the drug cyclophosphamide results in severe myelosuppression whereas low-dose therapy augments immunity through a number of mechanisms (1-3). The immunomodulatory effects of other chemotherapeutic agents, such as doxorubicin and the vinca alkaloids, are less clearly dose-dependent. These agents have both suppressive and stimulatory effects at equivalent doses, suggesting that factors other than dose determine their immunomodulatory potential (4, 5).

Investigation of the impact of various chemotherapy agents on immunity has been driven by a growing emphasis on multimodality approaches to cancer therapy. As recently reviewed by Lake and Emens, combination of cancer vaccines with conventional chemotherapy may be particularly fruitful (2, 6). Studies in mice and humans show that cytotoxic tumor reduction with chemotherapy followed by cancer vaccine therapy can markedly augment antitumor immune responses (7-13). But at the same time, many of these studies also show that variables such as the timing of chemotherapy and tumor vaccination or the drugs used can hinder development of effective antitumor immunity. For example, low-dose cyclophosphamide administration given at the time of tumor vaccination of mice with mammary carcinoma enhanced cellular and humoral immunity, but CYC administered with or subsequent to vaccination induced tumor tolerance (10,

11). Therefore a better understanding of the impact of chemotherapy on the immune response has become increasingly important to the design of successful multimodality treatment protocols.

Another factor that may influence the outcome of combined chemotherapy and tumor vaccination is tumor evasion of host immunity. Evasion can be manifested in many ways, encompassing aspects of both innate and adaptive immune responses such as aberrant DC and CTL function or tumor-driven recruitment of Treg. Many cancer patients, for instance, have abnormally high levels of Treg which may directly interfere with the development of cell-mediated antitumor immune responses (14-18). In contrast, cancer patients may also have abnormally low levels of mature DCs and consequently a diminished ability to mount an effective response to tumor vaccination (19, 20). Thus, the potential consequences of cancer on host immunity will also need to be addressed during investigation of the immunomodulatory properties of chemotherapy agents in the cancer patient.

The effects of chemotherapy on adaptive immune responses in cancer-bearing dogs have not been evaluated in a prospective manner, nor has the evaluation of canine Treg previously been described. There have been a limited number of studies of the impact of chemotherapy on lymphocyte numbers and antibody titers in dogs with cancer; the majority of these studies have focused on dogs with lymphoma (21-23). In a recent study of dogs undergoing chemotherapy for lymphoma, for example, a > 50% reduction in numbers of total lymphocytes was observed. However, lymphocyte numbers and subsets were not evaluated in these dogs prior to the onset of chemotherapy making differentiation of the effects of chemotherapy on lymphocyte numbers from those of

lymphoma impossible (21). Another investigation of lymphocyte subsets in dogs with lymphoma found that, compared to healthy dogs, numbers of CD4+ and CD8+ T cells were significantly lower in the cancer-bearing dogs, while B-cell numbers were considerably higher. During the course of chemotherapy, CD4 and CD8 lymphocyte levels increased and B cell levels decreased towards those of the control dogs (23). No assessment of B or T cell function or consideration of tumor immune evasion was undertaken in either of these studies.

Investigation of the effects of chemotherapy agents on rodent and human Treg is in its early stages. In these species CYC has been shown to selectively deplete Treg through a number of mechanisms (1, 3, 24, 25). Recently, the drug fludarabine has also been shown to inhibit Treg function although the mechanism for this effect is not yet understood (26). Treg have been identified in cats with FIV based on surface expression of CD25 in CD4+ T lymphocytes (27, 28). In these studies the investigators found that CD4+CD25+ Treg existed in a continually activated state during FIV infection and played a role in suppression of anti-viral CTL responses. Although these endogenously activated CD4+CD25+ T cells exhibited a phenotype similar to natural Treg, definitive differentiation from activated non-regulatory T cells was not described. There have been no reports investigating the impact of chemotherapy on Treg in companion animals with cancer.

In the first part of this chapter we investigated the effects of two common chemotherapy protocols on T and B cell numbers and humoral immune responses to *de novo* vaccination with the primary goal of better understanding the impact of chemotherapy on adaptive immune responses in dogs with cancer. Our first hypothesis

was that chemotherapy would not cause persistent lymphopenia but instead would transiently suppress T and B cell numbers. Secondly, we hypothesized that adaptive immune responses might differ in dogs treated with a multi-agent chemotherapy protocol compared to those treated with single-agent chemotherapy, thus we compared T and B cell numbers and antibody responses between treatment groups.

In the second section of this chapter a flow cytometry-based assay was used to preliminarily assess canine Treg levels by evaluating cell-surface expression of the IL-2 receptor (IL-2R) in CD4+ lymphocytes. We surmised that Treg would be present in dogs and would likely be higher in cancer patients than in healthy control dogs. Although a better method for canine Treg identification was developed after completion of these studies (described in Chapter 3), the initial assay was used to monitor CD4+IL-2R+ T-cells during the course of chemotherapy for the cancer-bearing dogs. These studies served as an important first step in assessing the impact of cancer and chemotherapy on adaptive immunity and Treg status in dogs with lymphoma and osteosarcoma.

MATERIALS AND METHODS

Inclusion Criteria

Owners of dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) were eligible for enrollment in this prospective study. Dogs with cytologically or histopathologically confirmed lymphoma (LSA) or osteosarcoma (OSA) were included. Dogs were excluded from the study if they had received previous chemotherapy or steroids within 2 weeks of initiating chemotherapy. Dogs receiving doxorubicin chemotherapy were treated at a dose of 30 mg/m^2 body surface area for a

total of 5 treatments administered over a 15 week period. Dogs receiving multi-drug therapy were treated with cyclophosphamide, vincristine, prednisone, doxorubicin and L-asparaginase (CHOP), according to a previously described protocol (29, 30). All chemotherapy administrations and sample collections were performed at the CSU-VTH. Eight healthy dogs of various breeds belonging to staff members of the CSU-VTH formed a control population. Protocols for all dogs enrolled in this study were approved by the Animal Care and Use Committee at Colorado State University and all owners were provided with informed consent.

Sample Collection

Baseline blood samples were collected immediately prior to initiation of chemotherapy. Subsequent blood samples were obtained at week 1, week 3, month 3 and month 6 of treatment. Ten to 15 mls of whole blood was collected into EDTA tubes at each time point for isolation of peripheral blood mononuclear cells (PBMC). Blood samples were also collected from healthy control dogs. Blood samples were mixed with PBS and then separated by Ficoll density centrifugation. The separated PBMC were washed and resuspended in cell freezing medium, frozen to -80°C overnight in a controlled rate cell freezer (Nalge Nunc International, Rochester, NY) and then transferred to liquid nitrogen for long-term storage. Complete blood counts were submitted at each time point for determination of absolute lymphocyte numbers. All flow cytometric analyses were performed on previously frozen PBMC specimens which had been aliquotted to approximately 5 x 10^6 cells/ml prior to freezing.

Dogs were vaccinated with 100μg of keyhole limpet hemocyanin (KLH) (Sigma-Aldrich Chemical Co, St. Louis, MO). The KLH antigen was added to a vaccine adjuvant

comprised of cationic liposomes and non-coding plasmid DNA immediately prior to injection, as reported previously (31). The vaccines were administered intradermally 24 hours after administration of the first chemotherapy and again on week 3 and week 6. Serum was obtained pre-treatment and on week 3, month 3 and month 6 following initiation of chemotherapy. Serum samples were stored frozen at -80°C until analysis. The 8 healthy dogs were also vaccinated with KLH and serologically assessed at the same time points.

Flow cytometric evaluation of T and B lymphocytes

Peripheral blood mononuclear cell (PBMC) specimens were thawed and aliquotted to between 5 X 10^5 and 1 X 10^6 cells per well in round bottom 96-well plates for immunostaining. Cells were stained with directly conjugated antibodies diluted to appropriate dilutions in FACS buffer (PBS with 2% FBS and 0.1% sodium azide). The antibodies and fluorochromes used for multi-color flow cytometric analysis included the following: FITC-conjugated anti-dog CD4 (clone YKIX302.9; Serotec, Raleigh, NC), PE-conjugated anti-dog CD8 (clone YCATE55.9, Serotec), PE-conjugated anti-dog B cells (clone CA2.1D6, Serotec), and a cross-reactive PEcy5-conjugated anti-mouse CD44 antibody (clone IM7, eBiosciences, San Diego, CA). Cells were washed after antibody incubation, then fixed for 30 minutes in 1% paraformaldehyde solution and stored in FACS buffer at 4°C until prior to analysis. Samples were analyzed with a Cyan MLE flow cytometer (Dako-Cytomation, Ft. Collins, CO) and analysis was done using Summit 4.0 software (Dako-Cytomation) on at least 20,000 events for each sample. Analysis gates were set on the live lymphocyte population based on typical forward and side scatter characteristics (32). The percentage of each lymphocyte subset was determined by

flow cytometry, and the absolute numbers were then calculated based on the total lymphocyte count determined on the same day from the complete blood count.

Flow cytometric evaluation of canine Treg

a) Immunostaining with recombinant human IL-2. The relatively high degree of sequence homology of the functional IL-2 receptor between dogs and humans has permitted identification of the IL-2R on canine lymphocytes although cloning of the alpha-chain of the IL-2R has not been reported (33). However, we reasoned that it might be possible to identify CD4+CD25+ T-cells by immunostaining with human IL-2. To this end, PBMC samples (from the patient population described in Part 1) were aliquotted to 1 x 10^6 per well in round bottom plates as previously described. Anti-dog CD4-FITC and anti-dog CD8-PE were added as before followed by 6 ng/ml of biotinylated human IL-2 (R&D Systems, Minneapolis, MN). The optimal concentration of the human IL-2 protein was determined based on the outcome of multiple titration experiments (data not shown). Control samples for specificity for the binding of biotinylated human IL-2 were prepared by adding a biotinylated irrelevant human protein (soybean trypsin inhibitor) in place of the IL-2 reagent. After incubation at 4^0C for 60 minutes and washing with FACS buffer, streptavidin-APC was added to each well and the cells incubated for an additional 20 minutes at 4^0C. Samples were then analyzed as above on a Cyan MLE with Summit 4.0 software and the percentage or absolute number of CD4+IL-2R+ or CD8+IL-2R+ cells was determined. In some experiments, PBMC were stimulated with conconavalin-A (conA) (Sigma-Aldrich Chemicals) at 5 *u*g/ml per 5 x 10^6 cells for 24 hours at 37^0C prior to immunostaining.

b) Immunostaining with additional cell surface markers of Treg. In an effort to identify additional cell surface markers associated with Treg identification in other species, we investigated whether any of the following antibodies (all purchased from eBiosciences) were cross-reactive in the dog: PE-conjugated Lag-3 (clone C9B7W), APC-conjugated anti-mouse GITR (clone DTA-1), biotinylated anti-mouse CTLA-4 (clone UC10-4B9), biotinylated anti-mouse CD122 (clone TM-b1) and PE-conjugated anti-mouse/human CD25 (clone PC61). In addition we also evaluated the use of an anti-feline CD25 antibody (a kind gift from Paul Avery, Colorado State University). For each antibody, an appropriate isotype control antibody was also used. Unfortunately, none of these reagents were useful in identification of these Treg-associated markers on canine lymphocytes.

Serology

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. Serial two-fold dilutions of serum from each sample were incubated in duplicate wells, starting at a dilution of 1:1000. Plates were washed, incubated with peroxidase-conjugated goat anti-dog IgG (Jackson ImmunoResearch, West Grove, PA), then washed again and incubated with ABTS substrate solution (KPL, Gaithersburg, MD). Absorbance was assessed at 405 nm using an automated plate reader and endpoint antibody titers were calculated using Ascent Software (Thermo Lab Systems, Franklin, MA).

Statistical analyses

Significant differences in lymphocyte and CD4+IL-2R+ T-cell numbers were calculated by first applying log transformation. After log transformation, two-tailed sample *t*-tests were used to determine significant differences. Significance was established at $p < 0.05$. Differences were further assessed using analysis of variance (ANOVA) and Tukey's multiple comparisons test. The mean (±SE) number of CD4+ T cells, CD8+ T cells, B cells and CD4+IL-2R+ cells was calculated pre- and post-chemotherapy for the following groups of dogs: 1) healthy dogs versus dogs with lymphoma, 2) healthy dogs versus dogs with osteosarcoma and 3) dogs with lymphoma versus dogs with osteosarcoma. For healthy dogs "pre" was defined as a sample taken at the initiation of the study while a "post" sample was obtained from the same dogs 6 months later. Statistical differences were assessed using ANOVA followed by Tukey's multiple comparisons test. Repeated measures ANOVA was used to further assess any changes over time. For analysis of KLH antibody titers, the mean (± SE) endpoint titer for each treatment group was calculated. Repeated measures ANOVA with Tukey's test was used to compare mean ELISA titers between groups at each time point.

RESULTS

Part 1: Adaptive Immune Responses

Patient population

Twelve dogs receiving doxorubicin single-agent chemotherapy and nine dogs receiving a multi-drug chemotherapy protocol for lymphoma were evaluated in this study. In addition, 8 healthy age-matched dogs were evaluated. Of the 12 dogs treated with doxorubicin only, 9 dogs were treated for osteosarcoma post-amputation and three dogs were treated for lymphoma. All 9 dogs receiving the multi-drug treatment protocol (CHOP) were treated for lymphoma. The median age of dogs with cancer in this study was 8 years (range 3-13 years). The median age of the 8 healthy dogs was 9 years (range 2-10 years).

Lymphocyte numbers and subsets

Total lymphocyte numbers and numbers of lymphocytes in subsets (CD4+, CD8+, and B cells) were compared between healthy age-matched control dogs and dogs with cancer (**Figure 1**). Dogs with osteosarcoma had significantly fewer total lymphocytes ($p = 0.003$) compared to healthy control dogs. In addition, dogs with lymphoma also had significantly fewer total lymphocytes ($p = 0.05$) than healthy control dogs (data not shown). When the T cell subsets were compared, all dogs with cancer had significantly fewer CD4+ and CD8+ lymphocytes, compared to healthy dogs. This was also true when the subsets of dogs with lymphoma or dogs with osteosarcoma were compared to control dogs ($p = 0.02$ and 0.03, respectively). However, pre-treatment total lymphocyte numbers or numbers of CD4+ or CD8+ T cells were not significantly different when dogs with lymphoma were compared to dogs with osteosarcoma. The

number of B cells was not significantly different between dogs with lymphoma and healthy control dogs or between dogs with osteosarcoma and healthy control dogs. However, dogs with osteosarcoma had significantly fewer ($p < 0.05$) B cells than dogs with lymphoma.

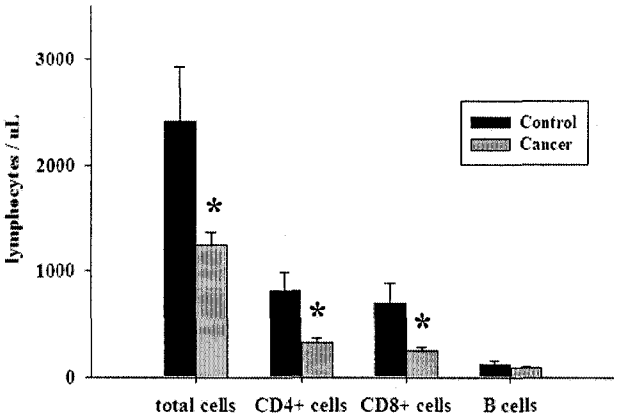

Figure 1: Dogs with cancer have fewer numbers of lymphocytes than healthy dogs. The total number of CD4+ and CD8+ T-cells and B-cells were compared between healthy dogs and those with either OSA or LSA. The mean values for total lymphocyte count, CD4, CD8 and B cell counts were then plotted for healthy control dogs versus dogs with cancer. Bars give standard error (SE) and * denotes statistically significant difference ($p < 0.05$).

Effects of chemotherapy on lymphocyte numbers

Lymphocyte subsets were evaluated for changes over time during and after treatment. In patients treated with doxorubicin only, significant differences in numbers of CD4+ T cells or CD8+ T cells were not observed at any time during or after treatment, when compared to pre-treatment values (**Figure 2**).

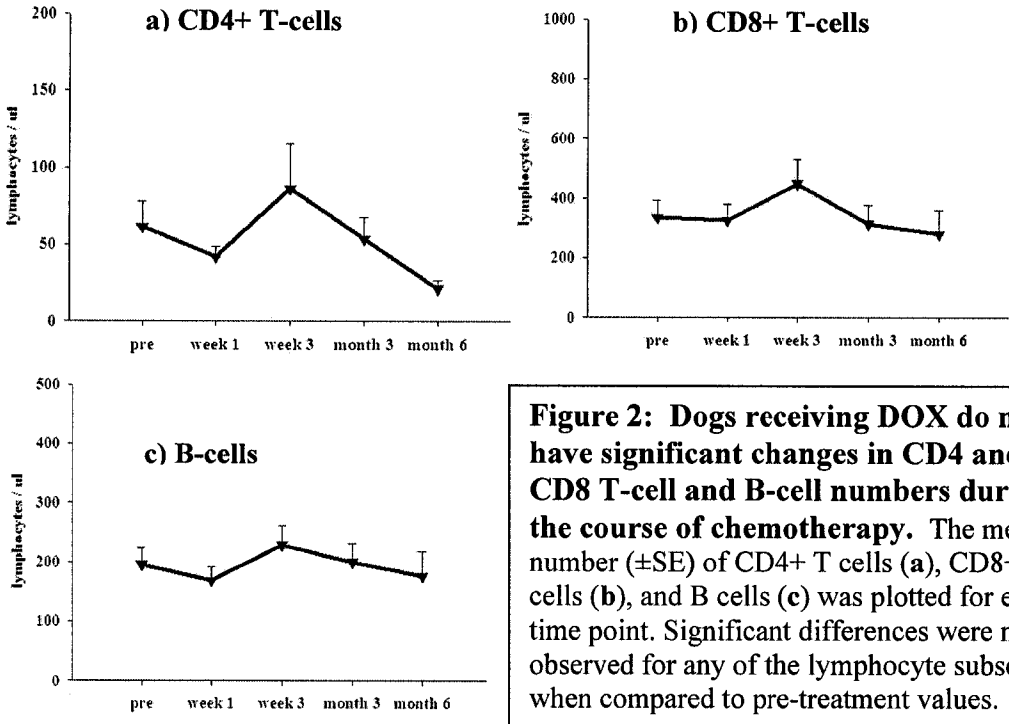

Figure 2: Dogs receiving DOX do not have significant changes in CD4 and CD8 T-cell and B-cell numbers during the course of chemotherapy. The mean number (±SE) of CD4+ T cells (**a**), CD8+ T cells (**b**), and B cells (**c**) was plotted for each time point. Significant differences were not observed for any of the lymphocyte subsets when compared to pre-treatment values.

