Myeloid Derived Suppressor Cells in Dogs with Cancer: Phenotype, Function and Clinical Implications

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Dedication

This thesis is dedicated to all of man's best friends. For the true love and friendship that only they can give to us. Dogs are pure and true and fill our hearts with joy. They know how to deeply touch our souls through simple but unforgettable demonstrations of love, friendship, affection and companionship… and all we wanted is that they be immortal.

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Abstract

Myeloid-derived suppressor cells comprise phenotypically heterogeneous population of myeloid cells at different stages of differentiation endowed with potent immunosuppressive activity. Abnormal accumulation of MDSC in tumor models and cancer patients produce profound immune suppression, severely impairing T cell antitumor immunity, contributing to angiogenesis, cell invasion and metastasis, and constitute a major hurdle in achieving successful immune-based therapies.

Understanding the mechanism that drives MDSC expansion and enhances function in humans and dogs is crucial for the development of efficacious immunotherapy.

Studies in dogs with several tumor types, including sarcoma, carcinomas, mast cell tumors and gliomas confirmed MDSC expansion in the peripheral blood of dog cancer patients. MDSC have been identified in dogs using the combination of three-marker phenotype $CD11b^{+}CD14^{-}MHCII^{-}$cells for granulocytic and $CD11b^{+}CD14^{+}MHCII^{-}$cells for monocytic subsets. Granulocytic MDSC accumulated in the peripheral blood of dogs with advanced sarcoma, carcinomas and mast cell tumors, co-purified with peripheral blood mononuclear cell (PBMC) fraction and expressed polymorphic mononuclear morphology. This subset of cells showed the ability to efficiently inhibit T cell proliferation and IFN-γ secretion of autologous T cells, as well as allogenic T cells from healthy dogs, and expressed ARG1, iNOS2, TGF-β and IL-10. Monocytic MDSC also

demonstrated potent ability to suppress T cell proliferation and preferentially accumulated in the peripheral blood of dogs with glioma. Elevated levels of arginase activity found in the serum of dogs with glioma could potentially be due to the presence of elevated numbers of MDSC. Evaluation of the anti-mouse Gr1 antibody for MDSC staining and identification revealed that does not cross react and therefore is not suitable for canine cells.

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List of Abbreviations

7AAD	7 aminoactinomycin D
APC	antigen presenting cell
ARG1	arginase 1
ATRA	all-trans retinoic acid
COX2	cyclooxygenase 2
CSF1R	colony stimulating factor-1 receptor
CTL	cytotoxic T cells
CTLA-4	cytotoxic T lymphocyte-associated-antigen 4
DC	dendritic cells
FasL	Fas ligand
FoxP3	Forkhead box protein 3
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
G-MDSC	granulocytic myeloid-derived suppressor cells
HLA	human leukocyte antigen
HPC	hematopoietic progenitor cell
HSCs	hematopoietic stem cells
IFNα	interferon-alpha
IFNγ	interferon-gamma
Ig	immunoglobulin
IL	interleukin

iNOS	inducible nitric oxide synthase
M-CSF	macrophage colony stimulating factor
MDSC	myeloid-derived suppressor cells
MHCI	major histocompatibility complex I
M-MDSC	monocytic myeloid-derived suppressor cells
MMPs	matrix metalloproteinases
NSAID	non-steroidal anti-inflammatory drug
NO	nitric oxide
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death protein-1
PDE-5	phosphodiesterase-5
PGE2	prostaglandin E2
RB	retinoblastoma
ROS	reactive oxygen species
SCF	stem cell factor
TADCs	tumor-associated tolerogenic dendritic cells
TCR	T cell receptor
TGF-β	transforming growth factor beta
TLR	tool-like receptor
TME	tumor microenvironment
Treg	T regulatory cells
TAA	tumor-associated antigens
TNF	tumor necrosis factor

VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2
5-FU	5-fluorouracil

Chapter I

Introduction

Overview on cancer in dogs and the canine cancer model

Approximately four million dogs are diagnosed with cancer each year in the United States, making cancer the leading cause of death in adult dogs and the major health care concern of pet owners in the United States, Australia, Japan and Europe (1).

Naturally occurring canine malignances often share a wide variety of biologic and clinical features often observed with human cancers. There are many similarities between human and canine malignances including spontaneous neoplasm development, tumor biology, genetics, incidence rates, histological appearance, and response to conventional treatments (1-5). In both humans and dogs, tumor initiation and progression can be influenced by similar factors such as age, nutrition, sex, reproductive status, and exposure to environmental risk factors. Spontaneous tumors in dogs evolve over long periods of time, interacting dynamically with the host immune system, which recapitulates the mechanisms of tolerance observed in human disease (3). These similarities, the ability to collect serum, urine, blood, biopsy tissue samples, and the use of advanced imaging diagnostic tests to monitor clinical parameters, tumor progression and efficacy of the therapy make dogs with spontaneous tumors a strong model for translational cancer research (2, 6, 7).

Although murine cancer models have proven to be extremely powerful to determine molecular pathways involved in cancer initiation, promotion, and progression, there are important limitations due to differences in size and physiology of this model. For instance, murine models do not reproduce some

essential features of cancer in humans; such as tumor grow over long periods, immune system function, tumor microenvironment and stroma interactions. More importantly, they have not been predictive of toxicity or efficacy of treatments in humans (2).

Unquestionably, an advantage of the canine model of spontaneous cancer includes the possibility to develop an almost identical treatment scheme to those used in humans, including surgery, radiotherapy, chemotherapy, and immunotherapy that may better predict the response in humans to novel therapies. For this reason, in the past few years, translational studies using pet dogs have been developed to assess novel therapeutic approaches for a variety of cancers (1, 2, 4).

Canine tumor models provide valuable tools for studying several aspects of human cancer such as identification of cancer-associated genes, the study of environmental risk factors, and better understanding of tumor biology and progression, pharmacokinetics/dynamics, toxicity, dosing, biomarkers/ endpoints, and adverse effects of new drugs (2, 3). Therefore, the use of canine cancer as model for the development of new cancer treatments has many advantages and makes the dog a very attractive model for oncology research and translational therapies (2, 3, 5).

Cancer immune surveillance and tumor-immune cell interactions

Understanding the relationship between the immune system and cancer began in the nineteenth century when the association between inflammation

against pathogens and its effect against tumors was established (8). Decades of studies using animal models led to the immunosurveillance theory, which postulated that the immune system is able to recognize and eliminate abnormal cancerous cells before they can develop into clinically apparent tumors (9-11).

Increased risk of tumor development in immunodeficient or immunosuppressed animal models and human patients, instances of spontaneous tumor regression, and the presence of tumor-infiltrating lymphocytes and NK cells in association with improved prognosis for a variety of tumor types offer a compelling evidence that the immune system is capable of identifying and destroying nascent tumors (10-13).

Anti-tumor immunity is accomplished by both innate and the highly specialized cellular and humoral components of the adaptive immune system (11, 13, 14). Cell mediated immune responses, especially cytotoxic T cells (CTLs), play a crucial role in killing neoplastic cells (13). Tumors are distinguished immunologically from normal tissue by the expression of self- and neo-antigens from aberrant genetic programs, so T cell immunosurveillance of malignant cells begin with recognition of these specific tumor-associated antigens (TAA). The binding of tumor-associated antigens to an antigen presenting cells (APC) and processing and presentation of these antigens to a cognate T cell receptor (TCR) are crucial points in the initiation of a T cell immune response.

Following uptake of tumor antigens, dendritic cells (DC), one type of APC, traffic to lymph nodes where they processes and present tumor peptides through

major histocompatibility complex I (MHCI) to prime naïve CD8 T cells. This presentation results in T cell activation, clonal expansion and differentiation into tumor-specific cytotoxic T cells (CTL) capable of infiltrating tumors and destroying cancer cells through activation of the apoptotic caspase pathway by releasing the cytotoxic proteins perforin-granzyme. Therefore, activation and infiltration of functional effector T cells within the tumor mass is important for successful immunosurveillance (12, 14).

Alternative mechanism of T cell killing occurs upon interaction between Fas, a member of the tumor necrosis factor (TNF) receptor family, expressed on the surface of tumor cells and the Fas ligand (FasL) on the surface of activated lymphocytes. Binding of Fas ligand to Fas leads to activation of caspases and apoptosis of tumor cells. However, several studies have demonstrated that many cancer cells developed resistance to FasL/Fas-mediated cell killing by expressing or secreting FasL and are therefore, capable of counteract and trigger the killing of activated effector T cells that infiltrate the tumor site (11, 12, 14, 15).

Despite the presence of immune surveillance, tumors do develop in immunocompetent animals and humans. There are several mechanisms that contribute to failure of the immune system to control tumor growth such as tumor immunoediting, the induction of T cell tolerance, the ability of tumors to induce the expansion and recruitment of immune suppressive cells into the tumor microenvironment, activation of negative regulatory pathways and secretion of inhibitory factors (9, 12, 16). These mechanisms will be further discussed below.

Tumor Immunosuppression

In the last decade, the majority of studies in the field of tumor immunology have focused on identifying tumor-specific antigens and novel treatment modalities to enhance immunologic responses against malignances (10, 17-21). Recently, an extensive amount of research is focused on understanding the mechanisms by which cancer cells counteract and escape the immune control, and therefore finding novel approaches to treat solid tumors (9, 12, 15, 17-19, 22).

Cancer immunoediting hypothesis describe that while the immune system can protect the host against tumor development, it may also serve to promote tumor growth and spread (11). According to this hypothesis, cancer development can be divided in three phases: elimination, equilibrium and escape (11-13). In the elimination phase, cells of the innate and adaptive immune systems detect and eliminate tumor cells. The second phase comprises the temporary state of dynamic equilibrium between immune system and tumor cells that have survived the elimination phase. In this phase, the immune response is strong enough to control, but not completely eradicate, the tumor that contains many unstable and genetically mutated cells. These interactions result in the selection of tumor cell variants capable to resist and evade the immune response. In the third phase, escape, less immunogenic and therefore, more resistant tumor cell variants develop into clinically apparent tumors (12, 13, 16, 23, 24).

There are multiple immunosuppressive strategies by which tumors can attenuate the effectiveness of T cell-mediated responses. Tumors are capable of

avoiding recognition and cell death through downregulation or complete loss of immunogenic antigens and/or MHC class I molecules (16, 18, 25), downregulation or inactivation of death receptors (CD95/FAS or TRAIL) and expression of either transmembrane decoy receptors with truncated non-functional domains or soluble decoy receptors missing the death domain (18, 25). Tumors can also induce T cell anergy and apoptosis by signaling through T cell co-inhibitory receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) or programmed cell death protein-1 (PD-1). Interaction of these receptors with members of the B-7 immunoglobulin superfamily, that are expressed primarily by DCs and are also upregulated in many tumor tissues, results in inhibition of T cell activation (16, 18, 26).

Another major method by which tumors protect themselves from immune mediated elimination is by creating a dominant immunosuppressive microenvironment. The tumor microenvironment (TME) is composed of immune cells, tumor cells, stromal cells and extracellular matrix. The interactions between cancer cells and the host immune system in the tumor microenvironment initiate an immunosuppressive network that induces immune tolerance and promotes tumor growth (9).

Tumor cells secrete multiple soluble suppressive factors that promote generation and recruitment of various immune suppressive cell types including myeloid-derived suppressor cells (MDSC), type 2 macrophages (M2), tumor-associated tolerogenic dendritic cells (TADCs), and T regulatory cells (Treg) (9, 16). Collectively, these tumor-recruited immune cells not only act to suppress an

anti-tumor immune response but also serve to promote tumor-dependent angiogenesis, invasion and metastasis (27, 28).

The most common suppressive immune cells are Tregs and MDSCs. Elevated levels of Treg are detected in the peripheral blood and tumors of cancer patients, and several studies reported that an increased number of Tregs is predictive of decreased survival time and poorer response to treatment (29-33). Tregs can be reliably identified in both human patients and mouse models by the expression of CD4 and CD25 surface makers and the intracellular transcription factor Forkhead box protein 3 (FoxP3) in both human patients and mouse models (34, 35). They exert suppression of T cell activity through secretion of the immunosuppressive cytokines transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), or inhibit T cell activation via modulation of APC function (31). Recent studies demonstrated that similarly to mice and humans, Tregs also accumulate in the peripheral blood and tumors in dogs with a variety of cancers such as, mammary carcinoma (30), melanoma (36), osteosarcoma (33) and they can be identified using the same markers (30, 37, 38).

The functional importance of MDSC suppressive effects on the adaptive immune responses has been recently appreciated and now MDSCs are widely accepted in the field of tumor immunology as a major contributor to immune suppression in cancer patients and tumor-bearing mice (39-41).

Origin of myeloid-derived suppressor cells

Myeloid cells are the most abundant hematopoietic cells in the body. They are

comprised of a group of distinct cell populations that have diverse functions such as immune protection against pathogens, elimination of dying cells and tissue remodeling (42). This lineage of cells originate in the bone marrow from the multipotent hematopoietic stem cells (HSCs) and further differentiate into various subsets of specialized myeloid cells such as DCs, monocytes, neutrophils, basophils and eosinophils. Bone marrow of healthy individuals continually generates immature myeloid cells that will normally develop into mature myeloid cells without causing evident immune suppression. On the contrary, cancer myelopoiesis is associated with defective myeloid cell differentiation, which results in the expansion and migration of immature myeloid cells to primary neoplastic lesions and metastases (39, 43-46). Thereby, MDSCs originate in the bone marrow from myeloid progenitor cells that do not differentiate into mature dendritic cells, granulocytes, or macrophages. Upon activation by tumor-derived-factors, they acquire immunosuppressive properties, migrate and accumulate in the circulation and lymphoid tissues, and finally enter the tumor site (39, 47, 48)

Accumulation of myeloid-derived suppressor cells in cancer

Expansion of MDSC has been detected in practically all tumor models and human cancers. The evaluation of MDSCs in mice and in patients with different solid tumors demonstrates that MDSC levels were significantly higher in tumor-bearing animals and cancer patients relative to healthy controls (39, 47, 49-53). These cells accumulate in the bone marrow, spleen, and peripheral blood, within primary and metastatic solid tumors, and to a lesser extent in lymph nodes of

tumor-bearing mice (39, 54-58). The majority of the studies in cancer patients evaluated MDSC in the peripheral blood (39, 52, 53, 59-62), however detection of this population of cells in the tumor site has also been reported (63). Elevated levels of circulating MDSC have been significantly correlated with clinical stage, metastatic burden and poor prognosis (52) and elimination of these cells dramatically improved immune responses in tumor-bearing mice and in cancer patients (52, 60, 64).

In clinical patients and animal models, MDSC recruitment is driven by tumor burden and a variety of tumor-soluble factors produced by neoplastic and stroma cells in the tumor microenvironment (27, 39, 52, 55). The crosstalk between neoplastic cells and tumor-associated stromal cells releases key tumor-derived factors that modulate the accumulation of immature cells and their further conversion into immunosuppressive cells. Several cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), vascular endothelial growth factor (VEGF), stem cell factor (SCF), transforming growth factor-β (TGF-β) and interleukin-3 (IL-3) are implicated in myelopoiesis and MDSC accumulation (43, 48, 55).

GM-CSF is a cytokine that stimulates the bone marrow to produce and secrete granulocytes (neutrophils, eosinophils, and basophils) and monocytes into the blood stream while G-CSF controls the proliferation and differentiation of granulocytes only. Several types of cells produce GM-CSF and G-CSF including immune cells, fibroblasts, endothelial cells and numerous types of tumors. The

overproduction of these cytokines is associated with MDSC formation (43, 65). Importantly, although GM-CSF is used as an immune adjuvant with tumor cells vaccines, there is strong evidence that this adjuvant can be efficacious at low concentrations, but can actually provoke immunosuppression at higher concentrations by stimulating MDSC accumulation (56).

Vascular endothelial growth factor (VEGF) is a growth factor secreted by many tumors, but it is also crucial in the formation and maintenance of normal blood vessels and blood cells. In addition to its well-characterized role in angiogenesis, VEGF has been shown to inhibit the activation of the transcription factor NF-κB in hematopoietic progenitor cells. This results in alterations in the development of multiple lineages of hematopoietic cells including inhibition of DC development and T cell formation, and most importantly leads to the accumulation of MDSC (65, 66). Gabrilovich et al. demonstrated that in vivo infusion of VEGF directly targeted pluripotent stem cells and led to a profound expansion of $Gr1^+$ immature cells in the spleen and a less profound, but still significant, increase of this cell population in lymph nodes of tumor-bearing mice (66). A later study by Larrivee et al. showed that accumulation of $CD11b^+Gr1^+$ cells in bone marrow and peripheral blood are mediated through VEGFR2 signaling and is dependent on VEGFR2-induced increases in GM-CSF. Through a series of experiments the authors concluded that VEGFR2 activation not only directly elicits the expansion of the $CD11b^+Gr1^+$ population in bone marrow and peripheral blood, but also the induction and secretion of GM-CSF by bone marrow stroma cells, which in turn exert paracrine effects on the myeloid

progenitor population. Therefore VEGFR2 signaling in combination with GM-CSF drives the rapid accumulation of $CD11b^{+}Gr1^{+}$ cells in bone marrow and peripheral blood (65).

Stem cell factor (SCF) is a growth factor important for the survival, proliferation, and differentiation of hematopoietic stem cells and other hematopoietic progenitor cells. It binds to the c-kit receptor (CD117) to induce signaling and is also known as kit ligand. Pan et al. showed that SCF is expressed by many human and murine tumors and plays a role in the MDSC accumulation associated with advanced malignancy. Large tumor burden generates increased levels of SCF that can simultaneously enhance myelopoiesis and decrease myeloid cell differentiation, thereby inducing expansion of the MDSC population. Furthermore, blocking the SFC/c-kit signaling pathway in tumor cells led to significant decreases in MDSC accumulation and suppressive function, resulting in enhanced anti-tumor responses, tumor regression and reversion of tumor-specific T cell tolerance (61).