In dogs treated with CHOP, the numbers of CD4+ T cells did not change significantly during the study, compared to pre-treatment values (**Figure 3**). The numbers of CD8+ T cells were, however, significantly decreased one week after the first treatment (p= 0.02), though the numbers of CD8+ T cells rebounded after week 1 and were not significantly different at any other time points (Figure 3). In dogs treated with doxorubicin only, significant changes in B cell numbers following treatment were not observed (Figure 2). However, in dogs treated with CHOP, B cell numbers declined significantly after the first treatment and remained significantly decreased throughout the 6-month period of study ($p < 0.05$) (Figure 3).

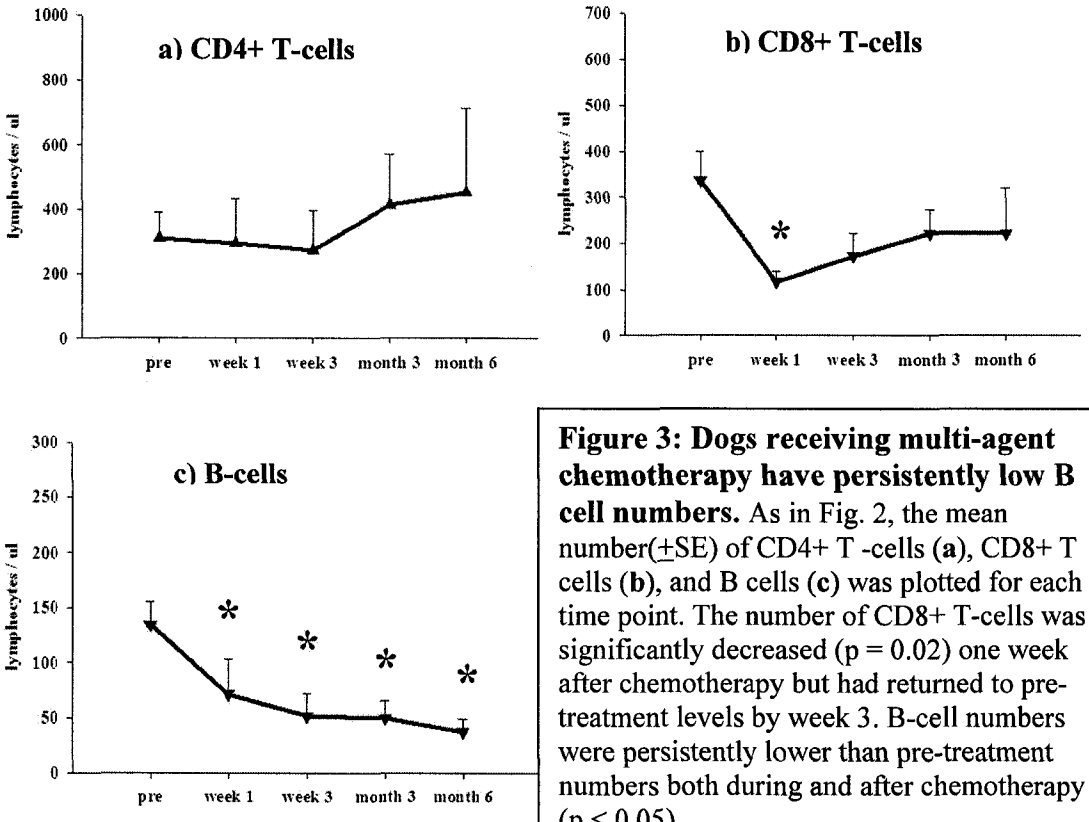

Figure 3: Dogs receiving multi-agent chemotherapy have persistently low B cell numbers. As in Fig. 2, the mean number(±SE) of CD4+ T -cells (**a**), CD8+ T cells (**b**), and B cells (**c**) was plotted for each time point. The number of CD8+ T-cells was significantly decreased ($p = 0.02$) one week after chemotherapy but had returned to pre-treatment levels by week 3. B-cell numbers were persistently lower than pre-treatment numbers both during and after chemotherapy ($p < 0.05$).

Serology

Dogs treated with either doxorubicin or CHOP and control dogs all mounted significant antibody titers following the 3 immunizations with KLH (**Figure 4**). The magnitude of the antibody responses was maximal at 3 months post-vaccination and declined by 6 months in all 3 groups. Antibody titers to KLH were numerically higher in CHOP treated dogs compared to control or doxorubicin treated dogs, but the differences were not statistically significant. When mean KLH titers at week 3, month 3 and month 6 were compared between all 3 groups, statistically significant differences were not observed.

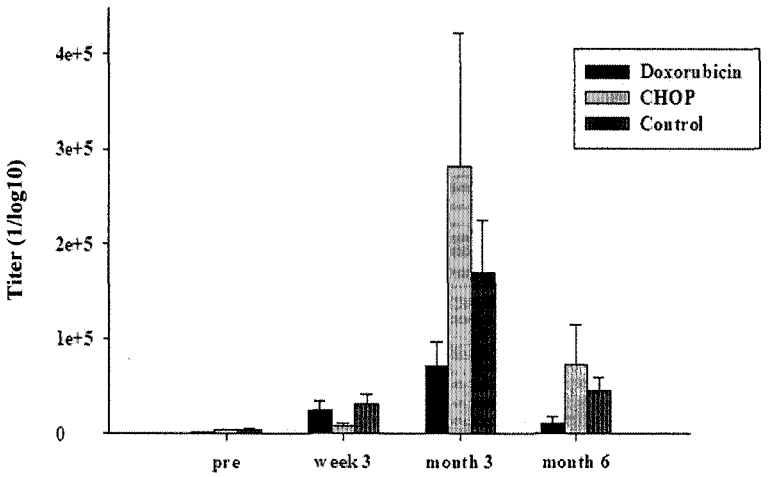

Figure 4: Dogs with cancer develop antibody titers similar to those of healthy dogs. Dogs were vaccinated during chemotherapy and compared to control vaccinated dogs. The mean titer (±SE) for each group of animals at each time point was calculated and plotted. Changes in mean antibody titer over time within or between the treatment groups were not statistically different.

Part 2: Preliminary Analysis of Regulatory T Cells

Preliminary Identification of Regulatory T Cells

Prior to analysis of PBMC specimens from the 21 study dogs, flow cytometric detection of the canine IL-2R on CD4+ lymphocytes was optimized using PBMCs obtained from 5 healthy dogs and 5 dogs with different types of cancer. Consistent detection of a small population of CD4+IL-2R+ T cells was possible in both normal and cancer-bearing dogs however, the percentage of cells expressing both markers was usually very low; generally ranging from 0.5 to 2.5% of the total CD4+ T cell population **(Figure 5A – upper right quadrant)**.

As demonstrated by immunostaining with the control protein (not shown), staining for the IL-2R was specific, however a population of CD4⁻IL-2R+ lymphocytes could also be detected in many of the samples **(Figure 5A- upper left quadrant)**.

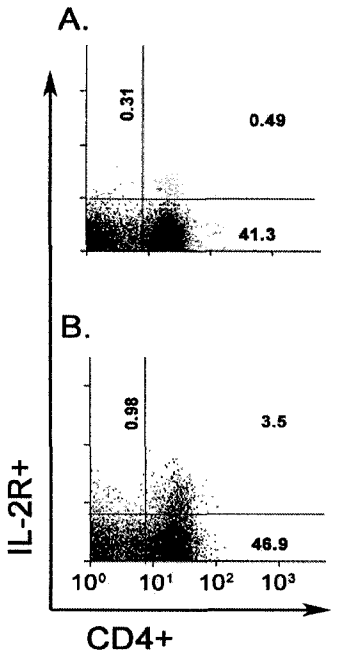

Figure 5: Identification of CD4+IL-2R+ T-cells by flow cytometry. Representative dot plots of IL-2R expression by CD4+ T-cells from **(A)** PBMCs and **(B)** following overnight incubation of the same PBMC sample with ConA as explained in the text. Note the large increase in CD4+IL-2R+ cells following stimulation. The percentages of lymphocytes contained within each quadrant are given.

Further investigation of this population revealed that the majority of these cells were CD8+ lymphocytes; however a small amount of IL2-R staining was also detected on CD4- and CD8-negative cells (data not shown). IL2-R staining on other cells such as B-lymphocytes, DCs or NK cells was not assessed.

Half of the PBMC samples run during the assay validation process were additionally cultured overnight in tissue culture medium containing ConA prior to immunostaining for CD4 and hIL-2R. As shown in **Figure 5B**, this led to an approximately 2 to 6-fold increase in the number of CD4+IL-2R+ and CD4⁻IL-2R+ cells. This result is most consistent with increased surface expression of the IL-2R on activated T cells and demonstrates the inability of this assay to discriminate between Treg and

activated CD4+ T cells. In an effort to solve this problem, we investigated whether other cell surface markers used to identify human and rodent Tregs could be used for canine Treg detection. As described in Material and Methods, PBMC samples were tested for cell surface expression of Lag3, CD122, CTLA-4, GITR, and CD25 (using a combination of human, murine and feline antibodies). Unfortunately none of these markers could be detected with these reagents in any of the samples.

Evaluation of CD4+IL-2R+ T cells in Healthy Dogs vs. Dogs with Cancer

Recognizing the limitations of the present assay to distinguish Treg from activated, non-regulatory CD4+ T cells, we compared pre-treatment numbers of CD4+IL2-R+ T cells in the cancer-bearing dogs to those of the healthy, age-matched control dogs. As shown in **Figure 6**, untreated dogs with lymphoma had significantly more CD4+IL-2R+ T cells than control dogs ($p = 0.01$). Although the dogs with osteosarcoma tended to have higher CD4+IL-2R+ T cell numbers than normal dogs, this difference was not statistically significant. No significant differences were found between the numbers of CD4+IL-2R+ T cells in dogs with lymphoma versus those with osteosarcoma.

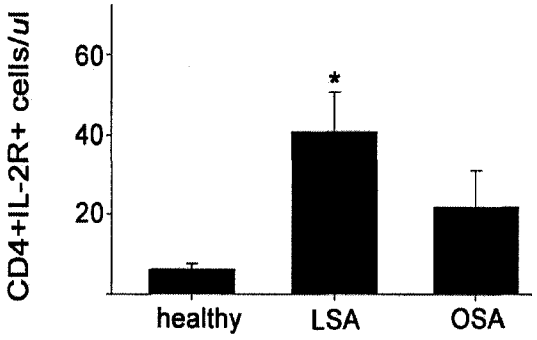

Figure 6: Dogs with lymphoma have increased CD4+IL-2R+ T-cells in their blood compared to healthy dogs. The absolute number of CD4+IL-2R+ cells ± (SE) is given, comparing values prior to beginning chemotherapy for healthy dogs to those with LSA or OSA, p= 0.01.

Effects of chemotherapy on CD4+IL-2R+ T cells

CD4+IL-2R+ T cells were next evaluated for changes over time during and after chemotherapy for each of the cancer-bearing dogs. Although dogs with lymphoma tended to have more CD4+IL-2R+ T cells at all time points compared to dogs with osteosarcoma, these differences were not statistically significant **(Figure 7A)**. In dogs treated with CHOP, the number of CD4+IL-2R+ T cells did not change significantly during the study compared with pretreatment values (**Figure 7B**). Similarly, for the dogs treated with doxorubicin only, no significant changes in CD4+IL-2R+ T cell numbers were observed during or after treatment when compared to pre-treatment levels. This was true for dogs with osteosarcoma as well as lymphoma, although the number of in this group was very small (n = 3).

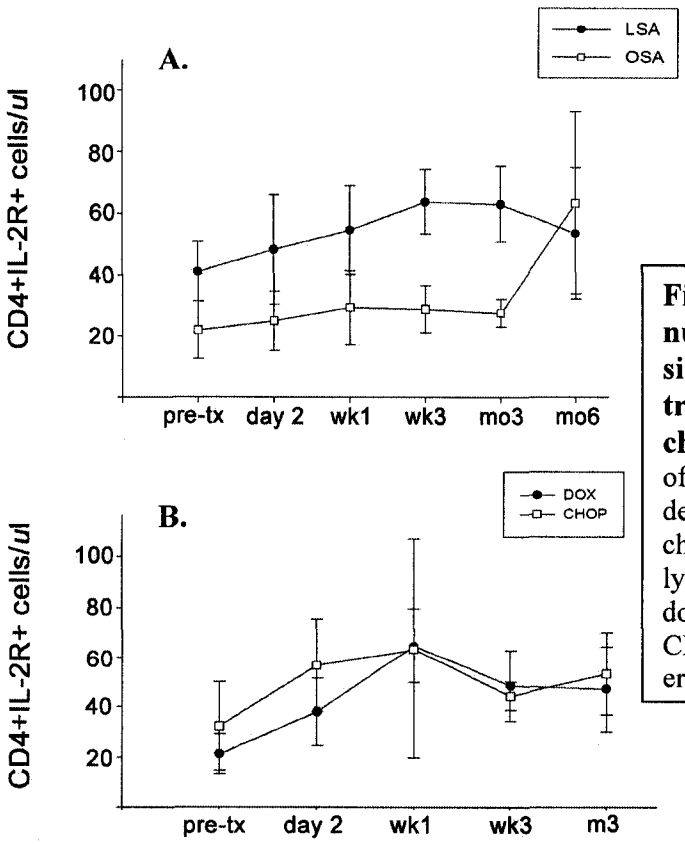

Figure 7: CD4+IL-2R+ T-cell numbers do not change significantly from pre-treatment levels during chemotherapy. The total number of CD4+IL-2R+ T-cells was determined at each time point during chemotherapy for **(A)** dogs with lymphoma or osteosarcoma and **(B)** dogs treated with either DOX or CHOP. Bars represent the standard error (SE).

DISCUSSION

The results of the studies in this chapter offer insight into the effects of cancer and chemotherapy on T and B-lymphocyte numbers in cancer-bearing dogs. One of the most striking observations that arose here was that neither administration of DOX as single-agent chemotherapy nor the CHOP multi-agent protocol elicited significant persistent changes in numbers of CD4+ and CD8+ T cells in any of the dogs during or after chemotherapy. Although functional analyses of CD4+ and CD8+ T cells were not performed, the normal antibody responses and lack of development of secondary infections in these dogs makes it unlikely that administration of either chemotherapy protocol was associated with a decreased ability to mount a cell-mediated immune response. The normal T-cell counts contrast, however, with the development of mild to moderate neutropenia in about 25 – 30% of the treated dogs in this study. In these dogs, the nadir generally occurred at 7 days following the initial treatment and had resolved by the 3-week sampling time point. Therefore, although chemotherapy may suppress part of the innate immune response (e.g. neutrophil counts) these studies suggest that cell-mediated adaptive immune responses are preserved.

The function of the humoral arm of the adaptive immune response also appears to be largely unaffected by repeated administration of chemotherapy agents. Thus, none of the dogs in this study demonstrated an impaired ability to mount an antibody response following vaccination with the KLH antigen. Interestingly, while total B cell numbers were persistently low in dogs treated with the CHOP protocol, this did not impact IgG responses as dogs receiving CHOP had antibody titers similar to those of healthy dogs and dogs treated with doxorubicin alone. This finding suggests that plasma cells and

memory B cells may be less sensitive to the effects of multi-drug chemotherapy than circulating peripheral B cells.

Another key finding to emerge from this study is that dogs with lymphoma or osteosarcoma have significantly lower numbers of CD4+ and CD8+ T cells prior to beginning chemotherapy than age-matched, healthy control dogs. These observations correlate with those of Winnicka et al. who also found that dogs with lymphoma had fewer T cells and T cell subsets when compared to normal dogs (23). Although low T-cell numbers have been described in human sarcoma patients they have not been previously reported in dogs with osteosarcoma (34). Possible explanations for our observations include stress-induced T-cell lymphopenia or tumor-induced T-lymphocyte suppression in dogs with lymphoma and osteosarcoma.

Definitive identification of Treg was not possible during the time period of this study. There were a number of limitations associated with the assay that restrict interpretation of CD4+IL-2R+ T cells as canine Treg. For example the percentage of CD4+IL-2R+ T cells in this study, expressed as a percentage of the total CD4+ T cell population, ranged from as low as 0.3% to as high as 12%. The vast majority of dogs with cancer, however, had CD4+IL-2R+ T cell percentages in the 1.0 to 3.5% range. In healthy humans and in mice, however, the circulating Treg population is reported to comprise from 5 – 10% of the CD4+ T cell subset and in cancer patients Treg levels of 10-25% are often described (35, 36). The abnormally low levels reported in this study suggest that: 1) the sensitivity of human biotinylated IL-2 for the canine IL-2R may be poor, 2) canine Treg do not express high levels of the IL-2R as is observed in rodents and humans, and/or 3) the IL-2-streptavidin-APC complex is lost from the surface of CD4+ T

cells during the staining process. The observation that overnight incubation of PBMCs with ConA leads to large increases in CD4IL-2R+ T cells, however, shows that CD4+IL-2R+ T cells can be identified when present in larger numbers. However, most of these cells would be expected to be activated CD4+ T cells rather than Treg.