MDSC accumulation is also associated with inflammatory processes and the inflammatory mediators produced during inflammation. Cyclooxygenase 2 (COX-2) and its downstream production of prostaglandin E2 (PGE2) has been implicated to promote tumor progression by inducing MDSCs accumulation and suppressive activity (9, 43, 67, 68).

PGE2 is an important mediator of inflammation and is involved in other biological processes, including angiogenesis, apoptosis, and immune

suppression. (68). Recently it was reported that PGE2 is a key factor in MDSCs development, accumulation and functional stability. PGE2 mediates the induction of COX-2 in MDSCs, that initiates a positive feedback loop resulting in enhanced production of endogenous PGE2 (55, 62). This positive feedback between active COX-2-induced and autocrine production of endogenous PGE2 proved to be essential for stabilizing the suppressive functions of MDSCs (69). Rodriguez et al. showed that lung tumors constitutively express COX-1 and COX-2 and produce high levels of PGE2. More importantly, that signaling through the PGE2 receptor E-prostanoid 4 expressed on MDSCs induced the production of arginase I contributing to T cell dyfunction (62). Fujita and colleagues demonstrated that gliomas express high levels of COX-2. They found that treatment with COX-2 inhibitors hindered systemic PGE_2 production by glioma cells and had a direct inhibitory effect on glioma cell growth in mice (58). Moreover, COX-2 blockade by non-steroidal anti-inflammatory drugs (NSAID) significantly decreases the numbers of $CD11b^{+}Ly6G^{hi}Ly6C^{lo}$ granulocytic MDSCs both in the bone marrow and the TME and led to increases in CXCL10 expression and CD8+ T-cell infiltration in the TME (58). Recent work in the field of transplantation research has shown that large numbers of functional human MDSC can be efficiently generated from monocytic precursors by supplementing peripheral blood-isolated monocytes cultures with PGE2 (70).

Pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6), as well as S100A8/A9 proteins, have also been implicated in MDSC accumulation and suppressive function (55, 71, 72). IL-1β plays a critical role in

the induction and maintenance of an inflammatory TME by contributing to the expansion of MDSCs (71, 72). Modulation of MDSC accumulation and suppressive function by IL-1β is indirect, since they do not have IL-1 receptors (IL-1R), it occurs through induction of multiple pro-inflammatory molecules that contribute to the inflammatory milieu in the TME (71). On the other hand, IL-6, one of several downstream mediators induced by IL-1β, drives MDSC accumulation via direct binding of tumor-secreted IL-6 to its receptor IL-6R (55). The S100 inflammatory proteins S100A8 and S100A9 induce MDSC expansion by blocking the differentiation of myeloid precursors via a STAT3-dependent mechanism. In addition, S100A8/A9 proteins serve as chemotatic factors, contributing to the recruitment and migration of MDSCs (55). Moreover, since MDSC are capable to synthesize and secrete these pro-inflammatory mediators into the TME, S100A8/A9 proteins also promote MDSC accumulation via an autocrine feedback loop (57).

Lastly, other important factors implicated in MDSC activation include the cytokines IFN-γ, IL-4, IL-10, IL-13 and TGFβ that are responsible for the induction of immunosuppressive pathways that commit immature cells to become functional MDSCs (43, 55, 57) and the chemotatic mediators CCL2, CCL12, CXCL5 that play a role in the recruitment of immature cells to the tumor stroma (43).

Characteristics of MDSC – phenotype and subsets

Myeloid-derived suppressor cells comprise a phenotypically

heterogeneous population of myeloid cells at different stages of differentiation that are endowed with potent immunosuppressive activity. MDSC regulate immune responses and tissue repair in healthy individuals and this population rapidly expands during many pathological conditions, including inflammation, infectious diseases, trauma, sepsis, and cancer (42, 64). Morphological, phenotypic, and functional heterogeneity is a hallmark of these cells. Due to their heterogeneity, they express a variety of lineage markers that overlap with other myeloid cells making their phenotypic characteristics broadly distinct. In mice, they are identified as cells with $CD11b^{+}$ and $Gr\text{-}1^{+}$ phenotypes (9, 39, 42, 55, 73) and consist of two major subsets: granulocytic MDSC (G-MDSC) identified by the phenotype $CD11b^{+}Ly6G^{+}Ly6C^{low}$ and monocytic MDSC (M-MDSC) that have the phenotype $CD11b^{+}Ly6G^{-}Ly6C^{high}$ (39, 41, 47, 73, 74). These two major subsets of MDSC differ not only in their morphology and phenotype, but also have different gene expression profiles, transcription factors activity and mechanisms of immune suppression (64, 75). G-MDSC is the predominant subset type (70–80 % of all MDSC), is moderately immunosuppressive and requires direct contact with T cells to promote tolerance. These cells suppress antigen-specific T cells primarily through arginase 1 (ARG1) and reactive oxygen species (ROS) mediated mechanisms. On the other hand, M-MDSC comprise 20–30 % of total MDSC, but are highly immunosuppressive, exert their suppressive effects in a non antigen-specific manner, and inhibit T cell function through a nitric oxide synthase (NOS)-mediated mechanism (42, 64, 76).

Recent research suggests that these subsets are not distinct, fully

differentiated populations. Youn et al. demonstrated that M-MDSC have the ability to dedifferentiate into immunosuppressive G-MDSC through the loss of expression of Retinoblastoma (Rb) protein; a process that is mediated by epigenetic silencing of the Rb gene (75).

In humans, the MDSC phenotype is less clearly defined, so combinations of various markers have been used to identify this population in the peripheral blood of cancer patients. They can be defined as cells that co-purify with peripheral blood mononuclear cells and lack expression of lineage specific cell markers of lymphocytes, natural killer cells and DCs, as well as markers of mature myeloid cells such as HLA-DR, CD40, CD80 and CD83. However they express the common myeloid markers CD33 and CD11b, and depending of the subset express the granulocyte marker CD15 or monocyte marker CD14, for human G-MDSC and M-MDSC, respectively (40, 52, 59, 77, 78).

Therefore variable phenotypes have been described in different types of human cancer to identify this heterogeneous and complex cell population. It has been suggested that human granulocytic MDSC can be identified as Lin^- $HLA\text{-}DR^-CD33^+$ or $CD11b^+CD14^-CD15^+$ while the monocytic subset expresses $CD11b^+CD14^+$ $HLA\text{-}DR^{-/low}$ or $CD14^+$ $HLA\text{-}DR^{-/low}$ (42, 55, 64, 77, 78).

An extensive amount of research is underway to better identify potential MDSC subsets and new ways to target these cells (17, 40, 49, 59, 61, 63, 68, 74, 75, 77, 79-90). Recently IL-4R alpha (IL4Rα) has been identified as a potential marker for highly immunosuppressive monocytic MDSC in mice and humans (84, 91). The activation marker CD66b has been described in granulocytic MDSC

subsets (59) and the VEGF-R can also be expressed by human MDSC (59, 60).

Therefore, the biology of MDCS is very complex and they express varied phenotypes and suppressive patterns, likely depending on the cytokine profile in the TME. The large number of candidate markers emphasizes the complexity in defining these cells, possible due to the dynamic plasticity of these cells and their ability to readily respond to tumor-derived factors (41, 43, 78).

Mechanisms of MDSC immunosuppression

Myeloid-derived suppressor cells have become the focus of intense study in recent years. This distinct population of cells is now widely recognized as critical mediators of tumor escape and progression in various solid tumors (39, 42, 54, 92). MDSC play a crucial role in the regulation of anti-tumor immunity, as well as contribute to angiogenesis, cell invasion and metastasis (93). Although heterogeneity is a hallmark of this population, all MDSC share a common trait, the extraordinary ability to suppress T cell responses (27, 39, 41, 42, 55, 93, 94). Several underlying mechanisms of MDSC immunosuppressive activity have been described and their overall biological role is now widely appreciated. Up-regulation of ARG1, inducible nitric oxide synthase (iNOS), and ROS has been implicated as a major factor in the immune suppressive activity of MDSC (42, 54, 94). Therefore, their immunosuppressive mechanisms can be separated into two groups: L-arginine-dependent, directly affecting T cell function, and L-arginine-independent, inducing expansion of other suppressive cells such as Tregs (42).

MDSCs express high levels of ARG1 and iNOS2 resulting in the rapid

depletion of the semi-essential amino acid, L-arginine. Decreased levels of L-arginine lead to loss CD3 ζ-chain expression and dysfunction of other signal transduction components, including tyrosine kinase and Janus-kinase-3, thereby blocking activation-proliferation signals and inhibiting tumor-specific T cell responsiveness (42, 73, 93, 95). In addition to L-arginine depletion, accumulation of nitric oxide (NO), a metabolite of iNOS2 activity, induces apoptosis of T cells through accumulation of p53 by FAS or caspase-independent signaling (42). Moreover, MDSCs induce nitration-nitrosylation of the TCR-CD8 complex through hyper-production of reactive oxygen species (ROS) and peroxynitrites during direct cell-cell contact. This change in the TCR-CD8 complex results in disruption of T cell function and promotes nitration of the chemokines such as CCL2 in the TME impairing trafficking of tumor-specific T cells to the tumor site. Furthermore, MDSC prevent T cell activation and function by decreasing the availability of extracellular cysteine, an amino acid required for T cell activation and function (42).

An indirect MDSC mechanisms by which MDSC inhibit T cell function is the development and expansion of T regulatory cells, mediated by secretion of immunosuppressive cytokines TGF-β and IL-10 (42, 61, 80, 93). Moreover, increases in IL-10 / TGF- β production are also associated with decreases in IL-12 production by macrophages, which induces NK and T cell anergy (93).

MDSC have also been demonstrated to support neoangiogenesis, tumor growth and metastasis (27). These cells have the ability to invade into the tumor and secrete pro-angiogenic factors, such as VEGF. In addition, MDSC produce

matrix metalloproteinases (MMPs) and chemoattractants, such as S100A8 and S100A9 pro-inflammatory proteins, which induce a pre-metastatic environment that leads to cancer invasion and metastasis (27, 55, 71, 93).

Given the variety of mechanisms employed by MDSC, they have a tremendous potential to suppress immune responses and assist in tumor progression and therefore, constitute an important and promising target to improve the efficiency of new cancer treatments, such as immunotherapy.

MDSC as therapeutic targets

Over the past two decades, it has become evident that tumor-associated immunosuppression contributes significantly to tumor progression and resistance to cancer immunotherapy (16, 17, 19, 96).

Since accumulation of MDSC in cancer patients severely impairs a T cell response and constitutes a major hurdle in achieving successful immunotherapy, several therapeutic strategies to block function, differentiation, expansion and accumulation of these cells have been developed (reviewed in (89)). Multiple methods to inhibit MDSCs are currently under investigation. They can be broadly categorized into methods that 1) functionally inhibit MDSC (phosphodiesterase-5 inhibitors, COX-2 inhibitors), 2) promote differentiation of MDSC into mature, non-suppressive cells (all-trans-retinoic acid, vitamin D), or 3) decrease MDSC levels (sunitinib, gemcitabine, 5- fluorouracil).

Therapeutic approaches to inhibit MDSC immunosuppressive function include ARG1 inhibitors, NO inhibitors, ROS inhibitors, and migration inhibitors.

Phosphodiesterase-5 (PDE-5) inhibitors, such as sildenafil, have been show to decrease expression of both ARG1 and iNOS2 and improve T cell function in patients with head and neck squamous cell carcinoma (HNSCC) and multiple myeloma (87). Based on encouraging preclinical results, several clinical trials using phosphodiesterase-5 (PDE-5) inhibitors have been initiated. Nitroaspirin has been demonstrated to effectively suppress the production of ROS in MDSC (89). COX-2 reduces the immunosuppressive function of MDSC by decreasing expression of ARG1, but is also associated with significant decreases in MDSC numbers (58, 62).

All-trans-retinoic acid (ATRA), a metabolite of vitamin A, induces MDSC maturation and expression of differentiation markers such as HLA-DR (82, 85). High doses of Vitamin D3 or A can induce maturation of myeloid cells and upregulation of HLA-DR increasing anti-tumor immunity. Moreover, CpG oligodeoxynucleotides (ODN) induce maturation of MDCS by stimulating the production of IFNα by cytoplasmoid DC (90).

Sunitinib is a FDA-approved multi-kinase inhibitor for the treatment of advanced renal cell carcinoma that has multiple targets including VEGFR and c-kit. Human studies demonstrate that sunitinib treatment significantly decreases circulating MDSC and Tregs in renal cell cancer patients, which resulted in increased T cell function (60).

Moreover, while some chemotherapeutic agents, such as doxorubicin and cyclophosphamide, have been shown to increase MDSC levels in peripheral blood of cancer patients (52), other conventional cytotoxic compounds such as

gemcitabine, cisplatin, 5-fluorouracil (5-FU) and docetaxel have been show to have cytotoxicity effects on MDSC (89). The use of genetic engineered molecules, such as IL4Rα-aptamer to specific target IL4Rα on MDSC, was demonstrated to efficiently induce MDSC apoptosis (97).

Therefore, there are multiple strategies that can be employed to target these immunosuppressive populations and new combination therapies utilizing these approaches to decrease the immunosuppression of cancer patients could potentially enhance the effectiveness of cancer immunotherapy.

Preface

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Chapter II

Identification of Myeloid Derived Suppressor Cells in Dogs with Naturally Occurring Cancer

Summary

Dogs with naturally occurring cancer represent an important large animal model for drug development and testing novel immunotherapies. However, poorly defined immunophenotypes of canine leukocytes have limited the study of tumor immunology in dogs. The accumulation of myeloid derived suppressor cells (MDSCs) is known to be a key mechanism of immune suppression in tumor-bearing mice and in human patients. We sought to identify MDSCs in the blood of dogs with cancer. Peripheral blood mononuclear cells (PBMCs) from dogs with advanced or early stage cancer and from age-matched healthy controls were analyzed by flow cytometry and microscopy. Suppressive function was tested in T cell proliferation and cytokine elaboration assays. Semi-quantitative RT-PCR was used to identify potential mechanisms responsible for immunosuppression. PBMCs from dogs with advanced or metastatic cancer exhibited a significantly higher percentage of $CD11b^{+}CD14^{-}MHCII^{-}$cells compared to dogs diagnosed with early stage non-metastatic tumors and healthy dogs. These $CD11b^{+}CD14^{-}MHCII^{-}$ cells constitute a subpopulation of activated granulocytes that co purify with PBMCs, display polymorphonuclear granulocyte morphology, and demonstrate a potent ability to suppress proliferation and IFN-γ production in T cells from normal and tumor-bearing donors. Furthermore, these cells expressed hallmark suppressive factors of human MDSC including ARG1, iNOS2, TGF- β and IL-10. In summary our data demonstrate that MDSCs accumulate in the blood of dogs with advanced cancer and can be measured using this three-

marker immunophenotype, thereby enabling prospective studies that can monitor MDSC burden.

We have found significant increases in arginase enzymatic activity in the serum of dogs with gliomas compared with healthy controls. Therefore we proposed that the elevated levels of arginase activity in these patients, could be due to the increased MDSC percentages

Introduction

Tumors in dogs progresses relatively faster than the same disease in humans, allowing questions related to treatment efficacy (progression and survival) to be addressed more rapidly in dogs. An important advantage of the dog model is the ability to test experimental therapeutics at human scale doses in the setting of minimal residual disease, which is difficult to do in a meaningful way in small rodents that have relatively rapid tumor growth kinetics. In addition, because the standard of care for most canine tumors is poorly established, there is much more flexibility in study design compared to human clinical trials. Collectively these features make the dog an outstanding platform for translational medicine.

Pet dogs with cancer are rapidly becoming an important tool used in drug development. One of the best examples of this is the recent parallel development of SU11654, a multi-targeted tyrosine kinase inhibitor, and sunitinib malate (SU11248). Both drugs are potent inhibitors of PDGFR, VEGFR, KIT, and FLT3. Studies in dogs with various solid tumors revealed that plasma concentration of SU11654, the mutational status of KIT, and the inhibition of KIT phosphorylation were strongly predictive of clinical efficacy. Optimal dosing parameters and toxicity were established in dogs as well. These pioneering studies greatly facilitated the further development of this entire class of drugs, most notably the approval of sunitinib malate by the U.S. Food and Drug Administration for the treatment of renal cell carcinoma (RCC) and gastrointestinal stromal cell tumors, which often contain similar KIT mutations (98). It was later recognized that

sunitinib markedly depletes MDSCs and restores T cell function in human RCC patients (60), an observation that could not have been made in dogs at the time because of limited canine reagents and poorly defined markers for canine leukocytes. We, and others, are testing novel immune-based therapies in dogs with various malignancies, but immune monitoring in these studies has been confounded by the same problem. To put the field in perspective, a surface immunophenotype for canine natural killer cells has not been defined, the MHC alleles are poorly understood, and many of the markers used rely on cross-reactive antibodies whereby specificity must be tested empirically. It is crucial that new reagents are developed and that the immunophenotypes of all major canine leukocytes subsets are determined. Laying this basic foundation will allow unique insights to be made as new small molecule drugs and immunotherapies are tested in dogs as a prelude to human trials.

The accumulation of MDSCs in tumor-bearing mice and humans with cancer is known to be a key mechanism of tumor escape from immune surveillance (52, 55, 73). MDSCs comprise a phenotypically heterogeneous population of myeloid cells in early stages of differentiation that expand in cancer and many other pathological conditions, and have a potent ability to suppress T cell function, especially T cell proliferation and effector cytokine production (64, 73, 95). MDSCs may be divided into monocytic and granulocytic subtypes. One source of controversy in this field is that MDSC heterogeneity has made comparisons between cancer patients and murine tumor models challenging (see reference (64) for excellent perspective).