Despite these limitations and the caution that must be used in interpreting the data, we found that dogs with lymphoma had higher numbers of CD4+IL-2R+ T cells than either dogs with osteosarcoma or normal dogs. The increased CD4+IL-2R+ T-cell numbers persisted throughout the course of therapy and were not dependent on the protocol used. This suggests that if CD4+IL-2R+ T-cells are indeed Treg, then peripheral Treg levels do not reflect remission status in dogs with lymphoma since nearly all of the dogs had achieved a complete remission based on physical examination by the 2^{nd} or 3^{rd} week of chemotherapy. Persistently elevated Treg levels may reflect the continued presence of microscopic disease rather than the response to chemotherapy. While we can not exclude the possibility that dogs with lymphoma experience chronic non-regulatory-CD4+ T-cell activation, the total number of CD4+ T cells did not change significantly at any point during the study period nor did the dogs demonstrate any indication of concurrent infection such as fever or elevated neutrophils counts.

Interestingly, chemotherapy did not appear to affect peripheral CD4+IL-2R+ T-cell numbers in either dogs with lymphoma or osteosarcoma. In people, treatment with low-dose cyclophosphamide results in a fairly rapid decline in peripheral Treg levels (1). In the present study, dogs receiving CHOP chemotherapy were given cyclophosphamide during the 2^{nd} and 7^{th} weeks of the protocol; following a similar dosing schedule to that used in human lymphoma patients. At the 3 week time point, however, CD4+IL-2R+

numbers were similar to pre-treatment values suggesting that cyclophosphamide does not lead to CD4+IL-2R+ T-cell depletion. Unfortunately, we can not exclude the possibility that CD4+IL-2R+ T-cells are not Treg nor can we be certain that the present assay is sensitive enough to detect changes in CD4+IL-2R+ T-cells over time.

In summary, the results of this chapter show that chemotherapy does not significantly suppress T-cell numbers and that, despite reduction in B-cell numbers with multi-agent chemotherapy, antibody responses to vaccination remained intact. These observations lend support for the concurrent administration of tumor immunotherapy and chemotherapy; studies of T-cell function are indicated to better assess the efficacy of combinatorial approaches to cancer therapy.

Unfortunately, it was not possible to draw firm conclusions from these experiments regarding the effects of chemotherapy on canine Treg levels. While it appears that dogs with lymphoma have abnormally high numbers of circulating CD4+IL-2R+ T-cells compared to those of healthy dogs and that chemotherapy does not decrease CD4+IL-2R+ T-cell levels, the potentially low sensitivity and specificity of the assay for Treg detection warrants caution in interpretation of the data. These results do, however, illustrate the need for a better method to identify canine Treg and suggest that Treg are likely to play a role in canine tumor pathogenesis just as they do in other species.

REFERENCES

1. Ghiringhelli F, Menard C, Puig PE, *et al.* Metronomic cyclophosphamide regimen selectively depletes CD4(+)CD25 (+) regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56(5):641-8.

2. Lake RA, Robinson BW. Immunotherapy and chemotherapy--a practical partnership. Nat Rev Cancer 2005;5(5):397-405.

3. Schiavoni G, Mattei F, Di Pucchio T, *et al.* Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. Blood 2000;95(6):2024-30.

4. Mihich E, Ehrke MJ. Immunomodulating effects of anticancer drugs: the example of adriamycin. Transplant Proc 1984;16(2):499-501.

5. Ujhazy P, Zaleskis G, Mihich E, Ehrke MJ, Berleth ES. Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells. Cancer Immunol Immunother 2003;52(7):463-72.

6. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 2005;65(18):8059-64.

7. Emens LA, Machiels JP, Reilly RT, Jaffee EM. Chemotherapy: friend or foe to cancer vaccines? Curr Opin Mol Ther 2001;3(1):77-84.

8. Eralp Y, Wang X, Wang JP, Maughan MF, Polo JM, Lachman LB. Doxorubicin and paclitaxel enhance the antitumor efficacy of vaccines directed against HER 2/neu in a murine mammary carcinoma model. Breast Cancer Res 2004;6(4):R275-83.

9. Livingston PO, Wong GY, Adluri S, *et al.* Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. J Clin Oncol 1994;12(5):1036-44.

10. Machiels JP, Reilly RT, Emens LA, *et al.* Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001;61(9):3689-97.

11. Nigam A, Yacavone RF, Zahurak ML, *et al.* Immunomodulatory properties of antineoplastic drugs administered in conjunction with GM-CSF-secreting cancer cell vaccines. Int J Oncol 1998;12(1):161-70.

12. Tong Y, Song W, Crystal RG. Combined intratumoral injection of bone marrow-derived dendritic cells and systemic chemotherapy to treat pre-existing murine tumors. Cancer Res 2001;61(20):7530-5.

13. Yu B, Kusmartsev S, Cheng F, *et al.* Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. Clin Cancer Res 2003;9(1):285-94.

14. Curiel TJ, Coukos G, Zou L, *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942-9.

15. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 2005;65(6):2457-64.

16. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN, Whiteside TL. Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 2005;92(5):913-20.

17. Shevach EM. Regulatory T cells in autoimmmunity*. Annu Rev Immunol 2000;18:423-49.

18. Viguier M, Lemaitre F, Verola O, *et al.* Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004;173(2):1444-53.

19. Almand B, Resser JR, Lindman B, *et al.* Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 2000;6(5):1755-66.

20. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4(12):941-52.

21. Gauthier MJ, Aubert I, Abrams-Ogg A, Woods JP, Bienzle D. The immunophenotype of peripheral blood lymphocytes in clinically healthy dogs and dogs with lymphoma in remission. J Vet Intern Med 2005;19(2):193-9.

22. Henry CJ, McCaw DL, Brock KV, *et al.* Association between cancer chemotherapy and canine distemper virus, canine parvovirus, and rabies virus antibody titers in tumor-bearing dogs. J Am Vet Med Assoc 2001;219(9):1238-41.

23. Winnicka A, Jagielski D, Hoffmann-Jagielska M, Lechowski R. Cytometric evaluation of peripheral blood lymphocytes in dogs with lymphoma during chemotherapy. J Vet Med A Physiol Pathol Clin Med 2002;49(6):303-6.

24. Ghiringhelli F, Larmonier N, Schmitt E, *et al.* CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34(2):336-44.

25. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;105(7):2862-8.

26. Beyer M, Kochanek M, Darabi K, *et al.* Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood 2005;106(6):2018-25.

27. Vahlenkamp TW, Tompkins MB, Tompkins WA. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. J Immunol 2004;172(8):4752-61.

28. Vahlenkamp TW, Tompkins MB, Tompkins WA. The role of CD4+CD25+ regulatory T cells in viral infections. Vet Immunol Immunopathol 2005;108(1-2):219-25.

29. Fan TM. Lymphoma updates. Vet Clin North Am Small Anim Pract 2003;33(3):455-71.

30. Rosenthal RC. Multimodality therapy: using the best available treatments together rationally. Vet Clin North Am Small Anim Pract 1996;26(1):1-8.

31. Mueller RS, Veir J, Fieseler KV, Dow SW. Use of immunostimulatory liposome-nucleic acid complexes in allergen-specific immunotherapy of dogs with refractory atopic dermatitis - a pilot study. Vet Dermatol 2005;16(1):61-8.

32. Faldyna M, Leva L, Knotigova P, Toman M. Lymphocyte subsets in peripheral blood of dogs--a flow cytometric study. Vet Immunol Immunopathol 2001;82(1-2):23-37.

33. Helfand SC, Modiano JF, Moore PF, *et al.* Functional interleukin-2 receptors are expressed on natural killer-like leukemic cells from a dog with cutaneous lymphoma. Blood 1995;86(2):636-45.

34. De Angulo G, Hernandez M, Morales-Arias J, *et al.* Early lymphocyte recovery as a prognostic indicator for high-risk Ewing sarcoma. J Pediatr Hematol Oncol 2007;29(1):48-52.

35. Kono K, Kawaida H, Takahashi A, *et al.* CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. Cancer Immunol Immunother 2006;55(9):1064-71.

36. Miller AM, Lundberg K, Ozenci V, *et al.* CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 2006;177(10):7398-405.

Chapter Three

Use of FoxP3 Expression to Identify Regulatory T Cells in Healthy Dogs and Dogs with Cancer

ABSTRACT

Regulatory T cells (Treg) are a distinct group of T lymphocytes with immunosuppressive properties that serve normally to prevent harmful autoimmune responses. However, Tregs can also interfere with beneficial immune responses such as anti-tumor and anti-viral immunity in humans and rodents. Given the overall importance of Tregs, it is likely that they play an important role in diseases of dogs as well. However, at present reagents required for identification of Tregs in dogs are not available. Therefore, we investigated whether expression of FoxP3, a transcription factor that is highly expressed in Tregs in humans and rodents could also be used to identify Tregs in dogs. We found that a cross-reactive FoxP3 antibody identified a subset of $CD4^+$ T cells in blood and lymph nodes of dogs. By flow cytometry, the mean percentage of $FoxP3^+CD4^+$ T cells in normal dogs was 4.3% in blood and 9.8% in the lymph nodes. In dogs with cancer, there was a significant increase in numbers of Treg in blood (7.1%) and tumor-draining lymph nodes (17.5%) compared to age-matched healthy control dogs. We also found that $FoxP3^+CD4^+$ T cells in dogs could be significantly expanded *in vitro* by TCR activation together with addition of TGF-β and IL-2. Treated

cells also significantly increased expression of TGF-β and IL-10 mRNA. We conclude from these studies that a cross-reactive FoxP3 antibody can be used to identify Tregs in dogs and that this reagent may serve as a useful tool for investigating the role of Treg in a variety of diseases of dogs.

INTRODUCTION

Regulatory T cells are a distinct subset of T lymphocytes that comprise approximately 5% to 10% of all $CD4^+$ T cells in rodents, cats and humans (1-3). Several types of Treg have recently been described, including naturally-occurring, thymic-derived Treg and those induced by exposure to antigen and activated in the periphery (4, 5). When stimulated through their TCR, endogenous $CD4^+$ Treg function to directly suppress the proliferation of harmful self-reactive T cells in a cell-contact dependent, cytokine-independent manner (6, 7). In contrast, antigen-induced Treg typically regulate immune homeostasis via production of cytokines such as IL-10 and TGF-β (8, 9). Despite functional and phenotypic differences between Treg subsets, the Treg population as a whole plays a critical role in the prevention of autoimmune disease and maintenance of peripheral tolerance.

In mice and humans Treg can be tentatively identified based on surface expression of high levels of the IL-2-receptor-α chain (CD25). Other cell surface molecules such as glucocorticoid-induced TNF-receptor (GITR), CD103, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have also been shown to identify a population of Treg (10, 11). In humans, $CD4^+$ T cells expressing low levels of CD127 have also been found recently to be highly enriched for functional Treg (12). However, it

is often difficult to distinguish true Treg from activated $CD4^+$ non-regulatory T cells using CD25 and other cell surface molecule expression, inasmuch as expression of these markers is also upregulated upon activation (13).

Recently, however, intracellular detection of the transcription factor FoxP3 has been shown to uniquely identify a highly enriched Treg population in rodents (14-16). Although FoxP3 expression is also highly enriched within the human $CD4^+CD25^{high}$ population and is currently the most specific Treg marker known, several investigators have demonstrated induction of FoxP3 expression in activated $CD4^+$ T cells that do not possess suppressive activity (17-19). Despite these potential species-specific differences in the regulation of FoxP3 expression, identification of $CD4^+FoxP3^+$ T cells has greatly facilitated the study of Treg. Treg have also been identified in cats using an antibody specific for feline CD25 (3).

Inhibition of the development of effective anti-tumor immunity by Treg is well-documented in humans and mice with cancer (4, 13). A number of clinical studies have demonstrated increased numbers of Treg in humans with a diverse range of malignancies such as melanoma, ovarian carcinoma, hepatocellular carcinoma and squamous cell carcinoma of the head and neck (20-23). Increased numbers of Treg, especially within tumor tissues and in tumor-draining lymph nodes, is a strong negative prognostic factor for some types of cancer. In some cases, the number of Treg predict survival and response to treatment better than traditional prognostic factors such as stage and grade of tumor (20, 24-26).

At present, there are no available antibodies to surface determinants such as CD25 in dogs that can be used to identify Tregs. Therefore, we hypothesized that a cross-

reactive anti-murine FoxP3 antibody could be used to identify Treg in dogs. We found that the FoxP3 antibody specifically recognized intracellular FoxP3 in canine $CD4^+$ T cells. In functional studies, we also found that FoxP3 expression was markedly upregulated in canine $CD4^+$ T cells by stimuli known to elicit expansion of human Treg in vitro. Our results suggest that FoxP3 expression occurs predominantly within the Treg population of $CD4^+$ T cells in dogs. Therefore, FoxP3 expression may serve as a useful marker for identification and quantitation of Tregs in dogs. Our preliminary data also suggest that Treg may play a role in dogs with cancer.

MATERIALS AND METHODS

Study population and sample collection

For analysis of Treg in normal dogs, blood and lymph node aspirates were collected from 10 healthy control dogs. For analysis of Treg in dogs with cancer, blood and lymph node aspirates were collected from patients (n = 10) at the Colorado State University Veterinary Teaching Hospital and from Veterinary Cancer Specialists in Englewood, CO. These studies were approved by Animal Care and Use Committees in place at both participating institutions.

Approximately 4 mls of anticoagulated whole blood was obtained via jugular venipuncture and PBMC were separated by Ficoll density centrifugation. Lymph node aspirates were obtained by fine needle aspiration and placed in complete tissue culture medium at 4°C prior to analysis by flow cytometry. For control dogs, lymph node samples were obtained from the submandibular lymph nodes. In dogs with cancer, where possible, lymph node aspirates were obtained from the nearest lymph node draining the

tumor. In addition, a second lymph node aspirate was obtained from a non-tumor draining lymph node, usually the submandibular lymph node.

Flow cytometric analysis

PBMC or cells obtained by lymph node aspiration were added at 5 x 10^5 to 1 x 10^6 per well in 96-well round bottom plates (Costar, Corning Corporation, Acton, MA.) and then immunostained for surface expression of CD4 and CD8, using appropriate concentrations of FITC-conjugated anti-dog CD4 (clone YKIX302.9; Serotec, Raleigh, NC) and PE-conjugated anti-dog CD8 (clone YCATE55.9, Serotec), as described previously (27). The staining was done at 4°C for 30 minutes, with antibodies suspended in FACS buffer (PBS with 2% FBS and 0.1% sodium azide).

After washing to remove unbound antibodies, intracellular detection of FoxP3 was performed using a cross-reactive, directly conjugated murine FoxP3 antibody (PE conjugated, anti-mouse/rat FoxP3, clone FJK-16s, eBioscience, San Diego, CA) and the permeabilization and fix/perm buffers that accompany the antibody (FoxP3 Staining Set, eBioscience). A directly conjugated rat IgG2a antibody (PE) was used as the isotype control (eBioscience). A series of experiments (data not shown) were done to determine optimal conditions for intracellular FoxP3 staining in which a range of FoxP3 antibody concentrations, as well as various incubation times and temperatures, were evaluated. Specifically, we found that optimal intracellular staining was achieved when the cells were resuspended in fix/perm buffer overnight at 4^0C following surface staining. The next day, cells were washed twice with permeabilization buffer and incubated with anti-mouse/rat FoxP3 antibody (1 *u*g per 1 x 10^6 cells) diluted in FACS buffer for 30 minutes

at 4^0C. Following 2 additional washes in permeabilization buffer, the cells were resuspended in FACS buffer and kept at 4^0C prior to analysis.

Flow cytometric analysis was done using a CyAn MLE flow cytometer (Dako-Cytomation, Ft. Collins, CO) and analysis was done using Summit software (Dako-Cytomation). Analysis gates were set on the live lymphocyte population based on typical forward and side scatter characteristics (28). For lymphocytes obtained from PBMC, the percentage of Treg was calculated based on the percentage of $FoxP3^+CD4^+$cells within the overall $CD4^+$ T cell population. Absolute Treg numbers in peripheral blood were calculated based on the total lymphocyte count determined on the same day by automated cell counter (Advia, Bayer Diagnostics, Tarrytown, NY).

Quantitation of canine Foxp3 by real-time PCR

Primers for FoxP3 detection were designed based on the canine FoxP3 sequence, using Primer3 software (Steve Rozen, Helen J. Skaletsky (1998) Primer 3.) (Code available at http://www-genome.wi.mit.edu/genome software/other/primer3.html). The forward primer sequence was 5'-AAACAGCACATTCCCAGAGTTC-3' (sense) and the reverse primer sequence was 5'-AGGATGGCCCAGCGGATCAG-3' (antisense). The sequences of the primer pair for the house keeping gene hypoxanthine-phosphoribosyltransferase (HPRT) were 5'-TGCTGCATTCCTGAAGTCTTTAT-3'(sense) and 5'-TCACAAGTCAAACAACAATCCAC-3'(antisense). Primers were purchased from Invitrogen (Carlsbad, CA) and were resuspended in distilled water and stored at -20^0C. For quantitative real-time PCR (RT-PCR) analysis, RNA was extracted from PBMC or lymph node aspirates (approximately 1-3 x 10^6 total cells) using an

RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First strand cDNA was generated using a kit containing M-MLV Reverse Transcriptase and First-Strand buffers (Invitrogen). For each PCR reaction, 3 *u*l of cDNA template was added to iQ SYBR Supermix (Bio-Rad, Hercules, CA) containing the primer pair for either FoxP3 or HPRT. Amplification was carried out in 40 *u*l volumes using the iQ iCycler system (Bio-Rad). Negative control reactions were performed using either mock RNA or water. All amplification reactions were performed in duplicate, and the average threshold cycle (C_t) numbers of the duplicates were used to calculate the relative mRNA expression of FoxP3 normalized to HPRT expression in each sample. Normalized FoxP3 expression was then compared between control and treated samples from multiple in vitro experiments.

Quantitation of canine TGF-β and IL-10 by quantitative real-time PCR.

For quantitation of canine IL-10 and TGF-β mRNA, primer sequences were obtained from published sources (29). For IL-10, the primer sequences were 5'-GTCCCTGCTGGAGGACTTTAAGA-3' (sense) and 5'-TGGTCGGCTCTCCTACATCTCG-3' (antisense). For TGF-β, the primer sequences were 5'-AGTTAAAAGCGGAGCAGCATGTGG-3' (sense) and 5'-GATCCTTGCGGAAGTCAATGTAGAGC-3' (antisense). RNA extraction, cDNA preparation and RT-PCR were done as described above.

In Vitro Expansion and Induction of Treg

Canine lymphocytes were activated and cultured in IL-2 and TGF-β, using protocols previously described for in vitro expansion of human and murine Treg (5, 30, 31). For these experiments, PBMC were obtained from blood collected from 3 healthy dogs. For each dog, PBMC were cultured at 2 x 10^6/ml in complete medium in 24-well plates (Costar). Tissue culture medium consisted of Modified Eagle's medium (Gibco-Invitrogen, San Diego, CA) supplemented with nonessential and essential amino acids (Gibco) with penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO) and 10% FBS (Gemini Bioproducts, West Sacramento, CA). Some cultures were placed in complete medium supplemented with recombinant human TGF-β and IL-2 (Peprotech, Rocky Hill, NJ) with or without the addition of ConA (Sigma-Aldrich) as a T cell activation stimulus. These culture conditions have been shown previously in experiments with murine T cells to favor the in vitro induction of Treg (30, 31). The following culture conditions tested were evaluated: Condition 1: cells cultured in complete tissue culture medium only; Condition 2: addition of 2 ng/ml rhTGF-β and 100 U/ml rhIL-2; Condition 3: addition of 5 *u*g/ml ConA only; and Condition 4: addition of 2 ng/ml rhTGF-β, 100 U/ml rhIL-2 and 5 *u*g/ml ConA. The cultures were harvested on day 5 and the samples were split for flow cytometric analysis of FoxP3 expression and quantitative RT-PCR analysis of canine FoxP3, TGF-β and IL-10 mRNA expression.

Statistical analyses

Differences between two groups were compared using the Mann-Whitney U test, which assumes non-Gaussian distribution of data. Comparisons between multiple groups

with small sample sizes were done using one-way analysis of variance for non-parametric data (Kruskal-Wallis test), followed by Dunn's multiple comparisons test. Comparisons between multiple groups with larger sample sizes were done by ANOVA with Tukey's multiple means comparison. Statistical analyses were done using Prism software (GraphPad, San Diego, CA). For all comparisons, a p-value ≤ 0.05 was considered significant.

RESULTS

Identification of canine Treg by intracellular expression of FoxP3

Given the critical role of the FoxP3 transcription factor in the development and function of regulatory T cells and the fact that the FoxP3 sequence is highly conserved between diverse species, we first investigated whether a cross-reactive murine FoxP3 antibody could detect the FoxP3 protein within $CD4^+$ canine lymphocytes (15, 32, 33). To address this question, staining protocols for FoxP3 detection were optimized and blood and lymph node aspirates from normal dogs and dogs with cancer were evaluated for CD4 and FoxP3 expression. In **Figure 1**, representative flow cytometry results for FoxP3 expression in $CD4^+$ lymphocytes from a dog with cancer are shown.

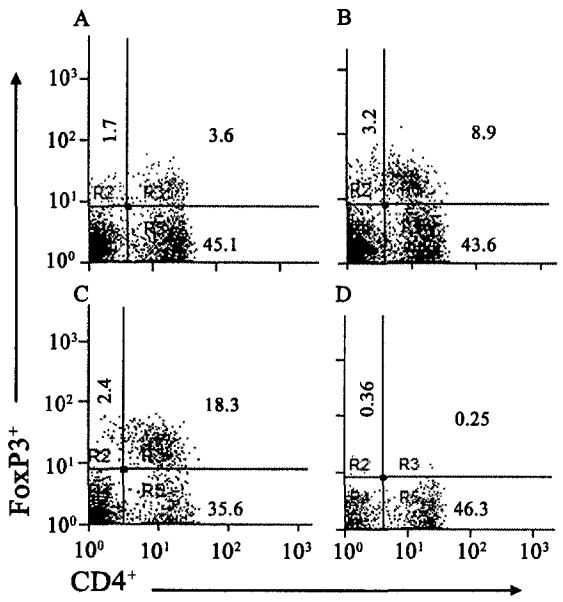

Figure 1. Identification of canine Tregs using FoxP3 antibody. Lymphocytes were obtained from blood and lymph nodes of a dog with oral melanoma and immunostained for expression of FoxP3 and CD4. Representative dot plots of FoxP3 expression by $CD4^+$ T cells from (**A**) blood; (**B**) an uninvolved lymph node; (**C**) tumor-draining lymph node are shown. In (**D**), staining of blood from the same dog with an irrelevant isotype-matched antibody (control for FoxP3 staining) is shown. The percentages of lymphocytes contained within each quadrant are given.

The specificity of FoxP3 staining was assessed by staining with an irrelevant isotype-matched antibody and also by assessing FoxP3 expression by non-$CD4^+$ T cells. Although staining for FoxP3 was consistently highest in $CD4^+$ T cells, there was some detectable FoxP3 expression in non-$CD4^+$ lymphocytes (see Figure 1). Further analysis revealed that the majority of the $CD4^-FoxP3^+$ population were $CD8^+$ (data not shown). This finding does not necessarily indicate non-specific FoxP3 staining, as $CD8^+FoxP3^+$ Treg have also been described in humans and in mice (33-35). Thus, these experiments indicated that the anti-murine FoxP3 antibody was cross-reactive with a protein expressed primarily in a subset of $CD4^+$ T cells of dogs.

In vitro expansion of dog $CD4^+FoxP3^+$ Treg using T cell activation plus TGF-β and IL-2

The preceding experiments indicated that FoxP3 expression could be detected specifically in dog $CD4^+$ T cells, but did not address the issue of whether these cells behaved functionally as Treg. For example, Treg in mice, cats, and humans can be enriched by cell sorting and added to cultures of activated T cells, where they exert a

strong inhibitory effect on proliferation and cytokine production. However, in the case of dogs, the absence of any available antibodies for detection of surface expression of CD25, GITR, CTLA-4, CD127, or CD103 make it nearly impossible sort cells and do these types of add-back experiments.

Therefore, we took a more indirect approach to demonstrating that FoxP3 expressing $CD4^+$ T cells in dogs behaved functionally as Treg. In mice and humans, Treg can be expanded *in vitro* from activated PBMC following addition of exogenous TGF-β and IL-2 (30, 31, 36). In mice, FoxP3 mRNA expression in activated T cells increases within 12 hours after cytokine addition and is maximal after about 96 hours of culture (31). Therefore, we set up in vitro cultures of dog PBMC under conditions that have been shown previously to induce expansion of Treg in mice and humans. For these cultures, we evaluated FoxP3 expression by flow cytometry and also FoxP3, TGF-β, and IL-10 mRNA expression by quantitative RT-PCR.

We found that culture of activated T cells in TGF-β and IL-2 led to a 2 to 3-fold increase in the percentage of $CD4^+FoxP3^+$ T cells as detected by flow cytometry (**Figure 2**). While there was an increase in FoxP3 expression in T cells that were activated only (i.e., exposure to ConA, but not TGF-β or IL-2), the increase in $FoxP3^+$ cells in cultures incubated with ConA plus TGF-β and IL-2 was significantly greater ($p = 0.01$, Figure 2C).

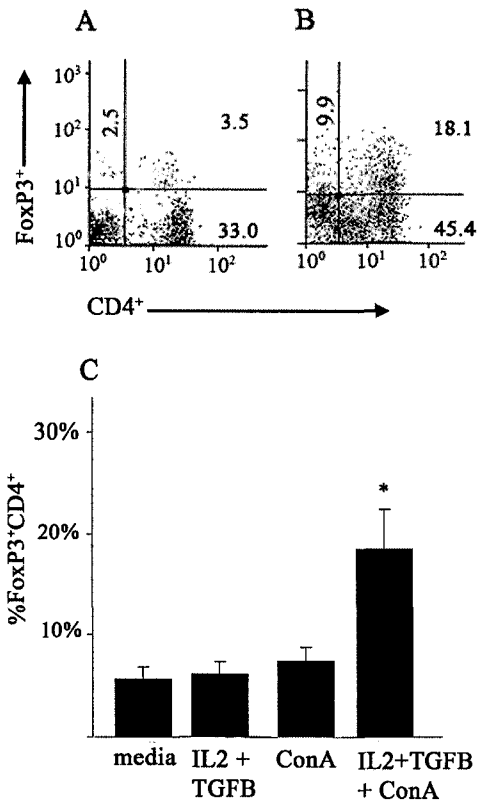

Figure 2. In vitro expansion of FoxP3$^+$CD4$^+$ T cells by T cell activation and culture in IL-2 and TGF-β. PBMC from normal dogs were cultured in medium alone, with IL-2 and TGF-β alone, with ConA alone, or with ConA plus IL-2 and TGF-β. Representative dot plots for flow cytometric analysis of cells cultured in medium alone (**A**) or medium plus ConA and IL-2 and TGF-β (**B**) are shown. In (**C**), the mean percentage of FoxP3$^+$CD4$^+$ lymphocytes present in cultures maintained under the indicated conditions was calculated and plotted. Culture in ConA plus IL-2 and TGF-β resulted in a significant increase ($p = 0.01$) in the percentage of FoxP3$^+$CD4$^+$ T cells, compared to other culture conditions, as determined by ANOVA. Bars represent standard error (SE).

We also observed a 45 to 250-fold increase in mRNA levels for FoxP3 in cultures containing ConA, IL-2 and TGF-β (**Table 1**). The increased expression of FoxP3 mRNA in these cultures was also accompanied by significant increases in mRNA expression for TGF-β (2 to 4-fold increase) and IL-10 (7 to 14-fold increase). Although increased expression of FoxP3 mRNA was detected in cultures containing ConA alone, the increase was significantly less than for cultures containing ConA plus IL-2 and TGF-β. Also, as expected from the results of rodent and human studies, cytokine addition alone without TCR activation did not result in significant increases in FoxP3 (protein or mRNA) or IL-10 or TGF–β mRNA expression (Table 1). These data indicated, therefore, that culture of canine T cells under conditions known to promote the *in vitro* generation of Treg in human T cells stimulates a significant increase in the number of canine FoxP3$^+$CD4$^+$ T

cells. This treatment was also associated with significant up-regulation of expression of TGF-β and IL-10 mRNA. These results provide support for our assertion that FoxP3 expression is associated with the Treg phenotype in dogs as well as in humans and rodents.

Table 1

Relative increase in mRNA during in vitro expansion of Treg

Culture Condition	FoxP3[a]	TGF-β[a]	IL-10[a]
Medium only	1	1	1
IL-2 + TGF-β	9.5 (+/- 6.1)	3.1 (+/- 1.2)	1.6 (+/- .75)
ConA only	11.7 (+/- 8.3)	2.6 (+/- 2.1)	1.9 (+/- 0.9)
ConA + IL-2 + TGF-β	147 (+/- 102)*	7.2 (+/- 2.0)*	14.0 (+/- 7.0)*

* denotes significant difference in mean fold increase in expression, compared to the other treatment groups. **a**) Mean (±SE) increase in mRNA expression as compared to medium alone control.

Dogs with cancer have increased Treg in their peripheral blood and tumor-draining lymph nodes

To assess the biological usefulness of Treg identification using the FoxP3 antibody, we evaluated Treg numbers in dogs with cancer. We chose this population of dogs to study because previous studies in mice and humans have shown that Treg numbers are often elevated in humans with cancer (4, 13). For this analysis, 10 dogs with several different common cancers of dogs were investigated and their results compared to those obtained from 10 age-matched, healthy control dogs. The percentages and numbers of $FoxP3^{+}CD4^{+}$ Tregs in lymph nodes and peripheral blood were determined using flow cytometry.

We found that the percentage of Treg and the absolute number of Treg were significantly higher in the peripheral blood of dogs with cancer than healthy dogs (**Figure 3**). For example, the mean percentage of Treg in peripheral blood in all 10 dogs with cancer was 7.5%, compared to 4.3% in the 10 normal dogs ($p < 0.01$).

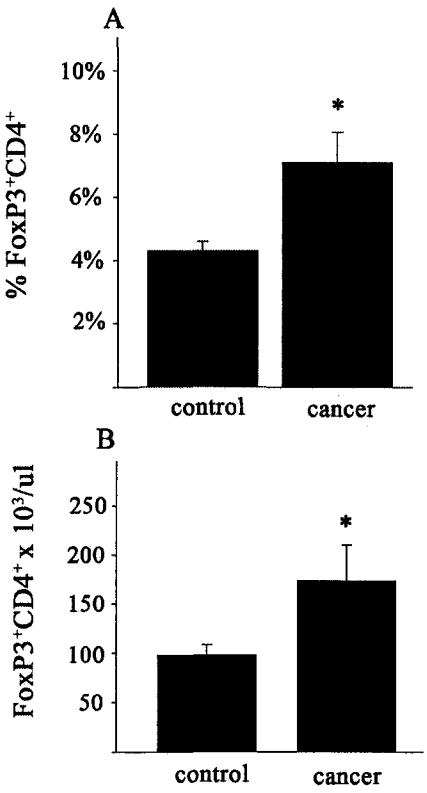

Figure 3. Relative and absolute numbers of Treg in peripheral blood in dogs with cancer and healthy control dogs. The mean percentage (± SE) of Treg in blood of normal dogs and dogs with cancer was plotted in (**A**) and the mean of the absolute numbers (± SE) of Treg was plotted in (**B**). The percentage of Treg in blood was significantly increased ($p = 0.01$) in dogs with cancer compared to healthy control dogs (4.3% vs 7.1%; $p = 0.0005$). The absolute numbers of Treg in blood were also significantly higher (98 X 10^3 vs 173 X 10^3/ul; $p = 0.04$) in dogs with cancer, compared to healthy control dogs.

The absolute numbers of Treg in peripheral blood of dogs with cancer were also significantly increased (mean of 173 cells per ul blood in dogs with cancer versus 98 cells per ul blood in control dogs, $p < 0.04$). These results are consistent with previous observations in humans and mice and provide further evidence for the specificity of using FoxP3 expression to identify canine Treg.

Previous studies had found higher numbers of Treg in lymphoid tissues of humans and cats than in blood (3, 37). Therefore, Treg populations in lymph node aspirates of

dogs were compared to blood Treg populations. We found that in both normal dogs and dogs with cancer the percentages of Treg were significantly higher in lymph nodes than in blood ($p < .001$) (Figure 3 and **Table 2**).

Table 2

Treg Levels in Healthy Dogs and Dogs with Cancer

Tumor Type	**Number of Dogs**	**% Treg in peripheral blood[a]**	**% Treg in Non-draining LN[a]**	**% Treg in draining LN[a]**
Healthy Dogs	10	4.3 (+/- 0.7)	9.8 (+/- 2.4)	ND
Oral Melanoma	4	11.1 (+/- 2.1)	12.0 (+/- 0.5)	19.5 (+/- 8.0)
Osteosarcoma	3	7.2 (+/- 1.9)	10.8 (+/- 0.5)	ND
Mast Cell Tumor	2	6.3	10.0	15.5
Soft Tissue Sarcoma	1	5.5	11.6	16.4

[a] Mean (+ SE) percentage of $FoxP3^{+}CD4^{+}$ T-cells; ND = not done

Next, we assessed whether lymph nodes draining tumors had more Tregs than lymph nodes not draining the primary tumor (non-tumor draining lymph nodes) in dogs with cancer. The percentage of Tregs in the tumor draining lymph nodes of 7 dogs with cancer (excluding 3 dogs with osteosarcoma where a tumor draining lymph node was no longer present due to limb amputation) was significantly higher ($p = 0.04$) than the percentage of Treg in the non-tumor draining lymph nodes of the same dogs (**Figure 4**). However, we found that the percentage of Tregs in the non-tumor draining lymph nodes of dogs with cancer (11.1%) was not significantly different from the percentage of Tregs in lymph nodes of healthy control dogs (9.8%; see Figure 4 and Table 2). Thus, it appears that Treg preferentially accumulate within the tumor draining lymph nodes of

dogs with cancer, as has been reported previously for humans with metastatic melanoma (23). These results also suggest the possibility that the major source of increased Tregs in the blood of dogs with cancer may be the Tregs that accumulate in the tumor draining lymph nodes.

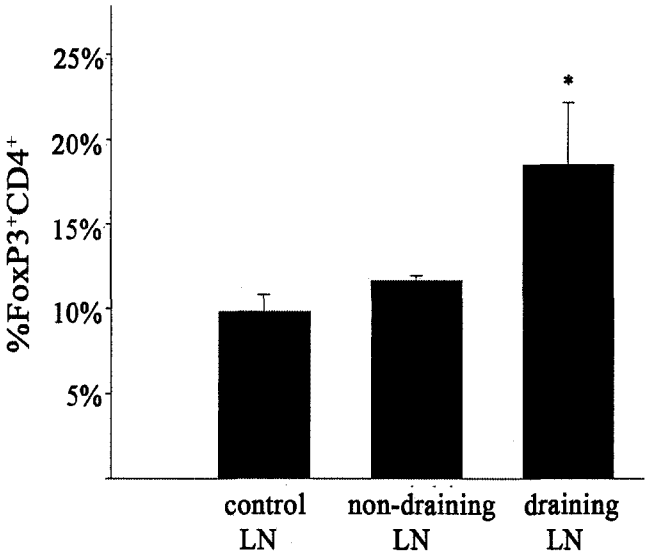

Figure 4. FoxP3$^+$CD4$^+$ T cells in lymph nodes of dogs with cancer and healthy dogs. The mean percentage of FoxP3$^+$CD4$^+$ T cells (±SE) was significantly higher ($p = 0.03$) in tumor-draining lymph nodes of dogs with cancer than in the non-draining lymph nodes from the same dogs. In addition, the mean percentage of FoxP3$^+$CD4$^+$ T cells was significantly higher in tumor-draining lymph nodes of dogs with cancer than in the lymph nodes of healthy control dogs, *($p = 0.01$).