The molecular mechanisms by which MDSCs inhibit T cell function are under investigation. Studies have implicated up-regulation of arginase 1 (ARG1), inducible nitric oxide synthase (iNOS2) and reactive oxygen species (ROS) as important factors for MDSC-mediated immune suppression (39, 41, 64, 95). ARG1 can profoundly impair T cell function at the tumor site by L-arginine depletion, triggering the amino acid starvation response and apoptosis in lymphocytes (55). Another mechanism of immune suppression is chemokine nitration, which blunts effector T cell infiltration into the tumor site (99). Furthermore, MDSC expansion is associated with downregulation of L-selectin on CD4+ and CD8+T cells. This reduces T cell trafficking to secondary lymphoid organs where tumor-reactive T cells can be primed (100).

Due to the ability of MDSCs to down regulate the immune response against tumors in mice and in humans, we hypothesized that these cells would also play an important role in tumor-induced immune suppression in dogs with cancer. Hence, the objective of this study was to identify surface markers that characterize the existence of MDSCs in dogs.

Materials and Methods

Study Population and sample collection

The description of all dogs in this study is summarized in Tables 1 and 2, with further detail provided in Tables S1 and S2. Table S3 is a summary of samples assayed in each figure. Clinical data were obtained from medical records. Control dogs were determined to be healthy based on physical

examination, owner observations, and complete blood count exams. For dogs with cancer, the diagnosis and tumor staging were based on complete physical examinations, histopathology of tumor biopsy specimens, blood work and specialized imaging tests, such as CT scans, ultrasound or radiographs, to assess tumor location and size, as well as the presence of metastatic disease. Dogs with large, necrotic or multiple masses, lytic or severe bone destruction (with osteosarcoma) or presence of metastasis, were placed into the advanced stage/metastatic group. Animals presenting with small masses or no metastatic nodules were placed into the early stage non-metastatic group. Tables S1 and S2 also list specifics about any treatment that dogs with cancer had received prior to or at the time of blood collection for this study. Blood samples from both cancer and healthy control dogs were obtained specifically for this study. Samples were collected in heparinized tubes by the Oncology and Community Practice Services of the Veterinary Medical Center at the University of Minnesota according to Institutional Animal Care and Use Committee guidelines. The samples were drawn after the owners signed the client consent form. The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the study entitled as ‘‘Flow Cytometric Immunophenotyping of Peripheral Blood Cells in Dogs’’ via designated member review under the code number 0912A75493. Unless explicitly stated otherwise, the cells being analyzed for this manuscript co-purified with peripheral blood mononuclear cells (PBMCs) of dogs with cancer or age matched healthy controls that were isolated using Ficoll (Sigma) gradient centrifugation as follows. Heparinized peripheral blood was

diluted 1:3 with sterile PBS (Invitrogen) and layered over Ficoll-Histopaque (Sigma). Samples were centrifuged at 400-xg for 30 min. The PBMCs collected at the interface were transferred to a fresh tube, washed twice with PBS, and resuspended with freezing solution consisting of 90% fetal bovine serum (Invitrogen) 10% Dimethyl sulfoxide (DMSO) (Sigma) and then frozen at $^{-}$80°C. Lastly, PBMCs were thawed for 2 minutes in a 37°C water bath before staining and analysis. For analysis of fresh samples, PBMCs were isolated as above, resuspended in FACS buffer, stained with antibodies, and immediately analyzed by flow cytometry or FACS as indicated.

Flow Cytometric Analysis

PBMC samples were isolated from fresh blood or thawed and resuspended in FACS buffer. Nonspecific antibody binding was blocked by pretreatment of cells with 10 mg/mL canine gamma globulin (Jackson Immunoresearch) for 20 min at room temperature. Cells were first labeled using indirect staining with 0.1 mg of unconjugated mouse anti-dog CD11b antibody (clone CA16.3E10, AbD Serotec) or IgG1 isotype control (AbD Serotec) and 0.5 mg of PE-conjugated goat F(ab')2 anti-mouse IgG (Abcam) secondary antibody at 4°C for 30 min in a dark room. Following indirect staining, cells were washed twice and stained with 0.3 mg of FITC-conjugated rat anti-dog MHCII (clone YKIX334.2, AbD Serotec) and 0.15 mg of the cross-reactive, Alexa fluor 647-conjugated mouse anti-human CD14 antibody (clone TU¨ K4, AbD Serotec) or isotypes controls at 4°C for 30 min in a dark room according to manufacturer's

protocol. Antibody-labeled cells were washed twice and re-suspended in FACS buffer. Cells were incubated for 10 minutes at room temperature in the dark with 7-amino-actinomycin D (7AAD, final concentration of 1 mg/mL; Calbiochem) and then analyzed on a Becton Dickinson Canto three-laser flow cytometer. Data were further analyzed with FlowJo software (Tree Star). Analysis gates were set based on the 7AAD negative population. The percentage of MDSCs was calculated based on the percentage of $CD11b^{+}CD14^{-}MHCII^{-}$ cells within the overall live PBMC population. In one experiment (Figure S1), anti-mouse PE conjugated CD11b (clone M1/70 eBioscience) and anti-mouse APC-conjugated Gr-1 (clone RB6-8C5 eBioscience) antibodies were also used to verify cross reactivity with dog cells.

Isolation of MDSCs, PMNs and T cells

For functional assays, RT-PCR and cell morphology analysis, fresh blood samples from a tumor-bearing dog were used for isolation of $CD11b^{+}CD14^{-}MHCII^{-}$ or $CD11b^{+}CD14^{+}MHCII^{-}$ cells, as indicated, using a BD FACS Aria cell sorter. For T cell isolation, PBMCs were isolated as previously described from fresh blood samples of healthy dogs and stained with 0.3 mg of FITC-conjugated mouse anti-dog CD3 (clone CA17.2A12, AbD Serotec), 0.15 mg of Pacific blue-conjugated mouse anti-dog CD4 (clone YKIX302.9, AbD Serotec) and 0.15 mg of Alexa700-conjugated mouse anti-dog CD8 (clone YCATE55.9, AbD Serotec) antibodies. Polymorphonuclear leukocytes (PMN) were purified from the cell pellet of a Ficoll gradient from healthy dog blood samples, after removal of the

PBMCs (at the top of gradient) and erythrocytes by RBC lysis buffer (eBioscience).

Ex Vivo Proliferation

Analysis of MDSC inhibitory activity on T cell proliferation was measured by ^{3}H-thymidine incorporation into DNA. Briefly, PBMCs from the indicated dogs were seeded into U-bottom 96- well plates ($5x10^{4}$cells/well) in medium consisting of RPMI 1640 containing L-arginine (150 mM) (Invitrogen) supplemented with penicillin/streptomycin (Invitrogen) and 10% heat-inactivated fetal bovine serum (Invitrogen) at 37°C, in a 5% CO2 incubator. $CD11b^{+}CD14^{-}MHCII^{-}$ or $CD11b^{+}CD14^{+}MHCII^{-}$ cells from a dog with cancer were sorted and added to cancer (autologous) or healthy responder PBMCs as indicated. Concanavalin A (5 mg/ml) (Sigma) and recombinant human IL-2 (10 IU/ml) (R&D systems) were used to stimulate T cell proliferation. Non-stimulated PBMCs were used as negative control. PBMCs or PMNs were co-cultured with healthy PBMCs to control for the effect of simply adding additional cells to the suppression assay as indicated. Plates were cultured for 72 h, and then pulsed with 1 µCi of ^{3}H-thymidine (Amersham Pharmacia Biotech) for 18 hrs at 37°C. Cells were harvested onto glass fiber filters (Perkin Elmer), washed, dried, and counted. Proliferative responses were measured by ^{3}H-thymidine incorporation into the DNA using a Matrix 96 Direct Beta Counter (Packard). All experiments were performed in triplicate.

IFN-γ Analyses

FACS-isolated $CD11b^{+}CD14^{-}MHCII^{-}$ cells from a cancer dog were co-cultured with PBMCs isolated from a healthy dog using the same method as the proliferation assay. After 72 hrs of incubation the cell culture supernatants were collected and measured using a Quantikine canine IFN-γ ELISA kit according to the manufacture's instructions (R&D systems). Samples were assayed colorimetrically, in triplicate, using a Microplate Reader Synergy2 (Biotek) and analyzed with Microplate Data Collection and Analysis Software Gen5 (Biotek).

Cytospin

FACS-isolated $CD11b^{+}CD14^{-}MHCII^{-}$ cells were stained using a modified Giemsa stain (Diff-quick, Astral Diagnostics Inc) for cell morphology evaluation and observed using a DME microscope (Leica) at 63X power magnification. Pictures were acquired with an EC3 camera (Leica).

RNA extraction and RT-PCR

RNA was extracted from FACS-isolated $CD11b^{+}CD14^{-}MHCII^{-}$ cells or healthy dog PMNs, using an RNAeasy plus Mini kit (QIAGEN) according to the manufacturer's protocol. RNA concentrations were evaluated using a ND (100) spectrophotometer (Nanodrop). To detect expression of ARG1 and iNOS2 enzymes, gene-specific primers were designed based on the canine ARG1 and iNOS2 sequence; primer sequences for housekeeping gene were designed from canine b-actin gene using Primer3Plus (http://www.bioinformatics.nl/cgi-

bin/primer3plus/ primer3plus.cgi). For detection of cytokines IL-10 and TGF- β primer sequences of IL-10 and TGF- β were obtained from published sources (37). The BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure primer specificity to the target gene. First strand cDNA synthesis was done using a QuantiTect Reverse Transcription kit (QIAGEN). The two-step PCR reaction was carried out in a 12.5-ml volume containing 26SYBR green master mix (Quanta Biosciences), 0.675U GoTaq Polymerase, 2 nM MgCl2 (Promega), 0.2 mM dNTPs (Stratagene), 0.2 mM of each primer pair and 50 ng of cDNA template. Reaction conditions consisted of initial denaturation at 94uC for 2min, then cycles of denaturation at 94uC for 30 s, annealing at 60uC for 45 s, elongation at 72uC for 45 s and final elongation at 72uC for 5 min in a DNA Engine Thermal Cycler (Bio-rad). The optimum annealing temperature for each primer pair was established prior to the study (see primer sequences in Table S4). PCR products were run on 2% agarose gels containing 0.5 ml ethidium bromide and imaged under 590 nm ultraviolet light on an Eagle Eye II image station (Stratagene). Negative control reactions were performed using RNA that was not subjected to reverse transcription PCR.

Statistical Analysis

The differences between two groups were analyzed using unpaired, two-tailed Student's t test. All tests were performed with Prism 4 software (Graph Pad Software, Inc). P values, 0.05 were considered to be statistically significant.

Results

Dogs with advanced cancer have elevated levels of granulocytic $CD11b^{+}CD14^{-}MHCII^{-}$ cells that co-purify with PBMCs

Peripheral blood samples from 45 dogs diagnosed with cancer and 18 healthy control dogs were collected (Tables 1 and 2). All dogs with cancer underwent clinical staging of their disease by performing complete physical examinations, blood work, imaging to assess tumor location and size and metastases, and histopathological diagnosis made from diagnostic aspirate or biopsy of the tumor. Among the 45 dogs diagnosed with cancer, 30 dogs were classified as having advanced or metastatic disease and 15 dogs were classified as early stage/non-metastatic or low grade disease based on clinical staging. Each group was further subdivided according to histological diagnosis into sarcomas, carcinomas or mast cell tumors (detailed in Tables S1 and S2).

The percentages of putative MDSCs in dogs with cancer and healthy dogs were evaluated by flow cytometry. PBMCs from dogs with advanced or metastatic cancer showed a marked increase in the $CD11b^{+}CD14^{-}MHCII^{-}$ fraction of cells, which accounted for the majority of the cells in the live cell gate, compared to dogs diagnosed with early stage non-metastatic tumors or healthy dog controls (Fig. 1A). This subset of cells exhibited a polymorphonuclear granulocytic morphology at heterogeneous stages of development (Fig. 1B), which resembles a granulocytic subset of MDSCs identified in mice (101) and humans (102).

Dogs with advanced or metastatic cancer had a significantly greater

fraction of putative MDSCs (36.04±2.542, mean±SEM) compared to dogs with early stage non-metastatic tumors (9.40±0.953, mean±SEM) and healthy control dogs (10.24±1.412, mean±SEM) (Fig. 2A). Moreover, this elevation in the $CD11b^{+}CD14^{-}MHCII^{-}$ fraction did not appear to be restricted to a specific tumor type. The differences were statistically significant in dogs with sarcomas, carcinomas, and mast cell tumors compared with healthy controls (Fig. 2B). Conversely, the percentage of $CD11b^{+}MHCII^{-}$ cells that did express CD14 was not significantly different among any group. Therefore, the frequency of $CD11b^{+}$ $CD14^{-}MHCII^{-}$ cells that co-purify PBMCs correlates with tumor burden. This finding is in agreement with previously published data regarding MDSC levels and tumor burden in mice and humans (53, 103).

$CD11b^{+}CD14^{-}MHCII^{-}$ cells are functionally defined as MDSCs

To test whether the $CD11b^{+}CD14^{-}MHCII^{-}$ subset was able to inhibit T cell function, we conducted a series of co-culture experiments. Purified $CD11b^{+}CD14^{-}MHCII^{-}$ cells from three different subtypes of cancer were co-cultured with autologous or healthy responder PBMCs. In all cases, $CD11b^{+}CD14^{-}MHCII^{-}$ cells exhibited a potent ability to suppress proliferative responses in a dose-dependent manner. Representative examples of proliferative suppression are shown using samples from a dog with tonsillar squamous cell carcinoma (Fig. 3A) and prostatic adenocarcinoma (Fig. 3B).

In order to determine if suppression was an artifact of using responders from tumor-bearing dogs, we assayed for proliferative suppression using normal

responders. The addition of $CD11b^{+}CD14^{-}MHCII^{-}$ cells, but not normal PMNs, impaired the proliferation of PBMCs from healthy dogs (Fig. 3C). Moreover, the amount of IFN-γ secretion was assessed in the conditioned medium from these co-cultures, revealing that $CD11b^{+}CD14^{-}MHCII^{-}$ cells, but not normal PMNs, suppressed the secretion of IFN-γ (Fig. 3D).

$CD11b^{+}CD14^{-}MHCII^{-}$ cells suppress both $CD4^{+}$ and $CD8^{+}$ T cells

To further interrogate the direct effect on T lymphocytes, purified $CD11b^{+}CD14^{-}MHCII^{-}$ cells from a dog with osteosarcoma were co-cultured with purified $CD4^{+}$ and $CD8^{+}$T cells from a healthy dog for 72 h. Non-stimulated cells and $CD4^{+}$ and $CD8^{+}$cells co-incubated with healthy PBMCs were used as controls. As expected, $CD11b^{+}CD14^{-}MHCII^{-}$ cells inhibited the proliferation of $CD8^{+}$ (Fig. 4A) and $CD4^{+}$T cells (Fig. 4B) while PBMCs from a normal dog did not. Taken together, these data demonstrate that $CD11b^{+}CD14^{-}MHCII^{-}$ cells are indeed functionally defined as canine MDSCs.

$CD11b^{+}CD14^{-}MHCII^{-}$ cells express hallmark MDSC-derived immunosuppressive factors

It has been shown that MDSCs can inhibit T cell function by the production of soluble factors such as arginase-1, reactive oxygen species, nitric oxide, and TGF- β (8–10). In order to assess whether $CD11b^{+}CD14^{-}MHCII^{-}$ cells from dogs with cancer could possibly utilize these mechanisms to mediate T cell suppression, we evaluated the expression of ARG1 and iNOS2, as well as the

immunosuppressive cytokines TGF- β and IL-10, within this cell population and from PMNs isolated from peripheral blood of healthy dogs. PCR analysis of RNA extracted from FACS isolated $CD11b^{+}CD14^{-}MHCII^{-}$cells confirmed the expression of ARG-1, iNOS2 enzymes and immunosuppressive cytokines TGF-β and IL-10 mRNA (Fig. 5A). In contrast, normal dog PMNs did not express ARG1, although iNOS, TGF- β and IL-10 mRNA were detectable (Fig. 5B). Because mRNA for ARG-1, iNOS2, TGF-β and IL-10 were all found, we conclude that these factors could play a role in the inhibition of T cell proliferation and effector function. However, since PMNs isolated from healthy dogs did not express detectable ARG-1 mRNA or impair T cell function, suggesting that ARG-1 may be a tumor-induced mechanism that MDSCs could employ for T cell suppression. This finding was not unexpected and has been previously documented in human MDSC studies (59).

Discussion

The field of comparative oncology shows great promise to advance the development of novel therapeutics for pet dogs and human patients alike. However, the paucity of reagents and poorly defined immunophenotype of canine leukocytes has restrained our ability to understand tumor immunology in dogs with naturally occurring cancer.

Our data demonstrates the existence of MDSCs in the peripheral blood of dogs, which are elevated in all types of advanced or metastatic cancer analyzed compared to early stage non-metastatic cancer and healthy controls. With this

basic foundation of knowledge in place, it will now be possible to prospectively monitor MDSC burden in dogs treated with experimental drugs and immunotherapy.

The $CD11b^{+}CD14^{-}MHCII^{-}$ cell population that we defined as MDSC co-purified with PBMCs, had polymorphonuclear granulocytic morphology, suppressed T cell proliferation and effector function, expressed hallmark suppressive factors of human MDSC, and positively correlated with tumor burden. Proliferation assays revealed relatively weak proliferation in PBMCs from tumor-bearing dogs (Fig. 3A,B) compared to normal responders (Fig. 3C) in the absence of exogenous MDSC. This likely reflects elevated levels of endogenous (not experimentally added) MDSCs and regulatory T cells in the PBMCs from dogs with cancer. Furthermore, it is crucial to note that a second subset of MDSC that is more monocytic in nature is widely appreciated in murine and human tumor immunology. We found no evidence for selective expansion of a $CD14^{+}$monocyte-like cell in the blood of dogs with cancer. However, $CD11b^{+}MHCII^{-}$ cells that were purified from dogs with advanced cancer that were also $CD14^{+}$ potently inhibited T cell proliferation (Figure S2), revealing that although monocytic MDSC are not a dominant population in dogs with cancer, they are indeed present. This finding of preferential expansion of granulocytic MDSC is not surprising and is in agreement with similar studies carried out in murine tumor models (101).

Overall, our data are consistent with a global state of immune suppression in dogs with advanced cancer that is likely attributable to several mechanisms.

The practical deliverable of this study is a simple three marker surface immunophenotype that can be used to prospectively monitor MDSC burden in dogs. We have performed pilot studies to look for additional markers. Specific preliminary results that are worth noting are as follows. We have been unable to demonstrate successful staining using anti-human CD66b antibodies. CD66b is an activation marker expressed on some human MDSC (59). The most widely used marker for MDSC in the mouse is Gr-1, and an antibody against mouse Gr-1 was demonstrated to have cross-reactivity with canine cells, as does anti-mouse CD11b (Figure S1). Further studies will be required to determine if canine cells that are identified by anti-mouse Gr-1 and CD11b antibodies are indeed MDSCs.

One potential limitation of this study that many of the samples we analyzed were frozen, the thawed before analysis, which could have influenced cell viability. However, freeze-thaw did not significantly affect cell viability of either granulocytic or monocytic MDSC (Figure S3). We consider this a positive finding because canine MDSCs could be frozen from multiple time points in future prospective studies, then thawed and analyzed simultaneously to limit batch to batch variability. A second limitation is that the RTPCR analysis of immunosuppressive molecules was qualitative, was performed on a small number of dogs (Table S3), and was not a direct comparison to matched healthy cells. We were not able to obtain adequate viable $CD11b^{+}CD14^{-}MHCII^{-}$ cells from healthy dogs by FACS to directly compare to the same population from dogs with cancer due to their low frequency and apparently high rate of cell death following

FACS. For this reason, normal PMNs isolated by gradient centrifugation were used for comparison in our studies. Quantitative mechanistic studies should be conducted to dissect which of the candidate molecules studied herein mediate T cell suppression.

Additionally, some of the dogs had received treatment for their cancer. This is relevant because MDSC levels in human cancer patients have been shown to be influenced by prior therapy. It is also known that tumor burden and inflammation significantly affect circulating MDSC levels. Studies in mice have shown that accumulation and suppressive activity of MDSCs are regulated by the inflammatory milieu (72). Thus treatment, such as surgical excision of the tumor, chemotherapy, and nonsteroidal anti-inflammatory drug (NSAID) administration, can alter the levels of these cells in the peripheral blood. Evaluation of the medical records of dogs in our study revealed that many dogs received some therapy prior to blood sample collection, which could have affected the levels of MDSCs in these samples (see Tables S1 and 2S). However, Figure S4 demonstrates that treatment of dogs with advanced cancer did not significantly alter MDSC burden relative to dogs that had not been previously treated.

Therefore, our study provides evidence that expanded MDSCs are likely a robust, general feature of cancer in canines despite genetic heterogeneity and a range of previous treatments (or lack of previous treatment).

In summary, we have identified a granulocytic subset of cells with immunosuppressive function that are elevated in dogs with advanced cancer that can be characterized as MDSCs. Canine MDSCs may be a potential target for

therapeutic interventions in dogs with cancer. Furthermore, the study of MDSCs in dogs treated with experimental therapies should reveal unique insights into what might be expected in human patients. This cross-species comparison provides an attractive opportunity to move the field of translational medicine forward.

Table1. Characteristics of dogs with cancer in the study

Age (yrs) - Mean (Range)	**9 (2–14)**
Gender	
Male/Neutered	22
Male/Intact	2
Female/Spayed	21
Processed Samples	
Fresh	21
Frozen	24
Breed	
Labrador Retriever	12
Mixed Breed	5
Golden Retriever	3
Greyhound	2
Boxer	2
Border Collie	2
Beagle	2
Scottish Terrier	1
Bull Mastiff	1
Rottweiler	1
Dalmatian	1
Great Dane	1
Bernese Mountain Dog	1
German Wirehaired Pointer	1
German Shepherd Dog	1
West Highland White Terrier	1
Gordon Setter	1
Weimaraner	1
Rhodesian Ridgeback	1
Rat Terrier	1
Newfoundland	1
Miniature Poodle	1
Chow Chow	1
English Springer Spaniel	1
Total	45

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Table 2. Characteristics of healthy dogs in the study

Age (yrs) - Mean (Range)	**8 (2–13)**
Gender	
Male/Neutered	7
Male/Intact	1
Female/Intact	2
Female/Spayed	8
Processed Samples	
Fresh	6
Frozen	12
Breed	
Labrador Retriever	4
Golden Retriever	2
English Setter	1
Shih Tzu	2
Mixed Breed	2
German Shepherd dog	1
German Wirehaired Pointer	1
Red Tick Hound	1
Poodle	1
Cocker Spaniel	1
Catahoula Hound mix	1
Greyhound	1
Total	18

doi:10.1371/journal.pone.0033274.t002

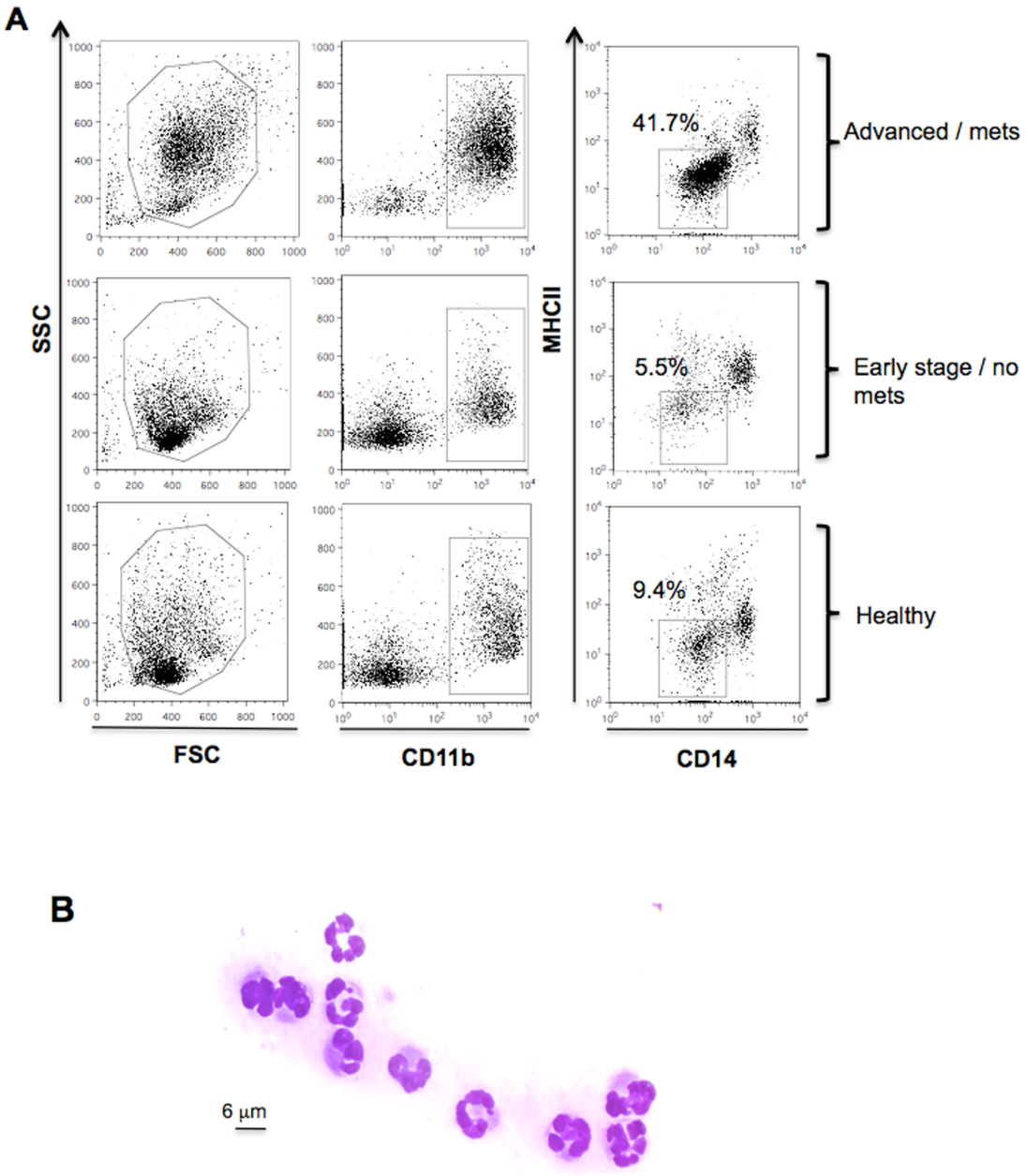


Figure 1. Immunophenotyping gating strategy and morphological analysis for MDSC identification in peripheral blood of dogs. PBMCs from healthy dogs and dogs with cancer were stained for the myeloid marker CD11b, monocytic marker CD14 and MHC II. (A) Representative flow cytometric analysis of forward and side scatter and gated $CD11b^{+}CD14^{-}MHCII^{-}$ cells from dogs with advanced or metastatic tumors compared to dogs with early stage non-metastatic tumors and healthy control dogs. Plots are representative of dog with advanced metastatic hemangiosarcoma (top), early stage bladder transitional cell carcinoma (middle) and a healthy dog. (B) FACS sorted $CD11b^{+}CD14^{-}MHCII^{-}$ cells were stained with diff-quick for cell morphology evaluation. A representative example of polymorphonuclear granulocyte morphology of $CD11b^{+}CD14^{-}MHCII^{-}$ cells is shown at 63× magnification.

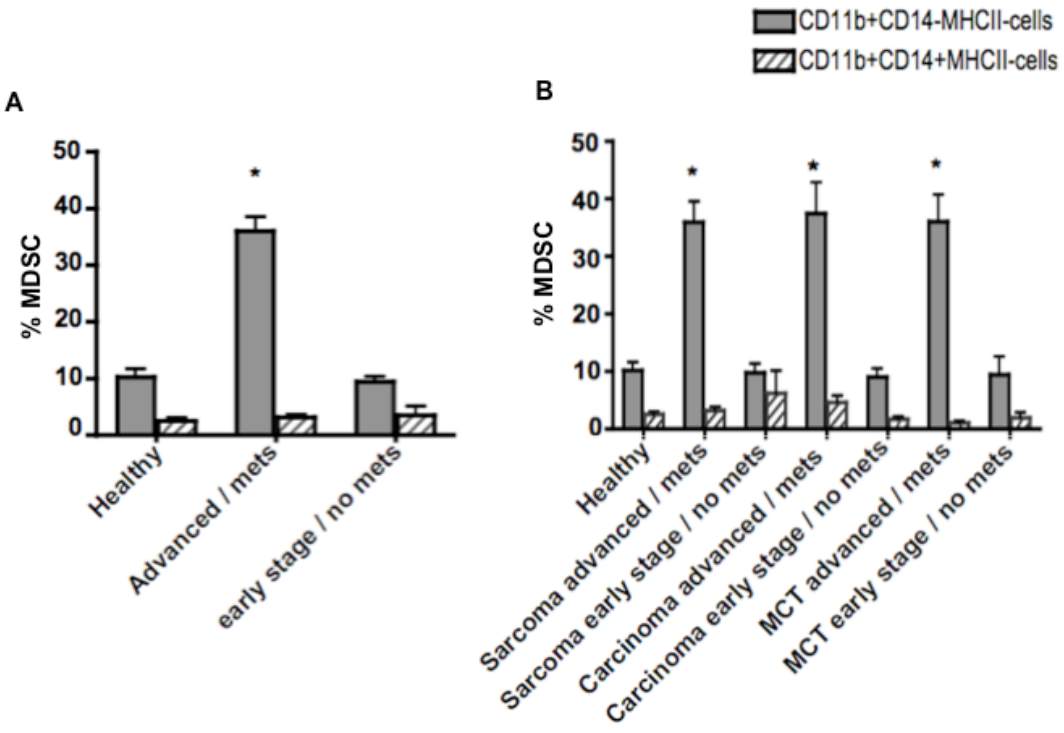


Figure 2. Percentage of circulating $CD11b^{+}CD14^{-}MHCII^{-}$ cells in dogs with cancer correlates with clinical tumor stage. (A) Analysis of average $CD11^{+}CD14^{-}MHCII^{-}$ population frequency in dogs with advanced stage or metastatic tumors (n = 30) compared with early stage non-metastatic tumors (n = 15) and control dogs (n = 18). There was a significantly higher percentage of $CD11b^{+}CD14^{-}MHCII^{-}$ cells in dogs with advanced cancer versus early stage non-metastatic tumors and healthy dogs (36.04% vs. 9.40% and10.24%, respectively. B) Average $CD11b^{+}CD14^{-}MHCII^{-}$ population frequency in the major cancer subtypes: advanced stage or metastatic sarcomas (n = 18), early stage non-metastatic sarcomas (n = 6), advanced stage or metastatic carcinomas (n = 7) early stage non-metastatic carcinomas (n = 7), advanced stage or metastatic mast cell tumors (n = 5) and early stage non-metastatic mast cell tumors (n = 2) compared with control dogs (n = 18). Significantly elevated percentages were detected in all advanced tumors subtypes relative to early stage tumors and healthy dogs. Percentages of $CD11b^{+}CD14^{+}MHCII^{-}$ cells were not significant between groups (* indicates $P<0.001$). Mean ± SEM are shown.

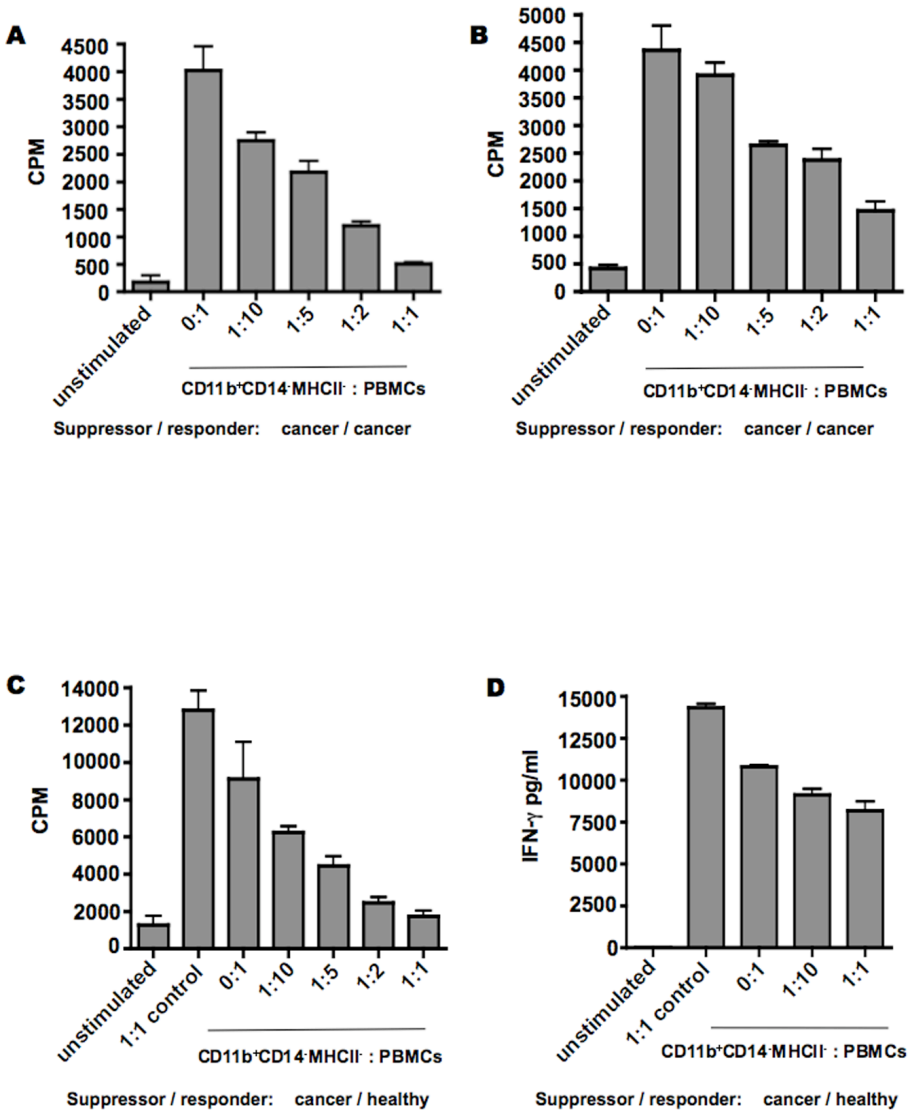


Figure 3. $CD11b^{+}CD14^{-}MHCII^{-}$ cells suppress T cell proliferation and cytokine elaboration. $CD11b^{+}CD14^{-}MHCII^{-}$ cells were sorted from peripheral blood sample of dogs with cancer and then co-cultured with autologous PBMCs (A, B) or healthy dog PBMCs (C) in the presence of mitogen for 72 hs. Representative examples from a total of eight dogs are shown. The graphs represent proliferative responses after addition of $CD11b^{+}CD14^{-}MHCII^{-}$ isolated from a single dog with squamous cell carcinoma (3A), prostatic adenocarcinoma (3B) and osteosarcoma (3C). Non-stimulated PBMCs were used as negative control and PBMCs stimulated in absence of $CD11b^{+}CD14^{-}MHCII^{-}$ cells were used as positive control for proliferation. PBMCs were also co-incubated with PMNs, to control for presence of additional cells (3C, 3D). Proliferative responses were measured by ^{3}H-thymidine incorporation. CPM, counts per minute. Amount of IFN-γ secretion in the co-culture was determined using canine specific IFN-γ ELISA assay (3D). All experiments were performed in triplicate. Mean ± SEM are shown.