Finally, we also conducted a preliminary assessment of whether particular tumor types might be associated with higher numbers of Tregs. For this analysis, we compared dogs with melanoma (n = 4) and dogs with osteosarcoma (n = 3) to healthy control dogs (n = 10). We found that dogs with melanoma had significantly ($p < 0.01$) more Tregs in their blood (11.2% versus 4.3%) than control dogs, as assessed by Kruskal-Wallis test and Dunn's multiple means comparison (**Figure 5**). When Tregs in aspirates from the same lymph node (submandibular lymph nodes) of all 3 groups of dogs were compared,

we also found that dogs with melanoma had significantly ($p < 0.05$) more Tregs (22.6%) than both control dogs (9.8%) and dogs with osteosarcoma (10.8%). However, it should be noted that the submandibular lymph node was the tumor draining lymph node for dogs with oral melanoma. Therefore, we also compared the percentages of Tregs in non-tumor draining lymph nodes for all 3 groups of dogs (Figure 4). In this case, we did not observe any significant differences ($p = 0.20$) in Tregs in lymph nodes between the 3 groups of dogs.

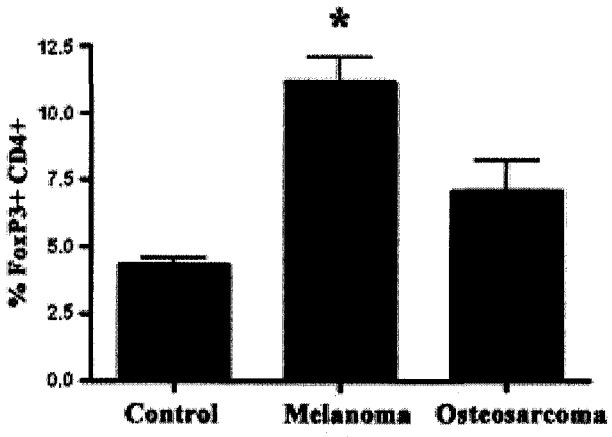

Figure 5. Comparison of Treg percentages in blood of dogs with melanoma or osteosarcoma and in healthy control dogs. Dogs with melanoma had significantly more Tregs than control dogs ($p < 0.01$), as assessed by the Kruskal-Wallis test. (* denotes significant differences relative to control dogs, bars represent SE).

DISCUSSION

The major findings to emerge from these studies were that a cross reactive FoxP3 antibody can also be used to detect dog FoxP3 and to identify canine Treg *in vivo*. We found that Treg numbers were significantly higher in peripheral blood of dogs with cancer and that Treg may preferentially accumulate within the tumor-draining lymph nodes of dogs with cancer. In addition, it appears that certain types of tumors in dogs, especially highly malignant tumors, may be associated with higher numbers of Tregs.

In mice, FoxP3 is specifically expressed by most Treg and can be used to distinguish $CD4^+CD25^+$ Treg cells from $CD4^+CD25^+$ non-regulatory T cells. These non-regulatory T cells upregulate CD25 expression, but not FoxP3 expression, upon activation (14, 34). Conflicting reports exist, however, concerning the specificity of human FoxP3 expression for identification of Tregs. For example Yagi et al., demonstrated that TCR stimulation of naïve human $CD4^+CD25^-$ T cells failed to elicit FoxP3 expression and that FoxP3 expression was limited to T cells with a regulatory phenotype (32). Other investigators however have shown that FoxP3 expression is increased in activated non-regulatory T cells (18, 19). Because of these discrepancies, evaluation of human Treg currently relies on analysis of expression of multiple Treg-associated cell surface markers, including CD25, GITR, and CTLA-4, in addition to assessment of intracellular FoxP3 expression. A recent study also suggests that evaluation of CD127 expression in conjunction with CD4 and CD25 expression may be even more specific than previous markers (12). The lack of antibody reagents for assessing expression of these molecules in dogs currently precludes Treg analysis using

multiple surface markers. Therefore, at present assessment of FoxP3 expression may be the best available method of enumerating Treg in dogs.

The definition of Treg also depends in part on demonstration of typical immunosuppressive functions. For example, studies of feline $CD4^+CD25^+$ Treg have shown that in vitro activation of purified Treg with LPS and rhIL-2 induced strong suppressive activity against ConA-stimulated $CD4^+CD25^-$ T cells (3). The lack of available antibodies to surface determinants such as CD25 on dog T cells, however, prevented us from performing these types of experiments. Therefore, as an alternative we investigated whether Treg expansion could be elicited in vitro by stimuli known to expand the numbers of Treg in human T cells. For example, in humans and mice naturally-occurring $CD4^+CD25^+FoxP3^+$ Treg can be expanded in vitro from PBMC when cultured in the presence of IL-2 and TCR stimulation (5, 38). Signaling through the high affinity IL-2 receptor CD25 has been recently shown to be critical to Treg growth and differentiation (39, 40). Addition of TGF-β to activated T cells may also be used to further expand Treg through induction of FoxP3 expression (30, 31). In our studies with dog lymphocytes, we observed an increase in FoxP3 mRNA and protein expression in activated lymphocytes cultured in IL-2 and TGF-β. Similar to observations described by Morgan and others, we found that TCR activation alone induced small increases in FoxP3 expression by $CD4^+$ as well as by some CD4- T cells (17-19). However, the expression was significantly greater when the activated T cells were also cultured in IL-2 and TGF-β (Figure 2). Thus, in contrast to murine Treg, our findings suggest that the regulation of FoxP3 expression in dog Treg is more similar to the regulation of FoxP3 expression in human Treg. Our assertion that FoxP3 expression occurs predominantly within the

canine Treg population is further strengthened by finding concurrent significant increases in IL-10 and TGF-β mRNA expression in appropriately activated cells, a result not expected if activation were to lead to the development of cells without a Treg phenotype.

The availability of a diagnostic reagent to reliably identify Treg in dogs should greatly facilitate future investigations into the role of Treg in important diseases of dogs, including autoimmune diseases and chronic infections and cancer. The observation that the dogs with cancer in this study had increased numbers of Treg in their peripheral blood and tumor-draining lymph nodes compared to healthy dogs is remarkably similar to findings for human cancer patients. Our preliminary observations suggesting that more malignant tumors may be associated with higher numbers of Tregs also indicates that evaluation of Treg numbers may have important prognostic value. Moreover, the results predict that the most informative sites to sample for evaluation of Treg numbers in dogs with cancer are the blood and the tumor draining lymph nodes.

We anticipate that further investigations into the role of Treg in various malignancies will offer important insights into immunological control of cancer. These studies may well lead to newer tools for assessing the immunopathogenesis of cancer in dogs and manipulating the immune system to control cancer.

Acknowledgements These studies were supported in part by National Institute of Health Grants CA86224-01 and T32RR00707. We also thank Drs. Amanda Guth and Susan Plaza and Ms. Julie Willer for technical support.

REFERENCES

1. Itoh M, Takahashi T, Sakaguchi N, *et al.* Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol 1999;162(9):5317-26.

2. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2(6):389-400.

3. Vahlenkamp TW, Tompkins MB, Tompkins WA. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. J Immunol 2004;172(8):4752-61.

4. Beyer M, Schultze JL. Regulatory T cells in cancer. Blood 2006;108(3):804-11.

5. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med 2001;193(11):1295-302.

6. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 2000;101(5):455-8.

7. Shevach EM. Regulatory T cells in autoimmmunity. Annu Rev Immunol 2000;18:423-49.

8. Hara M, Kingsley CI, Niimi M, *et al.* IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. J Immunol 2001;166(6):3789-96.

9. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med 2001;194(5):629-44.

10. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med 2001;193(11):1303-10.

11. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001;193(11):1285-94.

12. Liu W, Putnam AL, Xu-Yu Z, *et al.* CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203(7):1701-11.

13. Baecher-Allan C, Anderson DE. Regulatory cells and human cancer. Semin Cancer Biol 2006;16(2):98-105.

14. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4(4):330-6.

15. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol 2005;6(4):331-7.

16. Ramsdell F. Foxp3 and natural regulatory T cells: key to a cell lineage? Immunity 2003;19(2):165-8.

17. Allan SE, Passerini L, Bacchetta R, *et al.* The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. J Clin Invest 2005;115(11):3276-84.

18. Morgan ME, van Bilsen JH, Bakker AM, *et al.* Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. Hum Immunol 2005;66(1):13-20.

19. Walker MR, Kasprowicz DJ, Gersuk VH, *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003;112(9):1437-43.

20. Curiel TJ, Coukos G, Zou L, *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;10(9):942-9.

21. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF, Korangy F. Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 2005;65(6):2457-64.

22. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN, Whiteside TL. Characteristics of CD4+CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 2005;92(5):913-20.

23. Viguier M, Lemaitre F, Verola O, *et al.* Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004;173(2):1444-53.

24. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003;9(12):4404-8.

25. Alvaro T, Lejeune M, Salvado MT, *et al.* Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res 2005;11(4):1467-73.

26. Wolf D, Wolf AM, Rumpold H, *et al.* The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. Clin Cancer Res 2005;11(23):8326-31.

27. Walter CU, Biller BJ, Lana SE, Bachand AM, Dow SW. Effects of chemotherapy on immune responses in dogs with cancer. J Vet Intern Med 2006;20(2):342-7.

28. Faldyna M, Leva L, Knotigova P, Toman M. Lymphocyte subsets in peripheral blood of dogs--a flow cytometric study. Vet Immunol Immunopathol 2001;82(1-2):23-37.

29. Nuttall TJ, Knight PA, McAleese SM, Lamb JR, Hill PB. T-helper 1, T-helper 2 and immunosuppressive cytokines in canine atopic dermatitis. Vet Immunol Immunopathol 2002;87(3-4):379-84.

30. Chen W, Jin W, Hardegen N, *et al.* Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198(12):1875-86.

31. Fu S, Zhang N, Yopp AC, *et al.* TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. Am J Transplant 2004;4(10):1614-27.

32. Yagi H, Nomura T, Nakamura K, *et al.* Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004;16(11):1643-56.

33. Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol 2006;24:209-26.

34. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299(5609):1057-61.

35. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003;4(4):337-42.

36. Bacchetta R, Sartirana C, Levings MK, Bordignon C, Narula S, Roncarolo MG. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. Eur J Immunol 2002;32(8):2237-45.

37. Liyanage UK, Moore TT, Joo HG, *et al.* Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 2002;169(5):2756-61.

38. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 1998;188(2):287-96.

39. Almeida AR, Legrand N, Papiernik M, Freitas AA. Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. J Immunol 2002;169(9):4850-60.

40. Antony PA, Restifo NP. CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. J Immunother 2005;28(2):120-8.

Chapter Four

The Immunomodulatory Effects of Doxorubicin on Dendritic Cells

ABSTRACT

Immunotherapy-based approaches to the treatment of cancer, such as the administration of vaccines targeted to specific tumor antigens, will likely be most effective when given in combination with conventional therapies such as cytotoxic chemotherapy. Little is known, however, about the effects of chemotherapy on many aspects of immunity. For example, it is not known how chemotherapy affects the function of dendritic cells, which are central mediators of the innate immune system and critical to the development of effective antitumor immunity. Therefore, we investigated the effects of commonly used chemotherapy drugs on key aspects of dendritic cell function. We found that the drug doxorubicin was unique in its ability to activate dendritic cells both in vitro and in vivo. In vitro, exposure of bone-marrow derived dendritic cells to doxorubicin increased expression of CD40 and 4-1BBL, enhanced antigen presentation and stimulated the production of the p40 subunit of IL-12 and IL-23. Treatment of mice with doxorubicin led to significantly increased costimulatory molecule expression and antigen presentation of myeloid dendritic cells within 5 – 7 days following drug administration. In mice with B16 melanoma, treatment with doxorubicin increased the number of activated dendritic cells in tumor-draining lymph nodes and

within tumor tissues themselves. These findings demonstrate that DOX induces dendritic cell activation and expansion in vivo and therefore provide a rationale for the inclusion of doxorubicin in combined chemotherapy and immunotherapy protocols.

INTRODUCTION

Conventional cytotoxic chemotherapy has long been a part of standard cancer therapy, especially in the management of metastatic disease. Although progress has been made in maximizing the benefits of chemotherapy while minimizing its toxicity, many cancer patients experience side effects that significantly diminish their quality of life and most ultimately succumb to drug-resistant metastatic disease. One of the newer approaches to cancer treatment is the use of targeted forms of immunotherapy, such as vaccine strategies that actively elicit the development of antitumor immunity (1-3). Unlike cytotoxic chemotherapy, tumor vaccines can be targeted specifically to neoplastic cells, thus sparing normal tissues and potentially providing protection against relapsing disease. The results of recent clinical trials suggest, however, that the best use of tumor-specific immunotherapy is in the setting of minimal disease and as an adjuvant to more traditional methods of tumor reduction (4-7).

The limitations of chemotherapy and tumor immunotherapy as single treatment modalities as well as mounting evidence for the immunomodulatory potential of many chemotherapeutic agents, have led to investigation of combined treatment approaches (8-12). A number of mechanisms have been proposed for the immunostimulatory effects of certain chemotherapy drugs. For example, administration of doxorubicin (DOX) or gemcitabine to tumor-bearing mice induces immunostimulatory tumor apoptosis that

enhances tumor antigen cross-presentation to cytotoxic T-lymphocytes (CTLs) in vivo, significantly increasing the efficacy of adaptive antitumor immune responses (13-16). Other immunomodulatory effects, such macrophage activation by paclitaxel (PTX) and DOX, and inhibition of regulatory T cell function by cyclophosphamide (CYC) have also been described (17-23).

The majority of studies investigating combined chemotherapy and immunotherapy protocols have focused on the induction of tumor-specific T-cell responses, tumor cell apoptosis, and on clinical endpoints such as tumor growth and survival times. Although dendritic cells (DCs) are central to the development of antitumor immunity and effective immunotherapy hinges on their proper function, little is known about the impact of chemotherapy on DCs themselves. Therefore, we investigated the effects of commonly used chemotherapy drugs on key aspects of dendritic cell function. We hypothesized based on prior studies that DOX and paclitaxel would induce DC activation. We found that DOX was unique in its ability to activate DCs. Stimulation of DCs with DOX induced secretion of the p40 subunit of IL-12 and IL-23, increased cell-surface expression of several different costimulatory molecules, and enhanced antigen presentation.

We also hypothesized that treatment of tumor-bearing mice with DOX could activate DC function, despite the immunosuppressive effects of the tumor itself. Using the B16 melanoma model, we found that treatment with DOX significantly increased numbers of activated DCs in tumor-draining lymph nodes and tumor tissues compared to DCs isolated from untreated tumor-bearing mice. These results suggest that DOX chemotherapy may potentially augment antitumor immunity through stimulation of DC

function and provide an important rationale for further investigation of DOX in combination chemotherapy and immunotherapy protocols.

MATERIALS AND METHODS

Mice and B16 tumor model

Female ICR or C57BL/6 mice were used for experiments involving healthy or tumor-bearing animals, respectively. All mice were purchased from Harlan Sprague Dawley and used at approximately 10 – 18 weeks of age. To establish the B16 tumor model, C57BL/6 mice (4/group) were injected s.c. with 2 x 10^5 B16.F10 cells (provided by I. Fidler, MD Anderson Cancer Center, Houston, TX) and DOX chemotherapy (4 mg/kg i.v.) was administered 7 days later when most mice had palpable tumors. Tumor growth was monitored using calipers every 2 days until 7 days following DOX administration, at which time all mice were sacrificed. Animal protocols for these experiments were approved by the Animal Care and Use Committee at Colorado State University.

Chemotherapeutic agents

The following chemotherapy drugs were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO): doxorubicin, cisplatin, cyclophosphamide, chlorambucil, vinblastine, paclitaxel and dexamethasone. Each drug was diluted and stored as recommended by the manufacturer.

Preparation of lipid-DNA-complexes

A lipid-DNA-complex (LDC) was used as a positive control for stimulation of innate immunity in *in vivo* experiments as previously described (24, 25). To form the LDCs, sterile 10 mM solutions of the cationic liposomes comprising DOTIM (octadecenoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride) and cholesterol were prepared as previously described (26, 27). LDCs were prepared just before injection by gently mixing cationic liposomes with plasmid DNA at a ratio of 16 nmol lipid per 1.0 *u*g DNA in 5% dextrose in water. The final plasmid DNA concentration in the complexes was 100 *u*g DNA per ml.

Generation of bone marrow-derived DCs and determination of sensitivity to chemotherapeutic agents

DCs were cultured from bone marrow of ICR mice using a standard protocol (28). Bone marrow was flushed from femurs with Hank's balanced salt solution (HBSS) supplemented with 2% FBS (InVitrogen Life Technologies) and the cells were washed and RBC lysed with buffered ammonium chloride (NH_4Cl) solution. Viable cells were plated at 1 x 10^6/ml in 24-well plates in RPMI supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 1% non-essential amino acids, 50 IU/ml penicillin, 50 *u*g/ml streptomycin (RPMI/complete medium) and 3% B78-Hi supernatant (GM-CSF). The medium was changed every other day during the 7-day culture period. DCs generated in this way were routinely 60 – 70% $CD11c^+$/$DEC\text{-}205^+$ as assessed by flow cytometry.

A standard MTT assay was used to determine the cytotoxicity of each drug (29). Bone marrow-derived DCs (BMDCs) harvested at day 7 of in vitro culture were plated at

4 x 10^5/well in triplicate in 96-well plates for every concentration of chemotherapy drug evaluated. Each drug was serially diluted across a wide concentration range (100 - 0.05 *u*g/ml) and added to BMDCs. Following a 24-hour incubation period at 37°C, the MTT reagent was added. The average percentage of DC killed by each concentration of chemotherapy drug was calculated relative to medium (0 *u*g/ml drug) control.

Cell preparations and chemotherapy of mice

For in vitro studies, BMDCs were harvested after 7 days in culture and plated at 5 x 10^5/well in 24-well plates containing RPMI/GM-CSF. Based on the results of the cytotoxicity analysis, each chemotherapy drug was added at a single, minimally cytotoxic dose to triplicate wells; LPS (5 *u*g/ml) was used as a positive control for all in vitro studies. Following a 24 hour incubation period, cells were washed in and resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline with 2% FBS and 0.1% sodium azide) before cytometric analysis, or in RPMI/complete medium before use in experiments assessing cytokine production and antigen presentation.