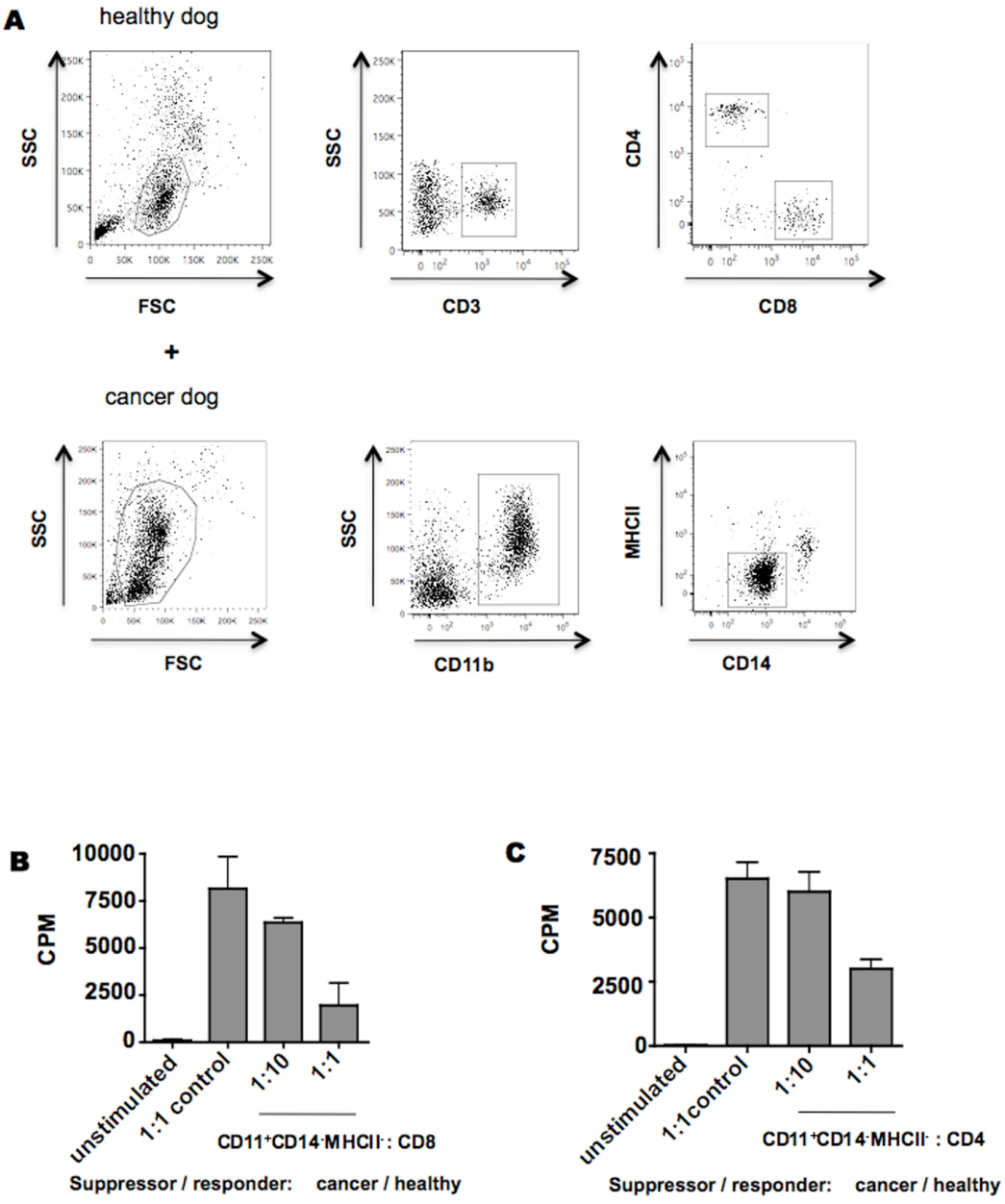


Figure 4. CD11b^{+}CD14^{-}MHCII^{-} cells suppress T cell proliferation.
Facs sorted CD11b^{+}CD14^{-}MHCII^{-} cells isolated from a dog with osteosarcoma or healthy PBMCs were co-incubated with mitogen-stimulated CD4^{+} and CD8^{+} T cells isolated from a healthy dog for 72 hrs. No stimulated cells were used as negative control. Proliferative responses were measured by ^{3}H-thymidine incorporation from experiments performed in triplicate. CPM, counts per minute. Mean ± SEM are shown.

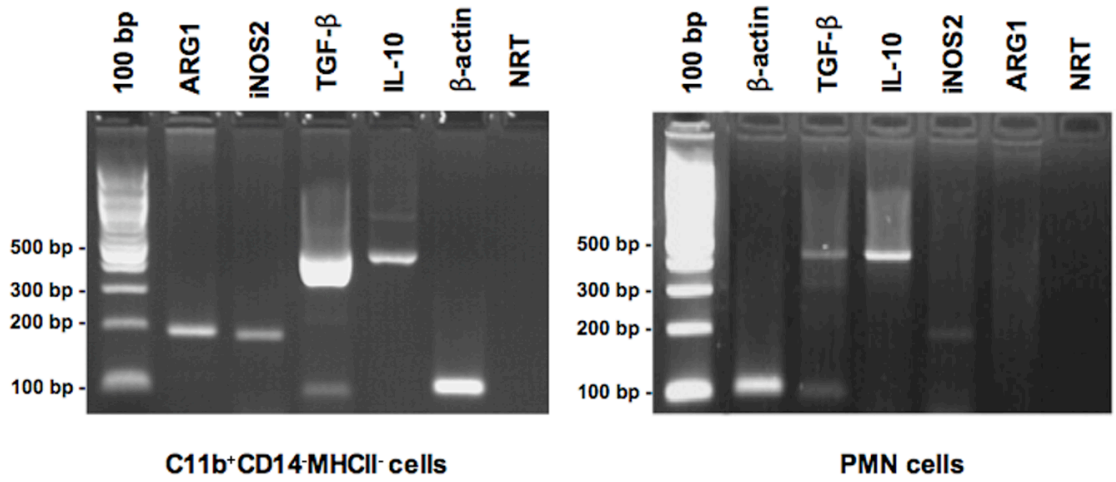


Figure 5. CD11b^{+}CD14^{-}MHCII^{-} cells express MDSC-derived immunosuppressive factors.
RT-PCR analysis of FACS purified CD11b^{+}CD14^{-}MHCII^{-} cells detected expression of ARG1 and iNOS2, as well TGF-β and IL-10 immunosuppressive cytokines. ARG-1 expression was not detected in normal PMNs. CD11b^{+}CD14^{-}MHCII^{-} cells were isolated from the peripheral blood of a dog with osteosarcoma and PMNs were isolated from a healthy dog. NRT, RNA template in the absence of reverse transcriptase. Results are representative three experiments.

Supporting Information

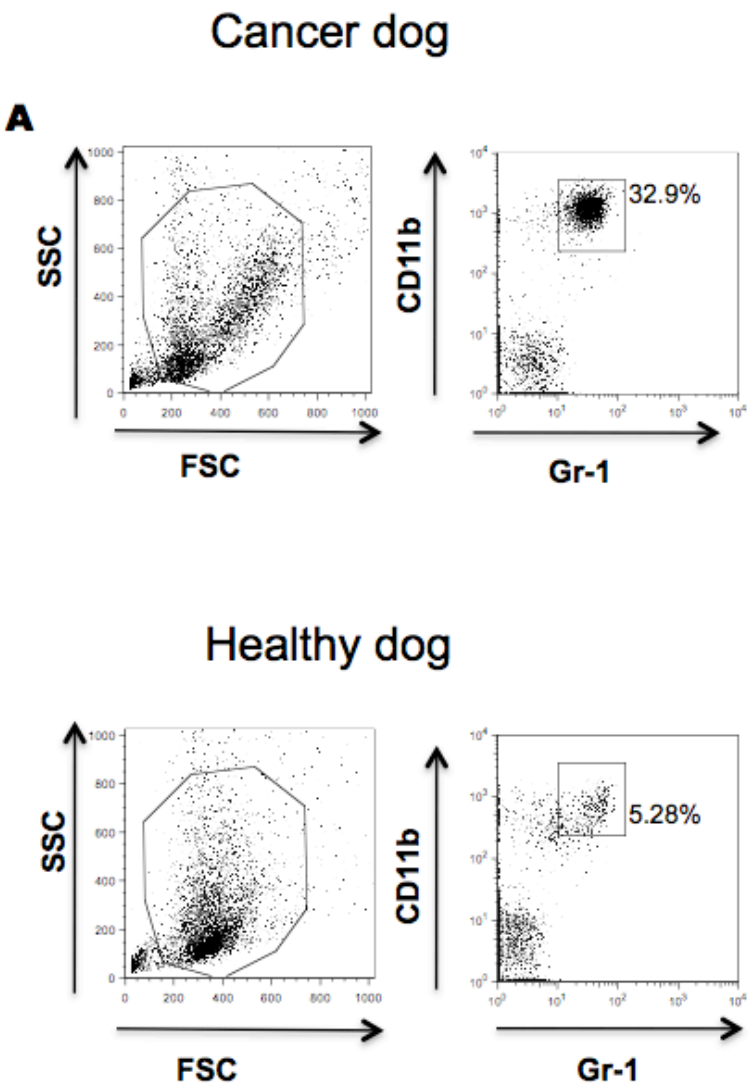


Figure S1. Mouse anti-CD11b and Gr-1 antibodies cross-react with canine samples. Fresh PBMCs from healthy dog and cancer patients were isolated by Ficoll, stained with anti-mouse CD11b and anti-mouse Gr-1 antibodies.

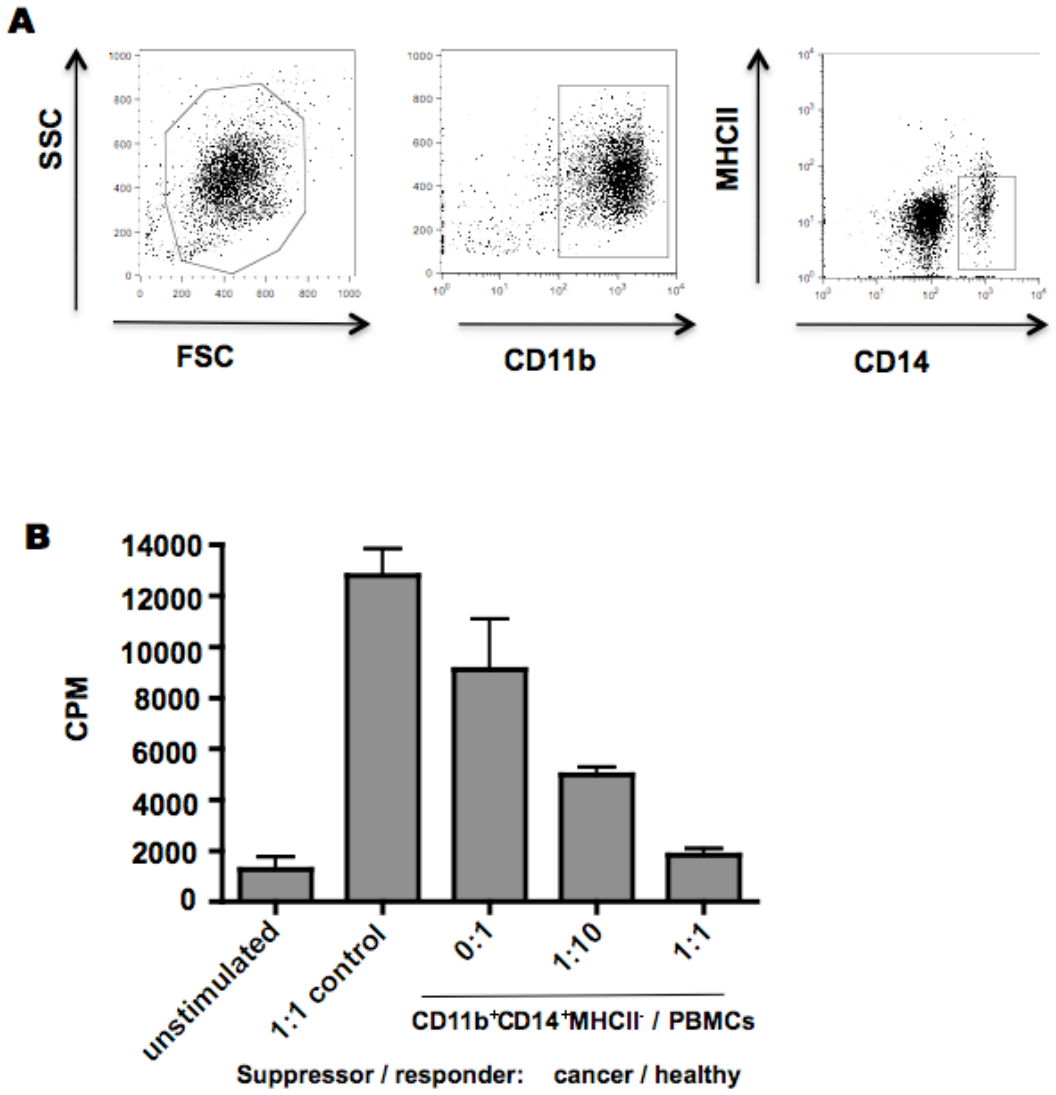


Figure S2. CD11b^{+}CD14^{+}MHCII^{-} cells demonstrate ability to suppress T cell proliferation. (A) CD11b^{+}CD14^{+}MHCII^{-} cells were sorted from peripheral blood sample of an osteosarcoma dog (B) and co-cultured with healthy dog PBMCs in the presence of mitogen for 72 hrs. Non-stimulated PBMCs were used as negative control and PBMCs co-cultured with healthy PMNs were used to control for the effect of adding cells to the assay. Proliferative responses were measured by ^{3}H-thymidine incorporation. CPM, counts per minute. The experiment was performed in triplicate. Mean ± SEM are shown.

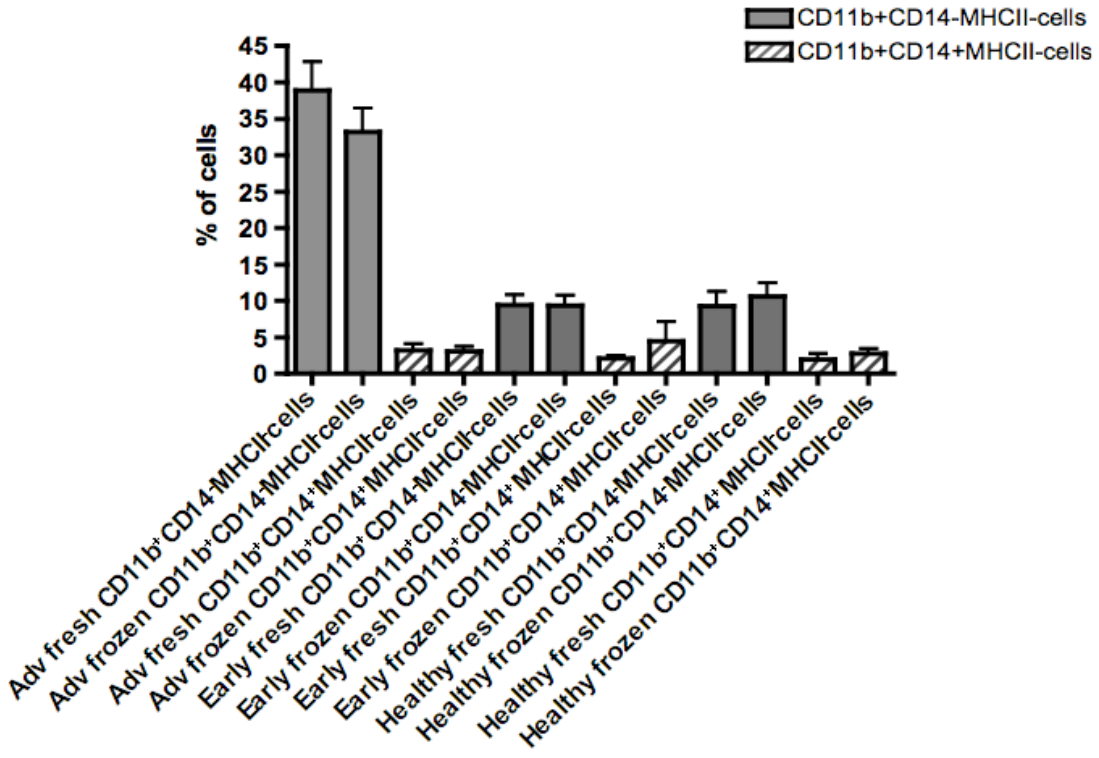


Figure S3. Frequency of MDSCs measured was not significantly altered by cryopreservation. MDSC percentages in fresh and frozen samples were assessed for comparison. Mean ±SEM are shown.

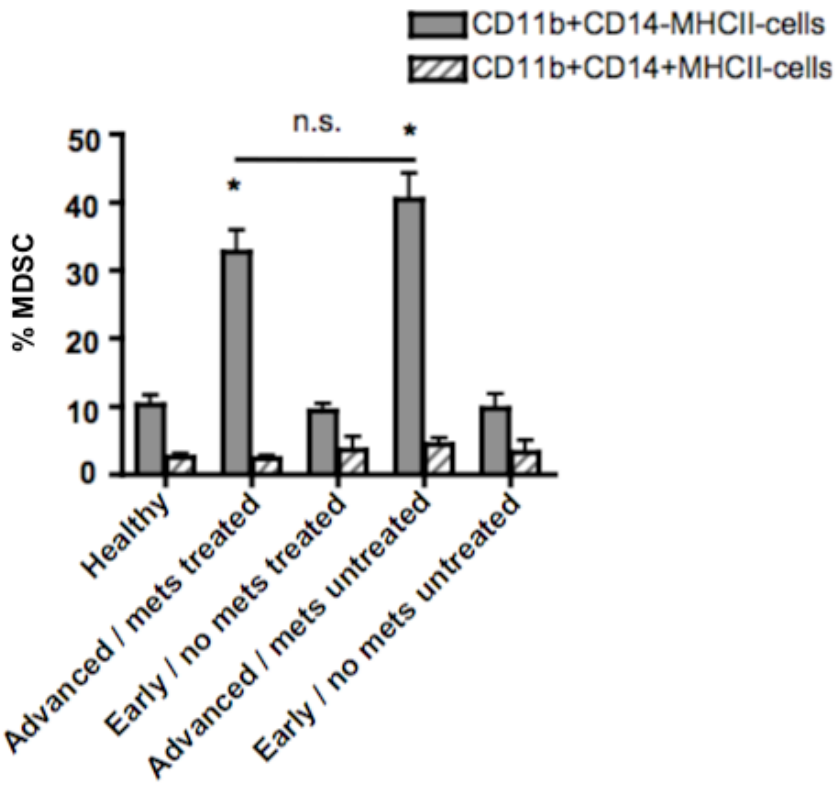


Figure S4. No significant effect of pretreatment on MDSC burden. Analysis of the average $CD11b^{+}CD14^{-}MHCII^{-}$ population frequency in treated (n = 17) or untreated dogs with advanced stage or metastatic tumors (n = 13) compared to control dogs (n = 18). There was a significantly higher percentage of $CD11b^{+}CD14^{-}MHCII^{-}$ cells in dogs with advanced cancer treated or untreated compared to healthy dogs (32.69±3.24%, 40.42±3.86% vs. 10.24±1.412%, respectively). N.S., not statistically significant (there was no significant difference between samples that had been treated compared to those from untreated samples). Mean ± SEM are shown (* indicates P<0.0001).

Table S1. Summary data for dogs with advanced stage or metastatic tumors

	Sample	Age / Gender / Breed	Tumor Type	Tumor Characteristics	Metastasis	% of $CD11^+$ $CD14^-$ $MHCII^-$	% of $CD11^+$ $CD14^+$ $MHCII^-$	Treatment
1	fresh	7y FS Labrador Retriever	OSA	Necrosis and venous tumor embolus, High grade	No	29.6	0.94	NSAID, T
2	fresh	8y FS Newfoundland	OSA	Lytic bone lesion	No	39	2.13	NSAID, T
3	frozen	10y FS Greyhound	OSA ∓	Lytic bone lesion	Yes. LG	19.1	5.66	Su, NSAID
4	frozen	7y MN Lab Retriever	OSA	Lytic bone lesion	No	11.7	2.99	NSAID, T
5	fresh	4y FS Mixed Breed	OSA	Ribs masses and pleural effusion	Yes. LG	50.5	7.26	NSAID, T
6	fresh	4y MN Mixed Breed	OSA	Mixed lytic proliferative bone lesion	No	39.9	1.51	NSAID, T
7	frozen	6y MN Greyhound	OSA	Mixed lytic proliferative bone lesion	No	20.6	0.99	NSAID, T
8	fresh	11y MN Golden Retriever	OSA	Mixed lytic proliferative bone lesion, severe destruction	Yes. LN	22	2.25	T
9	fresh	13y FS Lab. Retriever	HSA	Skeletal, lytic bone lesion, fracture	No	52,4	0.46	No
10	frozen	12y MN Border Collie	HSA	Oral	Yes. L, BL	39.3	2.25	No
11	frozen	8y MN Lab. Retriever	HSA ∓	Skeletal, large edema, serosanguineous. fluid	Yes. L, LG,S	41.7	1.98	Su
12	frozen	10y FS Boxer	HSA ∓	Splenic nodules	Yes. L	51.9	2.45	Su, NSAID
13	frozen	9y MN Mixed Breed	HSA ∓	Splenic, mast cell tumor	Yes. L, M	13.1	3.77	Su, CH (D,C)
14	fresh	10y MN Golden Retriever	HSA	Splenic multiple nodules	No	62	10.1	No
15	frozen	11y MI Rat Terrier	SCS ∓	Masses on axillary region/scapula and chest, rapid regrowth	Yes	42.1	1.02	Su
16	frozen	8y MN Bernese Mountain dog	Hist.Sarc ∓	Lung nodules	Yes. LG	52.8	1.36	NSAID, CH (D, C, Lo)
17	frozen	2y MI Golden Retriever	Hist.Sarc	Tumor extent from orbit through hard palate	possibly	30.9	1.31	No
18	frozen	12y FS Lab. Retriever	Soft T.Sarc	Multiple masses	Yes	20.3	8.96	No
19	fresh	10y MN Border Collie	SCC	Tonsillar, large neck mass	Yes. N	39.9	4.36	No
20	fresh	10y MN Scottish Terrier	AC	Prostatic, TCC bladder	Yes. L	19.9	1.52	NSAID
21	fresh	7y MN Bull Mastiff	AC	Thyroid, large mass on left side, small on right side	No	32.9	4.3	No
22	fresh	13y MN Mixed Breed	AC	Hepatocellular (large mass), bladder leiomyoma	No	29.1	0.82	No
23	frozen	9y MN English Spring Spaniel	AC	Anal sac, lymphadenopathy, hypercalcemia of malignancy	Yes. LN, LG	40	4.64	NSAID
24	fresh	5y MN Great Dane	AC	Thyroid, rapid growth with invasion, destruction of underlying bone	Yes. B	65.9	9.26	No
25	frozen	7y FS Dalmatian	AC	Mammary, multiple nodules	No	34.4	7.63	No
26	frozen	10y FS Rottweiler	MCT	Iliac and inguinal lymph node nodules	Yes. LN	29.1	1.21	AH
27	frozen	10y FS Lab. Retriever	MCT	Multiple nodes	Yes	32.8	0.6	AH
28	frozen	7y FS Boxer	MCT	Multiple nodes	Yes	38.2	0.6	No
29	fresh	9y FS German W. Pointer	MCT	Multiple nodes	Yes	53.1	2.42	No
30	fresh	10y FS Lab. Retriever	MCT	Multiple tumor	Yes. LN	27.1	0.47	No

∓ Recurrence after incompletely excised

FS, female spayed; MN, male neutered; MI, male intact; OSA, osteosarcoma; HSA, hemangiosarcoma; SCS, spindle cell sarcoma; Hist. Sarc, histiocytic sarcoma; Soft T.Sarc, soft tissue sarcoma; SCC, spindle cell carcinoma; AC, adenocarcinoma; TCC, transitional cell carcinoma; L, liver; LG, lung; BL, bladder; S, spleen; M, mesentery; B, bone; NSAID, non-steroidal anti-inflammatory drug; T, tramadol; Su, surgery; CH, chemotherapy; D, doxorubicin; C, cyclophosphamide; Lo, lomustine; AH, antihistamine

Table S2. Summary Data for Dogs with Early Stage non-metastatic tumors

	Sample	Age / Gender /Breed	Tumor type	Tumor Characteristics	Metastasis	% of $CD11b^{+}$ $CD14^{-}$ $MHCII^{-}$	% of $CD11b^{+}$ $CD14^{+}$ $MHCII^{-}$	Treatment
1	fresh	10y FS Labrador Retriever	Osteosarcoma	Moderate bone lysis distal radius/ulna	No	5.77	2.7	NSAID, T
2	frozen	11y FS Labrador Retriever	Spindle Cell Sarcoma ₸	Small masses on the neck	No	13	25	CST, T
3	frozen	8y FS Beagle	Spindle Cell Sarcoma	Carpal pad low grade tumor	No	7.38	0.92	NSAID
4	frozen	14y MN Mixed Breed	Soft Tissue Sarcoma ₸	Low grade tumor dorsal lumbar region	No	14.4	0.73	Su, R, Cs, P
5	frozen	9y MN Labrador Retriever	Histiocytic Sarcoma ₸	Small mass on left hock	No	12.5	6.72	No
6	frozen	8y MN Labrador Retriever	Spindle Cell Sarcoma	Small mass on left hindlimb	No	5.83	1.3	NSAID, T
7	fresh	11y FS Chow Chow	Spindle Cell Carcinoma	Mass on nasal planum	No	13.2	1.37	NSAID, T
8	fresh	13y MN Miniature Poodle	Transitional Cell Carcinoma	Bladder	No	5.36	1.04	NSAID
9	frozen	8y FS West Highland White terrier	Transitional Cell Carcinoma	Bladder, early stage, low grade	No	5.57	1.1	No
10	fresh	12y MN German Shepherd	Adenocarcinoma	Thyroid mass	No	11.1	1.9	No
11	frozen	12y FS Beagle	Adenocarcinoma	Hepatocellular	No	14.4	3.37	CST, P
12	fresh	12y MN Labrador Retriever	Squamous Cell Carcinoma	Small mass on nasal planum	No	8.63	2.87	NSAID, Ca
13	frozen	10y FS Gordon Setter	Transitional Cell Carcinoma	Bladder	No	5.03	0.24	NSAID
14	fresh	7y MN Weimaraner	Mast Cell Tumor ₸	Grade II	No	12.6	2.86	Su
15	frozen	8y FS Rhodesian Ridgeback	Mast Cell Tumor ₸	Grade II	No	6.25	1.05	Su, P

₸ Recurrence after incompletely excised tumor

FS, female spayed; MN, male neutered; NSAID, non-steroidal anti-inflammatory drug; T, tramadol; Su, surgery; CST, corticosteroid; R; radiation; D, doxorubicin; Ca, carboplatin. P, Palladia

Table S3. Table of canine cancer patient samples and the experiments in which the PBMCs were used

Sample Number	Experiment (s)	Figure (s)
Sample 3 - Healthy	Immunophenotyping	1A
Sample 5 - Advanced Stage	Tcell proliferation, cytospin	N.S
Sample 5 - Healthy	Tcell proliferation	N.S
Sample 6 - Advanced Stage	Tcell proliferation, RT-PCR	4, 5
Sample 6 - Healthy	Tcell proliferation, RT-PCR	4, 5
Sample 7 - Early Stage	Tcell proliferation, cytokine elaboration, cytospin	N.S
Sample 8 - Advanced Stage	Tcell proliferation, cytokine elaboration,	3C,3D
Sample 8 - Early Stage	Tcell proliferation, cytokine elaboration	N.S
Sample 9 - Early Stage	Immunophenotyping	1A
Sample 10 - Healthy	Tcell proliferation, cytokine elaboration	N.S
Sample 10 - Advanced Stage	RT-PCR	N.S
Sample 11 - Advanced Stage	Immunophenotyping	1A
Sample 12 - Healthy	Tcell proliferation, cytokine elaboration	3C,3D
Sample 13 - Healthy	RT-PCR, Tcell proliferation, cytokine elaboration	N.S
Sample 14 - Advanced Stage	RT- PCR	N.S
Sample 16 - Advanced Stage	RT- PCR	N.S
Sample 17 - Healthy	RT-PCR	N.S
Sample 18 - Advanced Stage	Immunophenotyping	S1
Sample 18 - Healthy	Tcell proliferation, cytokine elaboration	N.S
Sample 19 - Advanced Stage	Tcell proliferation	3A
Sample 20 - Advanced Stage	Tcell proliferation	3B
Sample 22 - Advanced Stage	Cytospin	1B
Sample 23 - Advanced Stage	RT- PCR	N.S
Sample 29 - Advanced Stage	RT-PCR, cytospin	N.S
Sample 30 - Advanced Stage	Tcell proliferation, cytokine elaboration cytospin	N.S

Table S4. Primer sequences for genes evaluated by semi-quantitative PCR

Gene		Primer Sequence (5'-3')	Size
IL-10	Forward	GTCCCTGCTGGAGGACTTTAAGA	443 bp
	Reverse	TGGTCGGCTCTCCTACATCTCG	
ARG-1	Forward	TGGCCTGCTGGAGAAACTTA	190 bp
	Reverse	CAGCACCAGGCTAGTCCTTC	
INOS-2	Forward	AAGTCCAAGTCTTGTCTGGGAGC	185 bp
	Reverse	TCCTTTGTTACTGCTTCCACCCT	
TGF-β	Forward	AGTTAAAAGCGGAGCAGCATGTGG	434 bp
	Reverse	GATCCTTGCGGAAGTCAATGTAGAGC	
β-actin	Forward	CCAGCAAGGATGAAGATCAAG	100 bp
	Reverse	TCTGCTGGAAGGTGGACAG	

Chapter III

Myeloid-derived suppressor cell accumulation in the peripheral blood of dogs with glioma

Summary

Glioma is a very aggressive and devastating malignancy of the brain that is associated with a dismal prognosis despite the use of aggressive standard treatment protocols. The poor prognosis for patients with high-grade glioma has led to the development of various immunotherapeutic strategies to induce specific immune response against the malignant tumors. Despite encouraging preclinical results, successful responses in human clinical trials have not been achieved in part due to tumor-induced immunosuppression. Systemic immunosuppression and elevated percentages of circulating and tumor-infiltrating MDSC have been reported in patients with malignant gliomas.

Similar to that in humans, malignant glioma in dogs is a disease with very dismal prognosis and survival. Due to the similarities between canine and human gliomas, we hypothesized that gliomas in dogs would demonstrate the same pattern of immunosuppression observed in humans. Therefore we assessed the percentage of MDSC in the peripheral blood of dogs with glioma.

Our results demonstrated that dogs with glioma have elevated percentages of circulating $CD11b^{+}CD14^{+}MHCII^{-}$ cells and elevated levels of serum arginase activity compared to control dogs without glioma. Moreover, increased percentages of $CD11b^{+}CD14^{+}MHCII^{-}$ cells were detected in all of the glioma subtypes analyzed including GBM, oligodendroglioma and astrocytoma. Importantly, we also evaluated the anti-mouse Gr-1 antibody as a potential marker of MDSC in dogs and the results of flow cytometry analysis and IHC

staining demonstrated that the anti-mouse Gr-1 antibody is not suitable for canine species.

Introduction

Glioma is a very aggressive and devastating malignancy of the brain that is associated with dismal prognosis. Despite the use of aggressive therapeutic regimens, median progression-free and survival times remain poor and a cure is still elusive. One hurdle in treating malignant brain tumors is the highly immunosuppressive microenvironment that favors the rapid spread of the glioma cells into the normal brain tissue (104).

The standard of care therapy for malignant gliomas involves surgical excision followed by radiation and chemotherapy. Nevertheless, surgical resection cannot completely eliminate the profusion of infiltrating neoplastic cells. Radiation and chemotherapy affect normal cells in addition to the cancer cells they target and have other limitations, such as the development of resistance by the tumor.

The poor prognosis for patients with high-grade glioma has led to the development of new therapeutic strategies, such as immunotherapy designed specifically to engage the immune system to recognize and attack the tumor cells (105, 106). Several studies have been developed to stimulate tumor-specific T cell responses in murine preclinical models (107-111). However, despite the relative safety and exciting preclinical results immunotherapeutic approaches

have shown, effective anti-tumor immunity against malignant gliomas has not been generated in the clinical setting (106, 112).

It is clear now that immunogenicity is not sufficient for generation of effective antitumor responses, and the negligible improvement in survival of patients treated with immunotherapeutic approaches is likely due an inability to successfully overcome tumor-induced immune suppression (105, 111, 112).

Systemic immunosuppression and defective Tcell immune responses in patients with malignant gliomas are well-documented (113), and significantly high percentages of circulating (50, 114) and tumor-infiltrating MDSC have been recently reported (63).

Similar to that in humans, dogs develop spontaneous brain tumors that carry a very poor prognosis regardless of therapeutic intervention. Due to the genetic, biologic and histological similarities between human and canine glioma, and the fact that these tumors evolve over long periods of time, interacting dynamically with an immunocompetent host, it is possible that canine glioma recapitulates the same mechanisms of resistance observed in human disease (1, 115). Therefore, the examination and quantification of the immune suppressive components in dogs with glioma may provide valuable information of the immunological status of these dogs and offer new insights into why some immunotherapies fail to induce an antitumor response and elicit survival benefit.

Given prior observations of T cell dysfunction and the documentation of elevated percentages of immunosuppressive myeloid cells in glioma patients, the objective of the present study was to evaluate MDSC accumulation in the

peripheral blood of dogs with glioma using the combination of the three surface markers CD11b, MHCII and CD14. In addition, we investigated the use of anti-mouse Gr-1 antibody as a potential marker of MDSC in dogs.

Materials and Methods

Patient Population

Blood samples from 26 canine patients diagnosed with glioma and 10 h control dogs without glioma were included in the study. Control dogs were determined to be healthy based on owner observations and a complete physical examination by a veterinarian. Analysis was performed using blood samples from dogs with newly diagnosed glioma prior to any definitive cancer treatment. All of the canine glioma patients included had a presumptive diagnosis of glioma based on MRI characteristics as well as a definitive histological diagnosis from biopsy tissues by a team of experienced neuropathologists. Blood samples were collected at the University of Minnesota Veterinary Medical Center after obtaining owner consent according to an approved protocol from the Institutional Animal Care and Use Committee guidelines.

Peripheral Blood Mononuclear Cells Isolation

Peripheral blood of dogs diagnosed with glioma and healthy donors were collected in heparinized tubes for peripheral blood mononuclear cells (PBMCs) isolation. Briefly, blood was diluted 1:3 with sterile phosphate-buffered saline (PBS, Invitrogen) and layered over lymphocyte separation medium (Cellgro).