For in vivo studies, groups of 4 mice were treated with a single dose of each chemotherapy drug using a dosage and route of administration based on the results of previous studies (30, 31). At the time of sacrifice, single-cell suspensions of spleen cells were prepared by mechanical disruption and screening through a 70-*u*m nylon mesh screen (BD Biosciences), followed by NH_4Cl lysis. In experiments with tumor-bearing mice, tumor-infiltrating cells and lymph node cells were prepared by lysis of RBC and screening through 70-*u*m screens as described for spleen cells. All cell preparations were resuspended in RPMI/complete medium prior to use in additional experiments.

Antibodies and flow cytometric analysis

Directly conjugated antibodies used for flow cytometric analyses were purchased either from Pharmingen (San Diego, CA) or from eBiosciences (San Diego, CA). The following antibodies in various combinations were used: anti-CD4 (FITC or APC-Cy7; clone RM4-5), anti-CD8a (APC; clone 53.6.7), anti-I-A/I-E-(MHC II), (biotin; clone M5/114.15.2), anti-CD11b (PE-Cy7 or APC-Cy7; clone M1/70), anti-CD11c (PE or APC; clone N418), anti-B220 (APC-Cy7; clone RA3-6B2), anti-NKG2D (biotin; clone CX5), anti-4-1BBL (biotin; clone TKS-1), anti-CD40 (PE; clone 1C-10), and anti-CD86 (FITC; clone GL1) and anti-DEC-205 (biotin; clone NLDC-145). Biotinylated antibodies were followed by staining with either SA-FITC or SA-PE. 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 mg/ml human immunoglobulin (IgG). Antibodies were diluted in FACS buffer for staining. Staining was carried out at 4^0C 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 4^0C before analysis. Flow cytometry was performed using a Cyan MLE cytometer (Dako-Cytomation, Ft Collins, CO). Gates were drawn to include live lymphocytes and APCS based on forward and side-scatter characteristics of spleen cells. DCs were defined as $CD11c^+/DEC\text{-}205^+$ cells that expressed variable levels of costimulatory molecules, including CD40, CD86 and 4-1BBL as well as MHC Class II.

Further division of DCs into myeloid, lymphoid and plasmacytoid subsets was based on detection of $CD11c^+$ cells expressing CD11b, CD8+ or B220, respectively. A minimum of 30,000 total events were collected for each sample; data analyses were

carried out using Summit Software (Dako-Cytomation). The percentage of each cell population was determined and then total cell numbers for each population were calculated from the total number of viable cells collected.

Antigen presentation assay

For in vitro analysis, BMDCs (harvested from ICR mice after 7 days of culture) were plated at 2 x 10^6/well in 24-well plates and exposed to DOX at 200 ng/ml. After 24 hours, DCs were harvested and added in graded doses to 5 x 10^4 purified splenic T cells (from C57Bl/6 mice) in 96-well round bottom plates. After another 3 days in culture, [^{3}H]-thymidine was added (1 uCi) for the final 12 hours of culture and the amount of incorporation measured.

For in vivo analysis, groups of 4 mice were treated with either a single dose of DOX (4 mg/kg i.v.), 200 *u*l of PBS i.v. (as a negative control), or 200 *u*l of LDC i.v. Seven days after treatment with DOX, or 24 hours following treatment with either LDC or PBS, the mice were euthanized and single cell suspensions were prepared from spleens. DCs were purified by first enriching for CD11c+ cells by using MACS MicroBeads (Miltenyi Biotec Inc., Auburn, CA), immunostained with anti-CD11c-biotin (Ebioscience, clone N4 18) and then separated from CD11c- cells using an LD Column according to the manufacturer's instructions (Miltenyi). Prior to cell sorting, the CD11c$^+$-enriched cell population was then immunostained with anti-CD3 (APC; clone 145-2C11), anti-CD19 (APC; clone MB19-1), anti-NKG2D (APC; clone CX5) and anti-CD11b (PE; clone M1/70). CD3$^-$/CD19$^-$/NKG2D$^-$/CD11c$^+$ DCs were sorted based on differential expression of CD11b using a MoFlo (DakoCytomation). This protocol is

routinely used in our lab and typically yields $CD11c^+/CD11b^+$ populations of greater than 95% purity. The sorted DCs were then added to purified, splenic T cells (obtained from C57Bl/6 mice) as before. Conconavalin (ConA) (Sigma-Aldrich) stimulated T cells, DCs or T cells alone were also included as positive or negative controls for both in vitro and in vivo assessment.

Determination of secreted cytokines

Culture supernatants were collected from in vitro studies and assayed for the presence of TNF-α, IL-1β, IL-6, IL-12p40, IL-10, and TGF-β by ELISA using commercially available kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). LPS (5 *u*g/ml) was used as a positive control for stimulation of pro-inflammatory cytokines while zymosan (3 *u*g/ml) was used to elicit IL-10. For detection of mouse IFN-α, an ELISA was developed as previously described (24, 32). A rat anti-mouse IFN-α capture antibody was purchased from PBL Biomedical Labs (Piscataway, NJ) and a detecting antibody (rabbit anti-IFN-α) was purchased from US Biologicals (Swampscott, MA). For IFN-β detection, a rat anti-mouse IFN-β capture antibody was purchased from Seikagaku (Cape Cod, MA) and a detection antibody (rabbit anti-murine IFN-β, PBL Biomedical) was used. For both cytokines, detection antibodies were followed with addition of a donkey anti-rabbit HRP antibody (Jackson ImmunoResearch) and then TMB substrate (Sigma-Aldrich). Sample analysis was performed using a Multiskan Ascent ELISA plate reader and Ascent software (ThermoLabsystems).

Statistical analyses

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

RESULTS

Determination of the sensitivity of BMDCs to common chemotherapy drugs

Because the goal of our initial studies was to assess the direct effects of chemotherapy on DCs without inducing significant cytotoxicity, we first established the sensitivity of BMDCs to drug-induced cytotoxicity in vitro. A panel of commonly used chemotherapeutics, each with a different mechanism of cytotoxicity, was selected for study; this information is summarized in **Table 1.**

Table 1: Chemotherapy Drugs Investigated in Preliminary Studies

Chemotherapy Drug	Abbreviation Used	Primary Mechanism of Action	Concentration Used In Vitro	Dose Used In Vivo
Dexamethasone	DEX	Inhibits DNA synthesis	15 *u*g/ml	2 mg/kg i.p.
Doxorubicin	DOX	Topoisomerase inhibitor	0.2 *u*g/ml	4 mg/kg i.v.
*Chlorambucil	CHLOR	DNA alkylator	35 *u*g/ml	2 mg/kg i.p.
Cisplatin	CIS	DNA intercalator	8 *u*g/ml	12 mg/kg i.p.
*Cyclophosphamide	CYC	DNA alkylator	NA	150 mg/kg i.p.
Vinblastine	VINB	Blocks mitotic spindle separation	35 *u*g/ml	1.5 mg/kg i.v.
Paclitaxel	PTX	Blocks mitotic spindle formation	60 *u*g/ml	30 mg/kg i.p.

* Both CYC and CHLOR are alkylating agents. CYC, however, must be activated in the liver to its cytotoxic metabolite while CHLOR is directly cytotoxic; thus CYC is used for in vivo studies but CHLOR is substituted for in vitro studies since metabolism of CYC to its cytotoxic form is not possible.

A standard MTT assay was then used to determine the concentration of each drug that resulted in less than 25% cytotoxicity to BMDCs and yet was clinically relevant to the treatment of human cancer patients (33-39). **Figure 1** presents the results for titration of DOX; similar calculations were performed for each of the other drugs (data not shown).

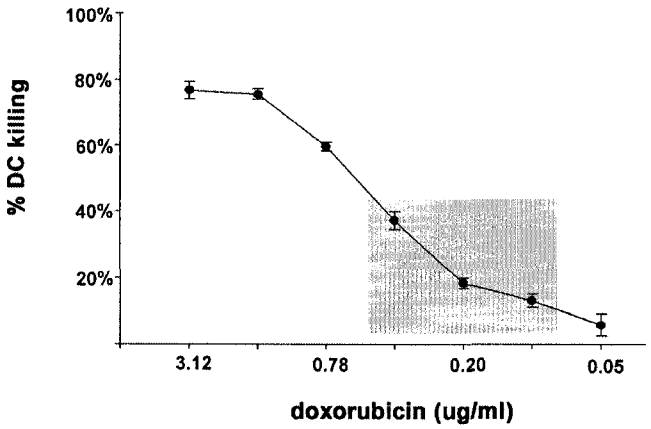

Figure 1: In vitro sensitivity of DCs to DOX. BMDCs were exposed to serial dilutions of DOX for 24 hours, then an MTT assay was performed. The concentration of DOX that induced less than 25% toxicity in vitro, but which was also clinically relevant, was determined for further in vitro studies. The shaded area represents the therapeutic dose range based on human pharmacokinetic studies. Bars give the standard deviation from the mean (SD).

Exposure to DOX and CIS induces CD40 expression on BMDCs

One important aspect of DC function is cell surface up-regulation of costimulatory molecules that facilitate optimal T cell communication. Therefore, we assessed cell surface expression of CD40, CD86, 4-1BBL and MHC Class II on BMDC using the information generated from the MTT assay to guide dose selection. We found that 24-hour exposure of BMDCs to DOX and CIS lead to significantly increased expression of CD40 ($p < 0.01$ for DOX; $p < 0.05$ for CIS) compared to BMDCs cultured in medium alone (**Figure 2**). Of the panel of drugs tested, DOX and CIS were unique in their ability to induce CD40 expression; however neither drug, nor any of the others, significantly increased expression of CD86, 4-1BBL or MHC Class II.

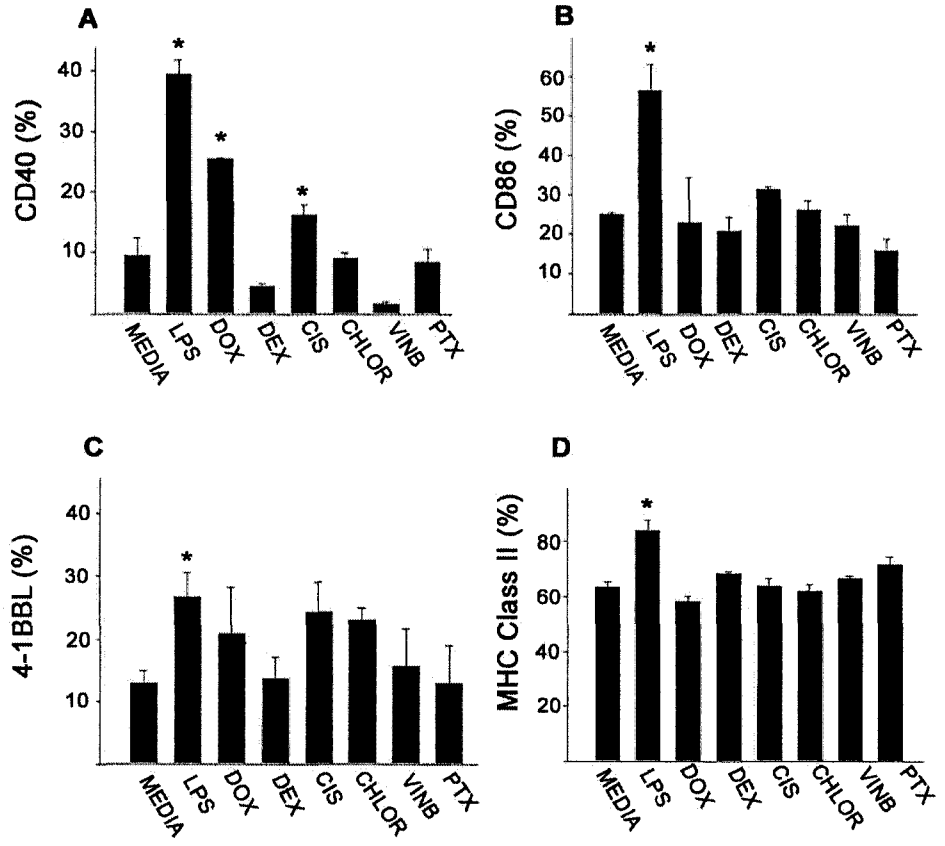

Figure 2: Exposure to DOX or CIS increases expression of CD40 on BMDC. BMDC harvested at day 7 of culture were exposed in triplicate to a single dose of each chemotherapy drug or to LPS for 24 hours. Cells were then immunostained for CD11c and costimulatory molecules and analyzed by flow cytometry. The percentage of CD11c+ DC expressing CD40 was significantly increased *($p < 0.01$ for DOX and $p < 0.05$ for CIS) following exposure to either DOX or CIS compared to control BMDC. No significant changes in expression of 4-1BBL, CD86 or MHC Class II were observed. Bars represent the SD.

Treatment of mice with DOX increases the

number of mature DCs in the spleen

The preceding results indicated that DOX, and to a lesser degree CIS, could stimulate CD40 expression on DCs in vitro. However, we hypothesized that

chemotherapy might have a different effect on costimulatory molecule expression in vivo, given the potential for DC interaction with other immune cells impacted by chemotherapy. Therefore, to investigate the effects of chemotherapy on DC phenotype in vivo, mice were treated with a single dose of each drug. As shown in **Figure 3**, total numbers of $CD11c^+$ DCs co-expressing CD40, CD86, 4–1BBL or MHC Class II were higher in the spleen of DOX-treated mice compared to DCs from PBS-treated control mice ($p < .01$).

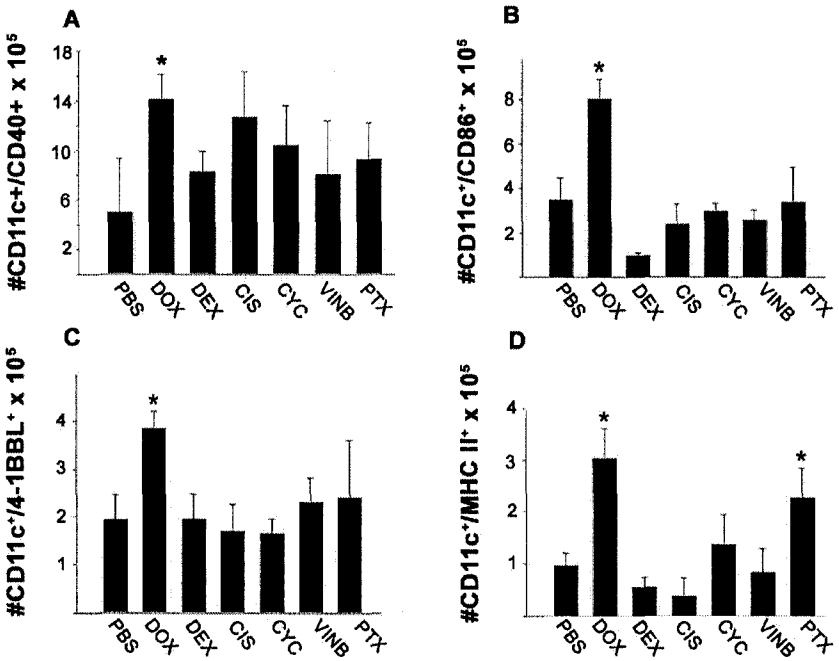

Figure 3: Treatment with DOX activates DCs in vivo. Mice receiving chemotherapy were sacrificed 7 days later and single cell suspensions were prepared from spleen. The number of CD11c+ DC co-expressing each marker was determined. Only mice treated with DOX demonstrated increased numbers of fully activated DCs * ($p < 0.01$). Bars give the SD.

Interestingly, although splenic DCs isolated from mice receiving paclitaxel exhibited significantly increased levels of MHC class II ($p < .05$ versus control mice), they did not demonstrate increased expression of CD40, CD86 or 4-1BBL. This observation is in line

with that of a previous study which found that although PTX induced MHC Class II expression on DCs in vitro, antigen presentation was markedly impaired (40). Administration of the other chemotherapy agents to healthy mice did not result in significant differences in costimulatory molecule or MHC Class II expression on CD11c+ splenic DCs compared to levels observed in PBS-treated mice.

Closer analysis of the effects of DOX chemotherapy on different subsets of splenic DCs revealed that mice receiving DOX had significantly higher absolute and relative numbers of myeloid DCs ($CD11c^+CD11b^+$ DCs) 7 days following drug administration ($p < 0.001$) (**Figure 4**).

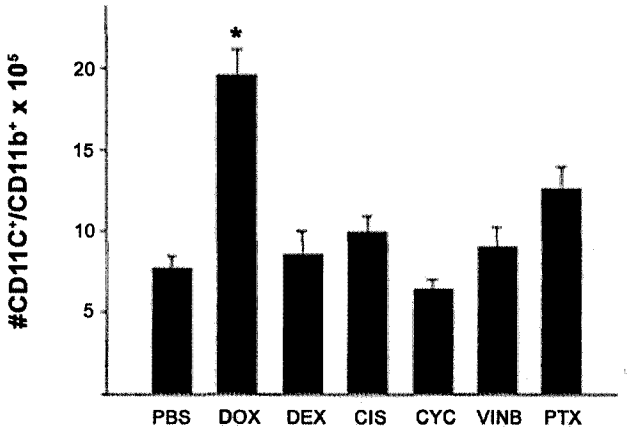

Figure 4: Doxorubicin significantly increases the total number of myeloid splenic DCs. A panel of chemotherapy drugs was given to groups of 4 healthy mice. Only mice receiving DOX had significantly increased numbers of $CD11c^+CD11b^+$ DCs in their spleen 7 days following drug administration *($p. < 0.001$). Bars give the SD.

No differences in the numbers of CD11c+CD8+ DCs or CD11c+B220+ DCs were observed in DOX-treated mice (data not shown).

Because of the apparent potential for DOX chemotherapy to activate and expand the splenic myeloid DC population, we more closely examined the kinetics of this

response in DOX-treated mice. Surprisingly, we found that the increase in $CD11c^{+}CD11b^{+}$ DCs was even greater at 5 days ($p < 0.001$ vs. control) following DOX administration than it was at day 7 ($p < 0.01$) and had returned to normal levels by 14 days post treatment (**Figure 5**). Analysis of costimulatory molecule expression during the expansion phase (24 hour, 3, 5, and 7-days post treatment), showed that expression of CD40 and 4-1BBL was significantly increased by 3 days post-treatment ($p < 0.05$ vs. control), reaching a maximum level at 5 days ($p < 0.01$). Increased expression of MHC Class II and CD86 was also maximal by 5 days post-therapy ($p < 0.01$) but was not increased over untreated mice at the 3 day time point as was observed for CD40 and 4-1BBL (Figure 5). By 14 days following DOX administration the level of costimulatory molecule and MHC Class II expression was similar to that of untreated mice (not shown).