Samples were centrifuged at 400 x g for 30 min. PBMCs were collected from the interface, transferred to a fresh tube and washed twice in PBS. Cells were resuspended in freeze medium containing 10% DMSO (Sigma) in FBS (Invitrogen) and stored in liquid nitrogen. For phenotypic studies, PBMC samples from the canine patients with gliomas were thawed for 2 minutes in a 37°C water bath before being stained with antibodies and analyzed by flow cytometry. PBMCs collected from the control dogs were isolated as described above but rather than storing frozen, were resuspended in FACS buffer, stained with antibodies and immediately analyzed by flow cytometry.

Serum samples

Peripheral blood of dogs with glioma was collected into red-topped clotting tubes for serum isolation. The blood tubes were centrifuged at 2500 x g for 5 min, the serum was decanted, 200 µl aliquots were placed into cryovials and stored in a -80°C freezer.

Flow Cytometric Analysis of patient PBMCs

For the analysis of patient PBMCs for MDSC, samples were thawed, washed in RPMI and resuspended in FACS buffer. Non-specific antibody binding was blocked by pretreatment of cells with 10mg/mL of canine gamma-globulin (Jackson Immunoresearch) for 20 min at room temperature. Cells were stained with anti-mouse PE conjugated CD11b (clone M1/70 eBioscience), anti-human Alexa fluor 647-conjugated CD14 (clone TU¨ K4, AbD Serotec) and the anti-dog

FITC-conjugated MHCII (clone YKIX334.2, AbD Serotec) at 4°C for 30 min in a dark room according to the manufacturer's protocol. Stained cells were washed twice, resuspended in FACS buffer, and incubated for 10 minutes at room temperature in the dark with 7AAD (BD pharmingen) before analysis by flow cytometry. Results are expressed as the percentage of positive cells of the total, live-gated PBMC. Data were acquired using BD FACS Diva LSRII instrument and analyzed using FlowJo software (Tree Star).

Arginase activity assay

Arginase was measured using a chromogen arginase assay kit (Abnova). Briefly, serum samples were thawed, diluted at 1:5 in PBS, mixed with substrate buffer and manganese solution and incubated for 2h at 37°C. Following the addition of urea reagent, samples were incubated at room temperature for another 20min. Optical density (OD) was measured at a wavelength of 530nm in an iMark Microplate Reader (Bio-Rad). Arginase activity was calculated using the formula [OD sample - OD blank / OD standard – water x 10.4x dilution] Urea solution (1M) was used as the standard. Arginase activity was expressed as units per liter.

Gr-1 antibody staining and cell isolation

For functional analysis, fresh blood samples from tumor-bearing dogs were used for isolation of Gr-1$^+$ cells. Cells were magnetically labeled and isolated using the Myeloid-Derived Suppressor Cell Isolation Kit and LS magnetic

columns (Miltenyi Biotech) in accordance with the manufacturer's instructions. For flow cytometry analysis, samples were stained with anti-mouse PE conjugated CD11b (clone M1/70 eBioscience) and anti-mouse APC-conjugated Gr-1 (clone RB6-8C5 eBioscience) antibodies or isotype controls at 4°C for 30 min in a dark room according to manufacturer's protocol.

Ex Vivo Proliferation

PBMCs from dogs with glioma and control dogs were first labeled using CellTrace Violet Cell Proliferation Kit (Invitrogen Molecular probes) according to the manufacturer's instructions and seeded into U-bottom 96-well plates (1×10^5cells/well) in RPMI 1640 medium containing L-arginine (150 μM) (Invitrogen) supplemented with penicillin/streptomycin (Invitrogen) and 10% heat-inactivated fetal bovine serum (Invitrogen) at 37°C in a 5% CO_2 incubator. Cells stained with Gr-1 antibody were isolated from dogs with cancer as previously described and added at a ratio of 2:1 to cancer (autologous) or healthy responder PBMCs. Concanavalin A (2μg/ml) (Sigma) was used to stimulate T cell proliferation. Non-stimulated PBMCs were used as negative control. Proliferation of T cells was assayed by flow cytometry of dilutions of the CellTrace dye after 72hrs in culture. Acquired data were analyzed using FlowJo (Tree Star).

Immunohistochemistry (IHC)

For analysis of Gr-1 expression in canine tissues, 4μm formalin-fixed, paraffin-embedded sections of tissue were deparaffinized and rehydrated,

followed by pepsin treatment for 15 minutes at room temperature. Immunohistochemistry for Gr-1 was performed on a Dako Autostainer using rat anti mouse Gr-1 monoclonal antibodies (1A8 and RB6-8C5 clones) as the primary antibody (after blocking endogenous peroxidase and application of a protein block), detected using a rat-on-mouse HRP-polymer kit (Biocare) with diaminobenzidine (Dako) as the chromogen. Mayer's hematoxylin (Dako) was used as the counterstain. To verify the cross-reactivity of the anti-mouse Gr1 monoclonal antibodies on canine tissues, canine bone marrow and lymph nodes were stained as test tissues. Mouse spleen was used as the positive control tissue, whereas for the negative control, the primary antibody was substituted with rat serum (Biogenex).

Statistical analyses

Statistical analysis was performed using GraphPad Prism v5.0 (GraphPad). The differences between two groups were analyzed using Mann-Whitney test. P values <0.05 were considered to be statistically significant.

Results

Characteristics of the patients in the study

Blood samples from 26 dog patients with newly diagnosed glioma and 10 healthy controls were analyzed in the study. The average ages of dogs with glioma and healthy control dogs were 7.6 years and 6.8 years, respectively.

Boston terriers and Boxers accounted for 54% of the gliomas patients. Among the 26 dogs diagnosed with glioma, there were 11 dogs with glioblastoma multiforme (GBM), 6 dogs with high-grade oligodendroglioma, 4 dogs with anaplastic astrocytoma, 2 dogs with grade II astrocytoma, and 1 dog each with unspecified high-grade glioma, unspecified low-grade glioma and low-grade oligodendroglioma (Table1).

Dogs with glioma have elevated levels of CD11b+CD14+MHCII- cells

Blood samples from dogs with glioma and healthy controls were evaluated by flow cytometry for quantification of the percentages of MDSC. Gating strategy for analysis of granulocytic and monocytic subsets of MDSC is shown in Fig 1.

A significantly increased percentage of circulating $CD11b^{+}CD14^{+}MHCII^{-}$ cells was observed in the blood of glioma patients compared with health control dogs (P=0.0001) (Fig 2). Although not significant, the percentage of $CD11b^{+}CD14^{-}MHCII^{-}$ cells was also elevated. The significant increase in $CD11b^{+}CD14^{+}MHCII^{-}$ cells in the blood of glioma dogs compared with control dogs differs with the findings of our previously published canine MDSC study. In the previous study we detected significant increases in $CD11b^{+}CD14^{-}MHCII^{-}$ cells in the blood of dogs with advanced sarcomas, carcinomas and mast cells tumors relative to healthy controls but no significant differences in the $CD11b^{+}CD14^{+}MHCII^{-}$ population (116) .

We next determined whether there were differences in the percentage distribution of the two MDSC subsets among the subtypes of glioma. For this

analysis we grouped the glioma samples into 3 histological subtypes: GBM (11 dogs), oligodendrogliomas (n=7), astrocytomas (n=6). Two dog samples from the previous analysis were not included in this evaluation because there was no determination of specific subtype. We observed that the percentages of the monocytic MDSC subset were significantly increased in all of tumor subtypes, GBM (P=0.0008), oligodendroglioma (P=0.007) and astrocytoma (P=0.017) compared to healthy controls (Fig 3). The granulocytic population was also elevated comparing to healthy dogs, but only dogs with oligodendroglioma had a significant increase (P=0.0136). These results indicated that dogs with glioma tumors were unique relative to dogs with other types of solid tumors that have been examined with regard to their increase in percentage of the monocytic MDSC population.

Dogs with glioma have elevated levels of serum arginase1

It is known that MDSC can effectively suppress tumor-specific T lymphocyte function by secretion of soluble factors such as the enzymes ARG1 and iNOS2 (39, 41-43, 54, 73, 77, 92, 95). Excessive amounts of ARG1 rapidly reduce L-arginine from the microenvironment and profoundly impair T cell proliferation and function. Since elevated serum arginase activity has been documented in patients with several types of cancer including gliomas (50, 59, 62, 114), we sought to evaluate whether elevated levels of arginase would also be present in the serum of dogs with glioma. Our results demonstrated that, like

that in humans, dogs with glioma have significantly elevated serum arginase activity, possibly due to the greater number of $CD11b^{+}CD14^{+}MHCII^{-}$ cells (Fig 4).

Gr-1 marker in canine cells

MDSCs in mice are characterized by the expression of both myeloid cell markers Gr-1 and CD11b. Although there is no commercially available anti-canine Gr-1 antibody Krol et al study demonstrated that anti-mouse Gr-1 antibody was capable to stain metastatic canine mammary carcinoma tissues (117). In a previous study, we reported that anti-mouse Gr-1antiboby stained canine PBMCs, however we did not validate whether the anti-mouse Gr-1 antibody would specifically stain and identify canine MDSC (116). To address this question, we performed functional assays, immunophenotyping and immunohistochemistry analysis. First, we evaluated the ability of the cells stained with Gr-1 antibody to inhibit proliferation of responder PBMCs following stimulation with ConA. Since we did not have fresh glioma PBMC samples available at the time of this analysis, we used peripheral blood samples from dogs with transitional cell carcinoma and hemangiosarcoma for functional experiments. Our results demonstrated that cells isolated with Gr-1 magnetic beads from a dog with hemangiosarcoma inhibited proliferative responses of autologous and health responders (Fig 5). However, although our results demonstrated that cells isolated from a cancer patient efficiently suppressed

PBMCs proliferation, we observed that the magnetic isolation of cells stained with Gr-1 antibody was not very efficient and only small number of cells were being recovered after positive selection. Based on those results, for immunophenotype analysis we stained canine glioma PBMCs with CD11b and Gr-1 antibodies or isotype controls in the presence or absence of dog gamma-globulin pre-treatment for non-specific antibody blocking. Of note, pre incubation with dog gamma-globulin was not done prior to magnetically labeling the cells. We observed that antibody blocking completely abrogated anti-mouse Gr-1 antibody binding (Fig 6A) on canine cells.

To verify the binding and cross-reactivity of the Gr-1 in canine tissues we used canine bone marrow and lymph node tissue samples, and mouse bone marrow as a positive control. As expected, mouse bone marrow stained positively for Gr-1, however none of the canine tissues were positive for Gr-1, results that differ from those observed by Krol et al. that showed positive mouse Gr-1 antibody staining in canine tissues (117).

We conclude from these results that there is indeed a population of cells capable to suppress immune T cell function in the peripheral blood of dogs with cancer, however anti-mouse Gr-1 antibody is not an appropriated antibody to identify this population of cells. An possible explanation for this finding is that anti-mouse Gr-1 antibody bound in a nonspecific manner to canine cells which allowed us to isolate a small fraction of cells from the PBMC pool. However, due to a weak nonspecific binding to canine epitopes, the isolation procedure was not optimal.

Discussion

Tumor-induced immune suppression is a condition that accompanies tumor progression and occurs in many patients with cancer. Myeloid-derived suppressor cells (MDSC) have emerged as key suppressors of tumor specific T-cell responses in various tumor models and human malignancies becoming the focus of intense study. However MDSC in dogs have not been extensively characterized, so limited information is available.

In this study, we have examined circulating MDSC in 26 canine glioma patients identifying the phenotypes $CD11b^{+}CD14^{-}MHCII^{-}$ for granulocytic and $CD11b^{+}CD14^{+}MHCII^{-}$ cells for monocytic MDSC, as described in our previous study (116). Contrary to the earlier findings, in dogs with a variety of solid tumors, these results showed that dogs with malignant glioma have significantly increased numbers of the $CD11b^{+}CD14^{+}MHCII^{-}$cells, which we identified as the monocytic MDSC population, compared with healthy controls. Moreover, the increase was consistent elevated in all of the glioma subtypes analyzed in the study. Importantly, although we did not observe significant increases in this cell population in the peripheral blood of dogs with solid tumors in our previous study, $CD11b^{+}CD14^{+}MHCII^{-}$cells sorted from those dogs demonstrated ability to inhibit T cell proliferation (116). We also observed an increase in the granulocytic subset $CD11b^{+}CD14^{-}MHCII^{-}$ cells, however, this increase was significant only in the dogs with oligodendroglioma. It is possible that specific tumor types may uniquely upregulate specific MDSC subsets, perhaps by secretion of tumor

derived-factors (43).

The human monocytic $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}$cells have been identified in various human malignancies such as, melanoma, hepatocellular carcinoma, head and neck squamous cell cancers (HNSCC) and glioblastoma (63, 80, 118, 119). Additionally, correlation between expression of IL-4R and suppressive activity in the monocytic MDSC population has been documented in colon, melanoma and glioblastoma cancers (63, 84). Dunddel et al. demonstrated that the $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}$cells elevated in HNSCC patients displayed predominantly mononuclear features, expressed CD33 and CD34 markers and high levels of pSTAT3, ARG1 and ROS. Moreover, although this cell population was elevated in the peripheral blood, lymph nodes and tumor tissues from patients, with higher percentages observed in the tumor tissue and they have the ability to secrete ARG-1 and suppress T cell proliferation (119). Hoechst et al. showed that immune suppression by $CD14^{+}HLA\text{-}DR^{-/low}$cells was mediated through arginase activity and the induction of T Reg cells.

Kohanbash and colleagues recently demonstrated that myeloid cells heavily infiltrate malignant gliomas and the tumor microenvironment contains high levels of GM-CSF, which induces the differentiation of immature myeloid cells into ARG1-producing MDSCs via IL-4Rα signaling (63, 120). Furthermore, these cells express higher levels of TGF-β, ARG-1 and COX-2 and efficiently suppress the proliferation of autologous CD8 T cells and IFN- γ production (63). Rodrigues et al. demonstrated that normal human monocytes exposed to glioma cell-conditioned media acquire myeloid-derived suppressor cell-like properties

(121). However, elevated percentages of the lineage negative, $CD33^+$ $HLA\text{-}DR^-$ and granulocytic $CD33^+$ $HLA\text{-}DR^-CD15^-$ MDSC, have been found in the peripheral blood of patients with glioblastoma (50, 121).

In the present study we found significant increases in arginase activity in the serum of dogs with gliomas compared with healthy controls. Therefore we proposed that the increased arginase activity in these patients, could be due to the increased numbers of $CD11b^+CD14^+MHCII^-$ cells in peripheral blood samples, as was observed in human MDSC studies (63, 80, 118, 119). Although our current understanding of human MDSC is primarily derived from peripheral blood analysis, studies have evaluated the MDSC populations in human tumor tissues and compared them results from these studies indicate that the percentages of tumor infiltrating monocytic MDSC significantly differ from the peripheral blood. Kohanbash et al. demonstrated that infiltrating $CD14^+HLA\text{-}DR^-$ IL-4α monocytes are significantly elevated in the patient GBM tissues compared to circulating PBMCs (63). Comparable results were also found in HNSCC patients, where significantly percentages of $CD14^+HLA\text{-}DR^-$ cells were found in tumor tissue and lymph nodes compared with peripheral blood (119). While isolation and phenotyping of tumor-infiltrating MDSC were beyond the scope of our study, since there was an increase of $CD11b^+CD14^+MHCII^-$cells in peripheral blood of glioma dogs (Fig 2), cells with similar phenotype may also be elevated in the tumor microenvironment.

Although we demonstrated that both $CD11b^+CD14^-MHCII^-$ and $CD11b^+CD14^+MHCII^-$ cells were endowed with suppressive capabilities in

peripheral blood of dogs with advanced sarcomas and carcinomas (116), we acknowledge that is it important to evaluate and confirm the immunosuppressive function of these cells in the context of brain tumors. MDSC phenotypes and suppressive function are likely to depend on tumor-derived factors in the tumor microenvironment, different tumor types could induce the accumulation of different cell subsets.

We were unable to reliably analyze the immunosuppressive function of $CD11b^{+}CD14^{+}MHCII^{-}$ cells in this study because all of the patient samples evaluated were obtained from cryopreserved PBMCs. It is known that blood sample processing and cryopreservation can affect the viability of various blood cells, so functional studies of MDSC should always be performed in fresh blood samples. A recent study demonstrated that cryopreservation significantly impaired MDSC suppressive function and affected the frequency of MDSC subsets in human samples (122). MDSC that were thawed after cryopreservation completely lost the ability to suppress autologous T cells and to express the immunosuppressive markers ARG-1 and ROS. In addition, the granulocytic subset of MDSC was shown to be quite sensitive to the freeze-thaw procedure, while the monocytic appear to not be affected. Therefore it is possible that the frequency of MDSC, and in particular G-MDSC, may be underestimated during retrospective clinical analyses using frozen blood samples due to the sensitivity of this subset to cryopreservation (123).

Interestingly, we did not appreciate significant increases in the granulocytic subsets of MDSC in our canine glioma patients with the exception of

those diagnosed with oligodendroglioma. Therefore, further investigation is needed to determine if the reason for low numbers of G-MDSC in our canine glioma patients is due to the selective impact of cryopreservation on the viability and recovery of these cells or to intrinsic tumor-derived factors that preferentially select for M-MDSC.

We also investigated whether the anti-mouse Gr-1 antibody would cross-react with and be used to identify to immunosuppressive MDSC populations in dogs. Contrary to the results of Krol et al (117) our results demonstrated that anti-mouse Gr-1 antibody failed to specifically stain canine cells and tissues.

In conclusion, we found a significant increase in the frequency of cells with the $CD11b^{+}CD14^{+}MHCII^{-}$ phenotype in the peripheral blood of dogs with glioma, as well, elevated serum arginase activity. On the basis of these findings we proposed that $CD11b^{+}CD14^{+}MHCII$ cells might represent the major immunosuppressive MDSC population in canine glioma patients. Further investigations using fresh blood are required to assess the immunosuppressive function of MDSCs and to determine whether the granulocytic subset, $CD11b^{+}CD14^{-}MHCII^{-}$ is elevated in the peripheral blood of canine glioma patients.