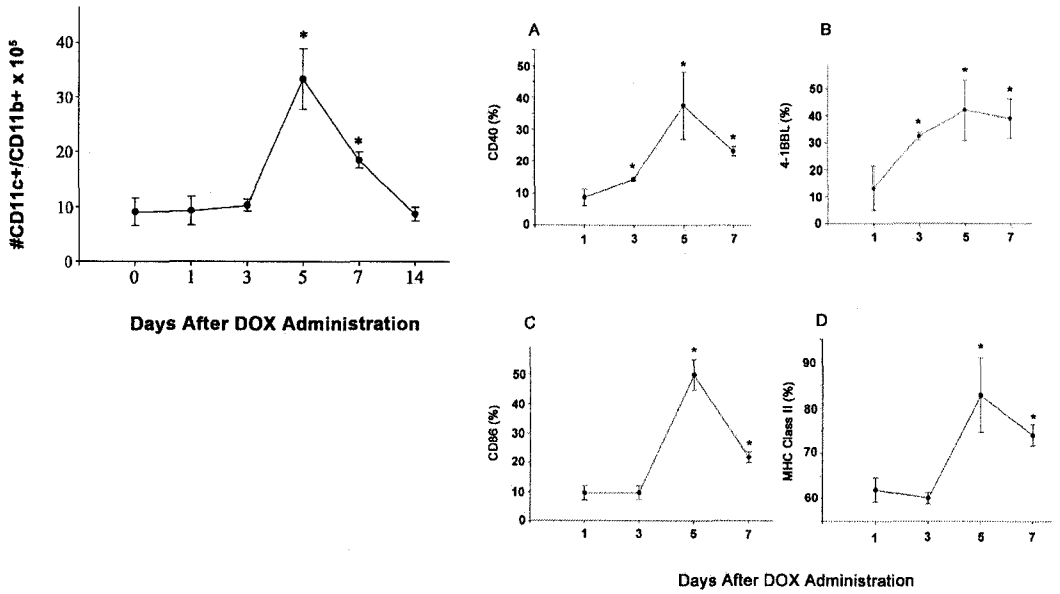

Figure 5: Stimulation of splenic myeloid DCs is maximal 5 days after treatment with DOX. Mice were sacrificed at the indicated time points after treatment with DOX and the total number of myeloid DCs and their activation phenotype was determined by flow cytometry. Expression of each marker was highest at day 5 after chemotherapy with DOX. Bars show SD, * $p < 0.05$.

Treatment of mice with DOX does not result in significant cytotoxicity to T-lymphocytes at day 7 post-administration

In people receiving conventional chemotherapy, cytotoxicity to rapidly dividing cells, such as lymphocytes and bone marrow stem cells, varies with differences in drug combinations, dosages and routes of administration. Patients receiving DOX at a dosage equivalent to the minimally immunosuppressive dose chosen for this study (approximately 20 – 30 mg/m^2 when given once every 3-4 weeks) generally do not experience high grade toxicity to bone marrow or gastrointestinal epithelial cells (41). Other investigations in mice using the 4 mg/kg dose of DOX that was used in this study have not reported significant leukocyte toxicity; in fact Casati et al. showed that administration of DOX at 6 mg/kg i.v. did not interfere with the development of antigen specific CD4 and CD8 T-cell responses in either healthy or tumor-bearing mice (14). However, to determine whether cell-mediated adaptive immune responses could potentially be limited by drug-induced cytotoxicity, we treated healthy mice with a single dose of DOX at 4 mg/kg i.v. and prepared single cell suspensions from the spleen and lymph nodes 7 days following administration of DOX. Total numbers of T-lymphocytes or T-cell subsets (CD4+, CD8+ T-cells) in the spleen or peripheral lymph nodes of DOX-treated mice were not significantly different than those of PBS-treated animals (**Figure 6**).

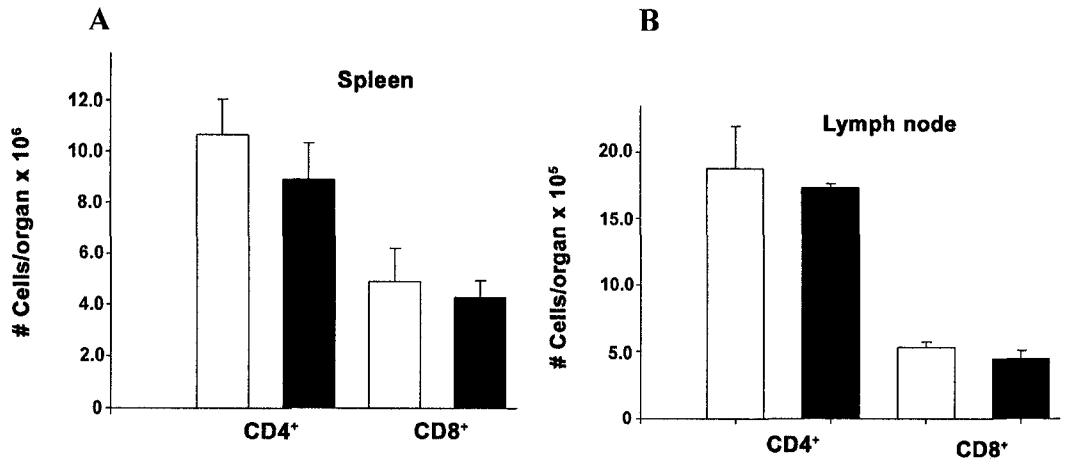

Figure 6: T-cell numbers are not significantly different in PBS-treated or DOX-treated ICR mice. The total number of CD4+ of CD8+ T-cells (±SD) were determined in the spleen (**A**) or lymph node (**B**) 7 days after treatment with DOX (black bars) and compared to those of PBS-treated mice (white bars). No significant differences were detected between groups.

DOX enhances antigen presentation by DCs

Although the studies described above suggested that DOX induces phenotypic maturation in myeloid DCs without eliciting significant cytotoxicity in the lymphocyte population, it was important to also determine whether other key aspects of DC function such as antigen presentation and cytokine production were also stimulated. Therefore, we next investigated whether treatment with DOX could increase antigen presentation by DCs. As described in Materials and Methods, BMDCs cultured with DOX or myeloid DCs purified from the spleen of DOX-treated mice, were added to cultures of allogeneic T-cells. For in vivo experiments mice were treated intravenously with DOX (4 mg/kg), PBS, or a liposome-DNA complex (LDC) containing 5 *u*g of empty vector DNA. In prior work in our laboratory we have shown that systemic administration of LDC triggers

strong activation of innate immune responses and can reliably be used as a positive control for DC activation (25, 42, 43).

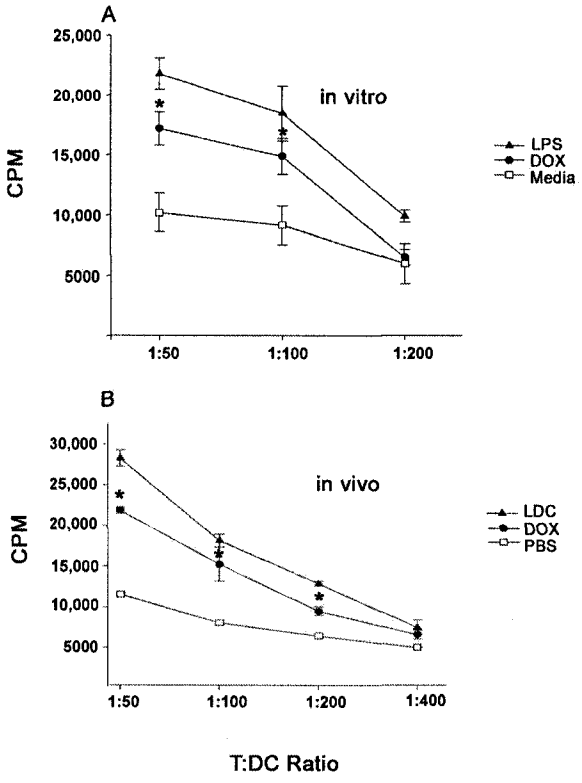

Figure 7: Doxorubicin enhances the antigen presenting ability of DCs. BMDC cultured with DOX **(A) or** DCs sorted from DOX-treated mice **(B)** showed significantly increased ability to stimulate T cell proliferation *($p < 0.01$). Bars represent the SD.

As illustrated in **Figure 7**, we found that DOX significantly enhanced antigen presentation both in vitro and in vivo; indeed the effects of DOX were nearly as pronounced as those elicited by treatment of mice with LDC or LPS stimulation of BMDCs. No increases in antigen presentation were observed in similar experiments with the other chemotherapy drugs under investigation; rather we found that dexamethasone,

paclitaxel and cisplatin significantly suppressed DC-mediated allogeneic T-cell proliferation while chlorambucil and vinblastine had neither suppressive nor stimulatory effects.

Exposure to DOX stimulates production of IL-12 from BMDCs

Efficient priming of Th1-mediated CD4+ and CD8+ T-cell responses by DCs is dependent on adequate production of pro-inflammatory cytokines. The nature of cytokine production by DCs from cancer patients is especially critical since tumor-induced defects in DC activation may result in secretion of immunosuppressive rather than pro-inflammatory cytokines that generate tumor tolerance rather than effective antitumor immunity (44-46). Therefore we conducted experiments to characterize cytokine production in BMDCs exposed to DOX. Despite increased expression of CD40, 24-hour culture of BMDCs with DOX did not trigger significant production of pro-inflammatory (TNF-α, IL-12, IFN-α, IFN-β or IL-1β) or immunosuppressive (IL-10, TGF-β) cytokines. In these experiments LPS (5 *u*g/ml) was use as a positive control for pro-inflammatory cytokine secretion while zymosan (3 *u*g/ml) was used to elicit IL-10 production. In addition, because the results of our in vivo studies showed that maximal stimulatory effect of DOX on DCs occurred 3-5 days after administration, we also examined cytokine production in DOX-pulsed BMDCs that were maintained in culture for several days following removal of the drug. Surprisingly, culture of DCs for an additional 4 days resulted in production of a small yet significant amount of IL-12 ($p < 0.01$) (**Figure 8**). Increased production of other cytokines, either pro-inflammatory or immunosuppressive, was not observed.

Analysis of costimulatory molecule expression after the extended culture period also revealed significantly increased levels of CD40 and 4-1BBL compared to DCs maintained in culture medium alone ($p < 0.001$), mirroring the early expression of these markers exhibited by splenic DCs in vivo (Figure 8). At this time point, however, the expression of CD86 and MHC Class II was not increased over the levels observed on control DCs (data not shown). Unfortunately we were not able to maintain sufficient viability in BMDC cultures past 4 days (despite replacement of the GM-CSF supernatant in the tissue culture medium every other day) and were therefore unable to assess costimulatory molecule expression or cytokine production past this point. Thus, the ability of DOX to fully activate DCs appears to be time-dependent both in vitro and in vivo.

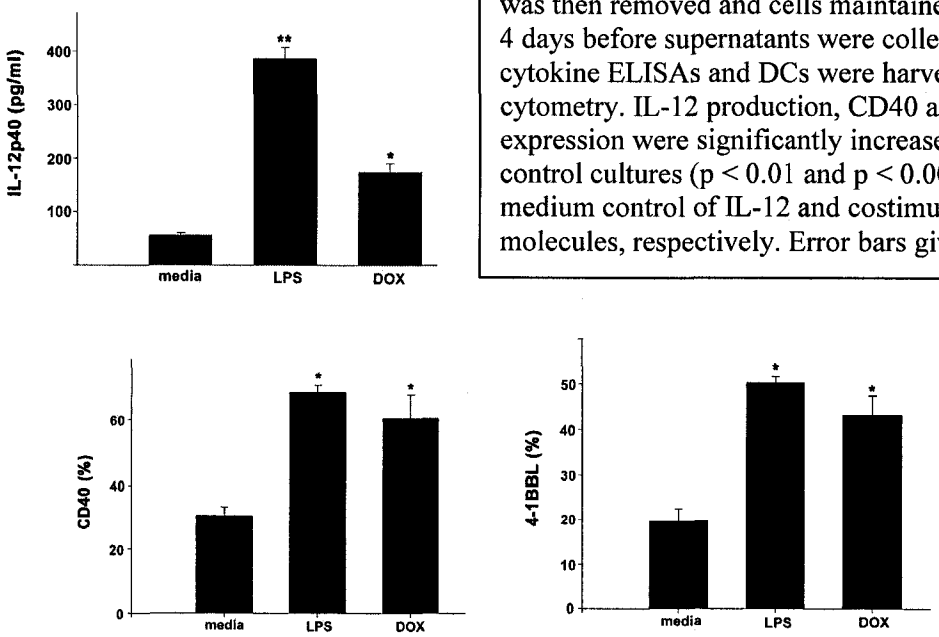

Figure 8: DOX induces IL-12 production and increased expression of CD40 and 4-1BBL. BMDCs were exposed to DOX for 24 hours, drug was then removed and cells maintained in culture for 4 days before supernatants were collected for cytokine ELISAs and DCs were harvested for flow cytometry. IL-12 production, CD40 and 4-1BBL expression were significantly increased compared to control cultures ($p < 0.01$ and $p < 0.001$) for DOX vs. medium control of IL-12 and costimulatory molecules, respectively. Error bars give the SD.

Treatment of tumor-bearing mice with DOX increases the number of activated DCs in tumor-draining lymph nodes and tumor-infiltrating lymphocytes

Despite our evidence for the ability of DOX chemotherapy to activate myeloid DCs in the spleen of healthy mice, previous investigations have shown that DCs from cancer patients are dysfunctional and may therefore respond poorly to activating stimuli (45). Therefore, to extend our findings to a more clinically relevant setting, experiments were conducted to assess the ability of DOX to activate DCs in tumor-bearing mice using the well-studied B16 melanoma model. In these experiments, B16 tumors were established in the skin of C57Bl/6 mice and DOX was administered when tumors were readily palpable (2-3 mm diameter) at about 7 days after tumor inoculation. Following i.v. administration of either DOX or PBS, mice were sacrificed 7 days later and single cell suspensions were prepared for FACS analyses from the spleen, draining and non-draining lymph nodes and tumor tissues.

As demonstrated in **Figure 9,** we found that mice treated with DOX had significantly more $CD11c^+$ DCs expressing $CD40^+$, $CD86^+$ or MHC Class II^+ within tumor-draining lymph nodes and the tumor infiltrating lymphocyte (TIL) population than in similar tissues of untreated mice. No differences were detected in the number of mature myeloid DCs in the spleen or non-draining lymph nodes between DOX-treated and PBS-treated mice (data not shown).

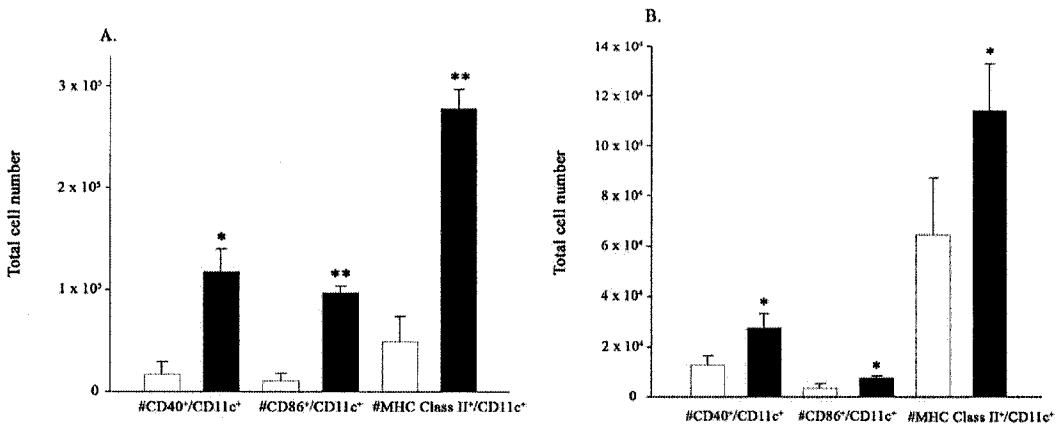

Figure 9: Treatment with DOX increases the number of mature DCs in mice with B16-melanoma. Mice receiving DOX (black bars) had significantly increased numbers of $CD11c^+$ DCs expressing CD40, CD86 or MHC Class II within tumor-draining lymph nodes, (*$p < 0.01$ and **$p<0001$), **(A)** and TILS, ($p < 0.04$), **(B)** than untreated mice (white bars). Error bars give the SD from the mean.

DISCUSSION

The primary objectives of the studies reported here were to better understand the effects of commonly used chemotherapeutic agents on DC function and to identify promising candidates for combination with tumor-specific immunotherapy. The key finding to emerge from this work is that the anthracycline derivative, DOX, augments several important aspects of DC function including costimulatory molecule expression, IL-12 production and antigen presentation. Furthermore, in mice with B16 melanoma, systemic DOX treatment expanded the number of mature DCs within tumor-draining lymph nodes and tumor tissues. These results suggest that DOX may be able to stimulate DC function in tumor-bearing animals as well as in healthy mice.

One striking and novel observation that arose from the studies reported here was the temporal nature of the immunostimulatory effects of DOX on DCs. Although increased levels of antigen presentation and CD40 expression were detected in BMDCs within a 24-hour incubation period, detection of increased expression of 4-1BBL and IL-12p40 production required an additional 72 hours once DOX had been thoroughly removed from the culture medium (Figure 8). This phenomenon contrasts with the rapid (i.e., $\leq$ 24 hr) production of multiple pro-inflammatory cytokines and wide array of costimulatory molecule expression induced through classic Toll-like receptor (TLR) signaling pathways (47, 48). Rather, our results suggest that DOX stimulates DCs in vitro through an as yet unidentified TLR-independent mechanism.

The pattern of costimulatory molecule and MHC Class II expression observed in splenic DCs from DOX-treated mice was similar to that of DCs in vitro. Thus, we found that CD40 and 4-1BB L expression were increased relatively early in DOX-treated mice (Figure 5) while CD86 and MHC Class II expression were not elevated above levels in control mice until 5 days following drug administration. One possibility, then, is that DOX may induce DC expression of TNF-receptor superfamily members more readily than other receptors such as CD86 and MHC Class II. Further analysis of the TNF-R signaling pathways in DCs exposed to DOX might contribute to a better understanding of the differential effects of DOX on costimulatory molecule expression.

A limitation of the studies described here is the inability to differentiate between indirect and direct effects of chemotherapy agents on DCs. Although the BMDC cultures used in our experiments contained approximately 60 – 70% CD11c+/DEC-205^{+} cells, the remaining cells were primarily CD11c^{-}/CD11b+/F480+ macrophages *(not shown)*. Given

the previously described ability of DOX expand and activate macrophages in vitro, DOX may simultaneously stimulate macrophages as well as DCs. A similar scenario might also occur in vivo; for example, the delayed expression of CD86 and MHC Class II on DCs from DOX-treated mice may be dependent on activation of other cell types such as NK cells and macrophages that further modulate DC function. Cell sorting and depletion experiments will need to be performed to aid in distinguishing between the direct and indirect effects of DOX on DCs.

Another intriguing finding to emerge from these studies was the marked expansion of the splenic myeloid DC population in DOX-treated mice (Figure 5). Analysis of the kinetics of this effect demonstrated a highly significant increase in the number of mature myeloid DCs 5 days after administration of DOX; a time period consistent with the rapid turn-over and proliferation of DCs in the murine spleen (49).

Although we suspect that the increase is secondary to DOX-induced stimulation of myeloid DC proliferation, an alternative explanation is that DOX drives differentiation of splenic DC precursors to the myeloid phenotype. Given the lack of increase in the number of $CD11b^{+}CD11c^{-}$ or $CD11b^{+}Gr1^{+}$ cells in the spleen of DOX-treated mice, this does not seem likely. Another possibility is that treatment with DOX favors accumulation of myeloid DCs in the spleen following their differentiation from precursors present in blood. Unfortunately, $CD11b^{+}CD11c^{-}$ or $CD11b^{+}Gr1^{+}$ cells were not enumerated in peripheral blood or lymph nodes, thus we can not rule out the potential contribution of precursor differentiation in these organs to the increased numbers of myeloid DCs observed in the spleen. Since the production of splenic DCs is largely under the control of the cytokine FLT3L (FMS-related tyrosine kinase-3 ligand) and the STAT3

(signal transducer and activator of transcription 3) transcription factor in mice, studies to determine whether DOX stimulates FLT3L production or increases STAT3 expression would contribute to a better understanding of the drug's effects on the splenic myeloid DC population (50).

In conclusion, our findings demonstrate the ability of DOX to stimulate multiple aspects of DC function, both in vitro and in vivo. Furthermore, our results suggest that DOX chemotherapy would be a logical prelude to administration of tumor vaccines, based on the ability of DOX to expand the number of activated DCs in the tumor-draining lymph nodes and TILs of mice with advanced B16 melanoma. Although the generation of more effective antitumor immunity in these mice awaits investigation, combination of DOX chemotherapy with vaccine-based immunotherapy for cancer merits continued investigation.

REFERENCES

1. Gilboa E. The promise of cancer vaccines. Nat Rev Cancer 2004;4(5):401-11.

2. Pardoll DM. Therapeutic vaccination for cancer. Clin Immunol 2000;95(1 Pt 2):S44-62.

3. Pardoll DM. Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol 2002;2(4):227-38.

4. Belardelli F, Ferrantini M, Parmiani G, Schlom J, Garaci E. International meeting on cancer vaccines: how can we enhance efficacy of therapeutic vaccines? Cancer Res 2004;64(18):6827-30.

5. Dalgleish AG, Whelan MA. Cancer vaccines as a therapeutic modality: The long trek. Cancer Immunol Immunother 2006;55(8):1025-32.

6. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10(9):909-15.

7. Yu Z, Restifo NP. Cancer vaccines: progress reveals new complexities. J Clin Invest 2002;110(3):289-94.

8. Broomfield S, Currie A, van der Most RG, *et al.* Partial, but not complete, tumor-debulking surgery promotes protective antitumor memory when combined with chemotherapy and adjuvant immunotherapy. Cancer Res 2005;65(17):7580-4.

9. Emens LA, Jaffee EM. Leveraging the activity of tumor vaccines with cytotoxic chemotherapy. Cancer Res 2005;65(18):8059-64.

10. Emens LA, Reilly RT, Jaffee EM. Breast cancer vaccines: maximizing cancer treatment by tapping into host immunity. Endocr Relat Cancer 2005;12(1):1-17.

11. Machiels JP, Reilly RT, Emens LA, *et al.* Cyclophosphamide, doxorubicin, and paclitaxel enhance the antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole-cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001;61(9):3689-97.

12. Nowak AK, Robinson BW, Lake RA. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. Cancer Res 2003;63(15):4490-6.

13. Casares N, Pequignot MO, Tesniere A, *et al.* Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med 2005;202(12):1691-701.

14. Casati A, Zimmermann VS, Benigni F, Bertilaccio MT, Bellone M, Mondino A. The immunogenicity of dendritic cell-based vaccines is not hampered by doxorubicin and melphalan administration. J Immunol 2005;174(6):3317-25.

15. Lake RA, Robinson BW. Immunotherapy and chemotherapy--a practical partnership. Nat Rev Cancer 2005;5(5):397-405.

16. Nowak AK, Lake RA, Marzo AL, *et al.* Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J Immunol 2003;170(10):4905-13.

17. Cohen SA, Ehrke MJ, Ryoyama K, Mihich E. Augmentation of the phagocytic activity of murine spleen cell populations induced by Adriamycin. Immunopharmacology 1982;5(1):75-84.

18. Ehrke MJ. Immunomodulation in cancer therapeutics. Int Immunopharmacol 2003;3(8):1105-19.

19. Ehrke MJ, Tomazic V, Ryoyama K, Cohen SA, Mihich E. Adriamycin induced immunomodulation: dependence upon time of administration. Int J Immunopharmacol 1983;5(1):43-8.

20. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Involvement of TLR4/MD-2 complex in species-specific lipopolysaccharide-mimetic signal transduction by Taxol. J Endotoxin Res 2001;7(3):232-6.

21. Li L, Okino T, Sugie T, *et al.* Cyclophosphamide given after active specific immunization augments antitumor immunity by modulation of Th1 commitment of CD4+ T cells. J Surg Oncol 1998;67(4):221-7.

22. Schiavoni G, Mattei F, Di Pucchio T, *et al.* Cyclophosphamide induces type I interferon and augments the number of CD44(hi) T lymphocytes in mice: implications for strategies of chemoimmunotherapy of cancer. Blood 2000;95(6):2024-30.

23. Wang J, Kobayashi M, Han M, *et al.* MyD88 is involved in the signalling pathway for Taxol-induced apoptosis and TNF-alpha expression in human myelomonocytic cells. Br J Haematol 2002;118(2):638-45.

24. Sellins K, Fradkin L, Liggitt D, Dow S. Type I interferons potently suppress gene expression following gene delivery using liposome(-)DNA complexes. Mol Ther 2005;12(3):451-9.

25. U'Ren L, Kedl R, Dow S. Vaccination with liposome-DNA complexes elicits enhanced antitumor immunity. Cancer Gene Ther 2006;13(11):1033-44.

26. McDonald RJ, Liggitt HD, Roche L, *et al.* Aerosol delivery of lipid:DNA complexes to lungs of rhesus monkeys. Pharm Res 1998;15(5):671-9.

27. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol 1997;15(7):647-52.

28. Inaba K, Inaba M, Romani N, *et al.* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176(6):1693-702.

29. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 1983;65:55-63.

30. Emens LA, Machiels JP, Reilly RT, Jaffee EM. Chemotherapy: friend or foe to cancer vaccines? Curr Opin Mol Ther 2001;3(1):77-84.

31. Nigam A, Yacavone RF, Zahurak ML, *et al.* Immunomodulatory properties of antineoplastic drugs administered in conjunction with GM-CSF-secreting cancer cell vaccines. Int J Oncol 1998;12(1):161-70.

32. Nguyen KB, Cousens LP, Doughty LA, Pien GC, Durbin JE, Biron CA. Interferon alpha/beta-mediated inhibition and promotion of interferon gamma: STAT1 resolves a paradox. Nat Immunol 2000;1(1):70-6.

33. Alberts DS, Chang SY, Chen HS, Larcom BJ, Evans TL. Comparative pharmacokinetics of chlorambucil and melphalan in man. Recent Results Cancer Res 1980;74:124-31.

34. Gianni L, Kearns CM, Giani A, *et al.* Nonlinear pharmacokinetics and metabolism of paclitaxel and its pharmacokinetic/pharmacodynamic relationships in humans. J Clin Oncol 1995;13(1):180-90.

35. Greene RF, Collins JM, Jenkins JF, Speyer JL, Myers CE. Plasma pharmacokinetics of adriamycin and adriamycinol: implications for the design of in vitro experiments and treatment protocols. Cancer Res 1983;43(7):3417-21.

36. Owellen RJ, Hartke CA, Hains FO. Pharmacokinetics and metabolism of vinblastine in humans. Cancer Res 1977;37(8 Pt 1):2597-602.

37. Schellens JH, Ma J, Planting AS, *et al.* Relationship between the exposure to cisplatin, DNA-adduct formation in leucocytes and tumour response in patients with solid tumours. Br J Cancer 1996;73(12):1569-75.

38. Struck RF, Alberts DS. Blood levels of alkylating metabolites of cyclophosphamide in the mouse after iv or oral administration. Cancer Treat Rep 1984;68(5):765-70.

39. Struck RF, Alberts DS, Horne K, Phillips JG, Peng YM, Roe DJ. Plasma pharmacokinetics of cyclophosphamide and its cytotoxic metabolites after intravenous versus oral administration in a randomized, crossover trial. Cancer Res 1987;47(10):2723-6.

40. Joo HG. Altered maturation of dendritic cells by taxol, an anticancer drug. J Vet Sci 2003;4(3):229-34.

41. Ackland SP, Ratain MJ, Vogelzang NJ, Choi KE, Ruane M, Sinkule JA. Pharmacokinetics and pharmacodynamics of long-term continuous-infusion doxorubicin. Clin Pharmacol Ther 1989;45(4):340-7.

42. Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. J Immunol 1999;163(3):1552-61.

43. Zaks K, Jordan M, Guth A, *et al.* Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 2006;176(12):7335-45.

44. Almand B, Resser JR, Lindman B, *et al.* Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res 2000;6(5):1755-66.

45. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 2001;166(9):5398-406.

46. Pinzon-Charry A, Maxwell T, Lopez JA. Dendritic cell dysfunction in cancer: a mechanism for immunosuppression. Immunol Cell Biol 2005;83(5):451-61.

47. Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. Current topics in microbiology and immunology 2006;311:17-58.

48. Tsan MF. Toll-like receptors, inflammation and cancer. Seminars in cancer biology 2006;16(1):32-7.

49. Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 2007;7(1):19-30.

50. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3L-dependent dendritic cell differentiation. Immunity 2003;19(6):903-12.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this dissertation focused primarily on the effects of chemotherapeutic agents on immune responses in healthy and tumor-bearing animals. As a whole, these studies contributed to a better understanding of the impact of chemotherapy on innate and adaptive and immune responses and set the stage for future investigations into multi-modality approaches to the treatment of cancer. It is anticipated that extension of this work will include clinical trials investigating chemotherapy combined with tumor vaccination in dogs with cancer as well as continued basic research using tumor models in mice.

Initial studies investigated the effects of two different chemotherapy protocols on adaptive immune responses in dogs with either lymphoma or osteosarcoma. The objectives of this work were to determine whether: 1) dogs with cancer have decreased T and B cell numbers in peripheral circulation compared to those of healthy dogs, 2) cancer-bearing dogs can mount an appropriate humoral immune response to de novo vaccination, and 3) lymphocyte counts or antibody responses change during the course of chemotherapy. Although not designed to investigate the impact of each chemotherapy drug *per se*, this study also compared the effects of combination versus single agent chemotherapy.

A key finding of this study was that chemotherapy did not significantly suppress T-cell numbers and that, despite reduction in B-cell numbers with multi-agent

chemotherapy, antibody responses to vaccination remained intact. Although these observations provide support for the concurrent administration of tumor immunotherapy and chemotherapy, studies of T-cell function are indicated to better assess the efficacy of combined treatment protocols.

A logical next step, therefore, would be to assess lymphocyte function in cancer-bearing dogs undergoing chemotherapy and immunotherapy. This can be accomplished through determination of delayed-type hypersensitivity reactions, lymphocyte proliferation, antibody titers, and cytokine-release assays such as the ELISpot assay for IFN-γ. These tests would be important in determining the nature of humoral and T-cell mediated immunity in patients treated with chemotherapy and immunotherapy and would help in understanding the significance of the observed lymphopenia in tumor-bearing dogs.

In human cancer patients pre-treatment lymphopenia is linked to a poor prognosis for some tumor types (1, 2). In addition, defective CTL function is well-recognized as an important reason for the failure of tumor vaccination to elicit clinically effective antitumor immunity (3-5). Based on the inability of chemotherapy to alleviate the abnormally low T-cell counts in the present study, it is suspected that chemotherapy might neither improve (nor suppress) T-cell function in cancer-bearing dogs.

The immunosuppressive effects of cancer may be manifested in a variety of ways including defects in lymphocyte and APC function or recruitment of inhibitory cells such as regulatory T cells. The samples collected during the chemotherapy study described above provided an opportunity to preliminarily assess of the effects of chemotherapy on Treg levels in cancer-bearing dogs. Although chemotherapy did not appear to either

negatively or positively impact the number of probable Treg (CD4+IL-2R+ T-cells), the low sensitivity and specificity of the assay made interpretation of the data difficult. However, dogs with lymphoma appeared to have persistently elevated levels of CD4+IL-2R+ T-cells that did not diminish despite induction of clinical remission. This suggests that Treg are likely to play a role in dogs with cancer as they do in people and that chemotherapy may not be sufficient to alleviate some mechanisms of tumor immune evasion.

The inability to distinguish between activated, non-regulatory CD4+ T-cells and Treg underscored the need to find a better method for Treg identification. Thus, as described Chapter 3, we developed an assay to identify canine Treg based on intracellular detection of the FoxP3 transcription factor. This approach is currently accepted as the most specific method for Treg identification in other species (6). With this assay we were able to evaluate Treg levels in dogs with different types of cancer and confirm that Treg are abnormally elevated in the blood and tumor-draining lymph nodes compared to Treg levels in normal dogs. This technique will now permit a more critical evaluation of the role of Treg in the canine cancer patient. It is anticipated that the results of this study will help to provide a foundation for future clinical trials in which Treg depletion can be explored as therapeutic modality in dogs with cancer.

In Chapter 4 we investigated the effects of chemotherapy on DCs. Despite the pivotal role that DCs play in the generation of antitumor immune responses there is surprisingly little information regarding the impact of drug therapy on DC function. Although the immunostimulatory properties of several chemotherapy agents have been described, previous investigations have focused largely on CTL responses or the

stimulatory effects of tumor cell death on APCs. Therefore, we systematically evaluated the effects of a panel of commonly used chemotherapy agents on multiple aspects of DC function in both in vitro and in vivo studies. The objectives of this work were not only to gain a better understanding of chemotherapy's impact on DCs, but to also identify a drug (or drugs) that might enhance DC function, especially when used in combination with tumor vaccines.

Through this work the ability of the chemotherapy drug DOX to activate DCs soon became apparent. We found that DOX stimulated multiple aspects of DC function including antigen presentation and increased costimulatory molecule expression. Administration of a single dose of DOX led to dramatic expansion of the number of mature myeloid DCs in the spleen within 5 to 7 days of treatment. Furthermore, in mice with B16 melanoma, treatment with DOX led to increased numbers of activated DCs within tumor tissue and tumor-draining lymph nodes suggesting that DOX therapy may stimulate DC function in tumor-bearing as well as healthy animals.

Based on these observations, we would next propose studies in a mouse tumor model (e.g. CT26 colon carcinoma) to determine if DOX chemotherapy can be combined with tumor vaccination to enhance antitumor immunity. Our hypothesis is that mice receiving DOX chemotherapy prior to tumor vaccination will demonstrate enhanced CTL function as well as delayed tumor growth compared to mice receiving the tumor vaccine alone. Clinical trials in dogs with spontaneous cancer could also be conducted to determine if administration of DOX chemotherapy in combination with an LDC-based tumor vaccine improves the response to treatment for diseases such as osteosarcoma and lymphoma.

Overall, the research described here has documented progress towards a better understanding of the impact of chemotherapy on innate and adaptive immune responses and led to initial studies of canine Treg using a novel diagnostic technique. This work represents a significant step towards the development of more effective approaches to anti-cancer therapy. We believe that it provides important groundwork for future investigations of combined treatment modalities that can readily be translated from the laboratory to the clinic. Thus, by using both companion animals with spontaneously occurring cancer and the tools of basic research, extension of the studies described here may help us to move forward down the path towards a cure for cancer.

REFERENCES

1. Lissoni P, Brivio F, Fumagalli L, *et al.* Efficacy of cancer chemotherapy in relation to the pretreatment number of lymphocytes in patients with metastatic solid tumors. Int J Biol Markers 2004;19(2):135-40.

2. Soygur T, Beduk Y, Baltaci S, Yaman O, Tokgoz G. The prognostic value of peripheral blood lymphocyte subsets in patients with bladder carcinoma treated using neoadjuvant M-VEC chemotherapy. BJU Int 1999;84(9):1069-72.

3. Lee PP, Yee C, Savage PA, *et al.* Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 1999;5(6):677-85.

4. Rubio V, Stuge TB, Singh N, *et al.* Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 2003;9(11):1377-82.

5. Stuge TB, Holmes SP, Saharan S, *et al.* Diversity and recognition efficiency of T cell responses to cancer. PLoS Med 2004;1(2):e28.

6. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol 2005;6(4):331-7.