Table 1. Summary data of dogs in the study

	Age - Gender	Breed	Tumor Type	Tumor Grade	% of $CD11b^+$ $CD14^-$ $MHCII^-$	% of $CD11b^+$ $CD14^+$ $MHCII^-$
1	10y FS	Soft Coat Wheaten Terrier	GBM	IV	44.3	2.9
2	4y MN	Boston Terrier	Anaplastic Oligodendroglioma	III	25	9.23
3	6y MN	Boston Terrier	Anaplastic Astrocytoma	III	19.2	17.4
4	12y FS	Golden Retriever mixed	Gemistocytic Astrocytoma	II	11.7	0.47
5	11y MN	Boston Terrier	GBM	IV	9.43	7.35
6	6y FS	Boston Terrier	GBM	IV	11	13
7	8y MN	Springer Spaniel	GBM	IV	9.5	3.52
8	9y MN	Boxer	GBM	IV	46.8	1.35
9	8y FS	Pembroke Welsh Corgi	Astrocytoma	II	37.6	4.51
10	11y MN	Boston Terrier	Low Grade Glioma	II	19.6	3.11
11	6y MN	Boxer	Anaplastic Oligodendroglioma	III	17.8	1.18
12	7y FS	American Sttafordshire	High Grade Glioma	III	15.9	0.46
13	9y MN	American Sttafordshire	Oligodendroglioma	II	47.8	3.55
14	7y MI	Boxer	GBM	IV	2.4	7.25
15	3y FS	Boston Terrier	GBM - cerebelar	IV	8.89	12.5
16	5y MN	French Bulldog	Anaplastic Oligodendroglioma	III	13.3	19.3
17	13y FS	English Bulldog	Anaplastic Astrocytoma	III	8.15	8.97
18	5y FS	French Bulldog	Anaplastic Astrocytoma	III	47.9	4.41
19	11y FS	Shih-Tzu	GBM	IV	2.87	11
20	6y FS	Boxer	Anaplastic Oligodendroglioma	III	16	16.7
21	7y MN	Boxer	Anaplastic Oligodendroglioma	III	17.8	2.0
22	6y MN	Boxer	GBM	IV	3.8	15
23	1y FI	American Staffordshire	GBM	IV	29.9	0.49
24	10y MN	Boston Terrier	GBM	IV	35.3	0.53
25	7y FS	German Shepherd	Anaplastic Oligodendroglioma	III	12.6	24.2
26	10y FS	Boston Terrier	Anaplastic Astrocytoma	III	5.58	5.11
27	7y MN	Golden Retriever	Healthy	-	6.6	0.19
28	5y FS	Boxer	Healthy	-	2.1	0.05
29	8y MN	Golden Retriever	Healthy	-	15.2	0.35
30	6y FS	Cocker Spaniel	Healthy	-	12.	0.24
31	1y MN	Shih-tzu	Healthy	-	8.6	0.26
32	10y FS	Dachshund Wire-Hair	Healthy	-	13.	0.48
33	9y FS	Pitbull	Healthy	-	14.5	1.99
34	8y FS	Mixed Breed	Healthy	-	3.79	0.048
35	11y MN	Pitbull	Healthy	-	13.4	3.4
36	11y FS	Dogo Argentino	Healthy	-	17.0	0.44

FS, female spayed; MN, male neutered; MI, male intact; FI, female intact; GBM, glioblastoma multiforme;

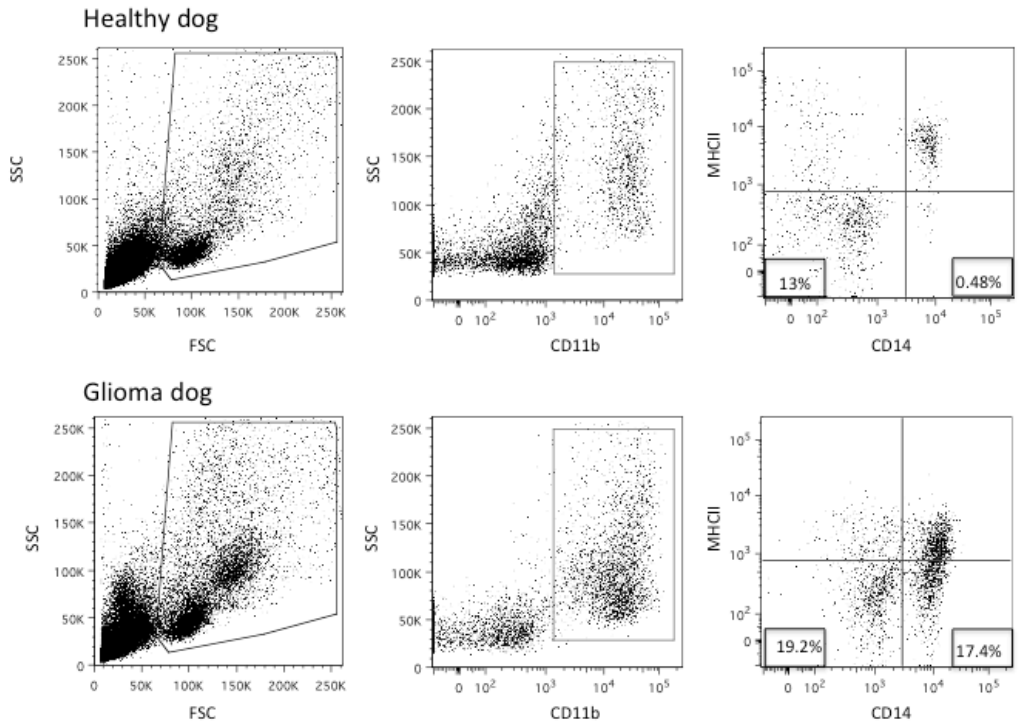


Figure 1. Flow cytometry analysis of circulating MDSC in dogs with glioma. PBMCs from healthy control dogs and dogs with glioma were stained with CD11b, MHCII and CD14 antibodies. Forward and side scatter gate was analyzed for $CD11b^{+}$ cells. Positive CD11b gate was then analyzed by the expression of MHCII and CD14 markers. Top panels are representative of healthy dog while lower panels represent dog with anaplastic astrocytoma.

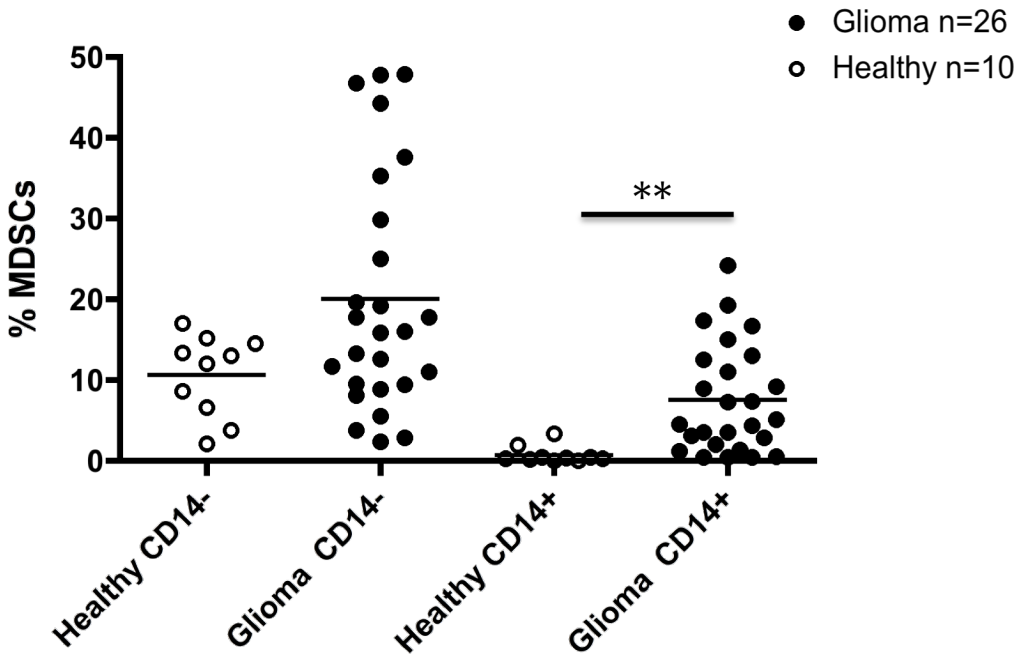


Figure 2. Dogs with glioma have significant increased percentage of $CD11b^{+}CD14^{+}MHCII^{-}$ cells.
Analysis of $CD11b^{+}CD14^{-}MHCII^{-}$ and $CD11b^{+}CD14^{+}MHCII^{-}$ subset percentages in glioma patients and healthy controls. A significantly higher percentage of $CD11b^{+}CD14^{+}MHCII^{-}$ cells were present in the peripheral blood of dogs with glioma. (** indicates $P<0.0001$).

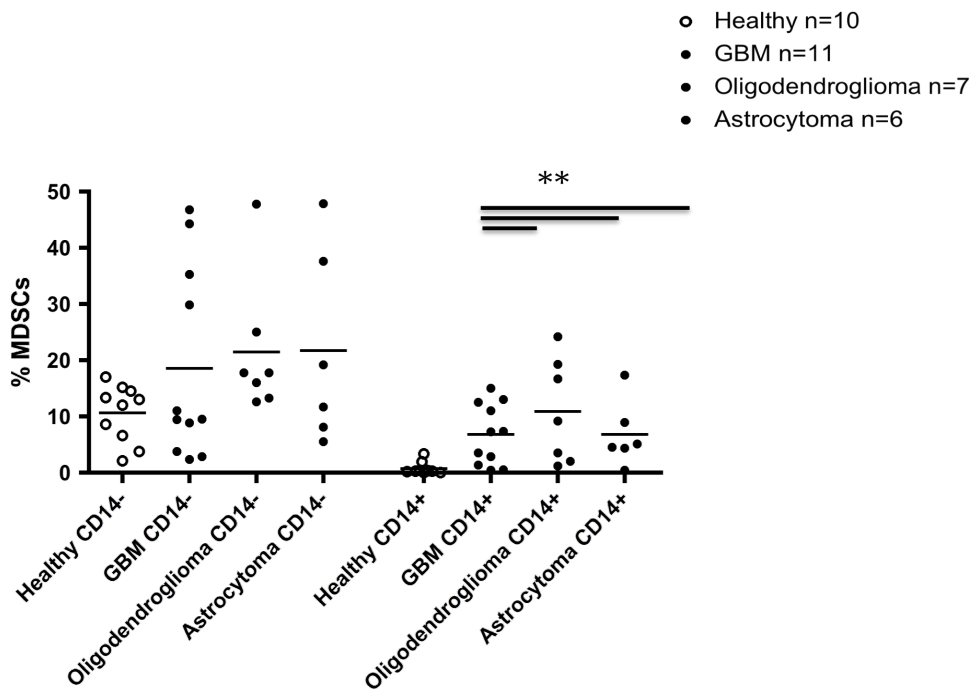


Figure 3. Percentage of CD11b^{+}CD14^{+}MHCII^{-} cells is significantly increased in all of the glioma subtypes.

On the left side of the graph, analysis of the percentages of CD11^{+}CD14^{-}MHCII^{-} cells in the peripheral blood of dogs with GBM, oligodendroglioma and astrocytoma compared to healthy dog controls. On the right, analysis of the percentages of CD11^{+}CD14^{+}MHCII^{-} cells in the peripheral blood of dogs with GBM, oligodendroglioma and astrocytoma compared to healthy dog controls A significantly higher percentage of CD11b^{+}CD14^{+}MHCII^{-} cells were detected in all of the glioma subtypes, GBM ($P<0.0008$), oligodendroglioma ($P<0.0007$) and astrocytoma ($P<0.0017$) compared to controls.

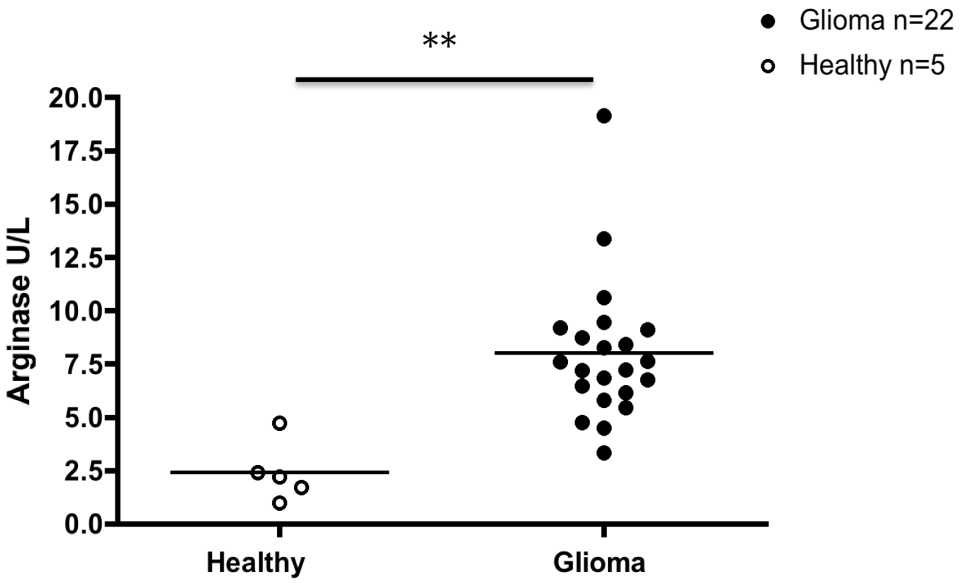


Fig 4. Dogs with glioma have elevated serum arginase activity. Analysis of serum arginase activity in healthy control dogs and canine glioma patients. Serum samples from canine glioma patients had a significantly higher level of arginase activity than healthy controls dogs. (P=0.012).

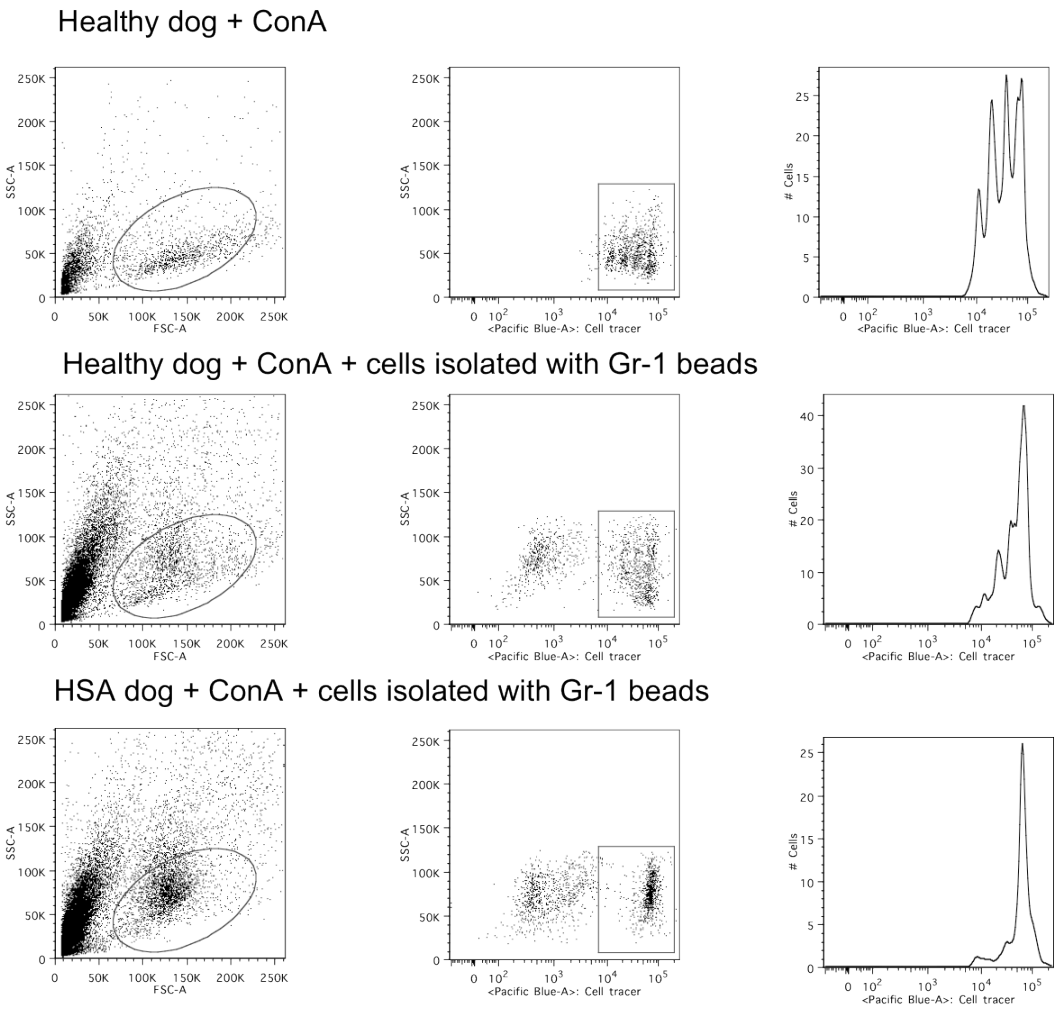


Fig 5. Cells isolated with Gr-1-magnetic coated beads inhibit T cell proliferation in health PBMCs and autologous PBMCs.
Cells were magnetically isolated with Gr-1 beads from peripheral blood sample of dog with HSA and then co-cultured with both autologous or healthy PBMCs labeled with violet dye in the presence of ConA for 72 hrs. Non-stimulated PBMCs were used as negative control. Proliferative responses were measured by dilution of the dye after 72 hrs in culture. The experiment was performed in duplicate.

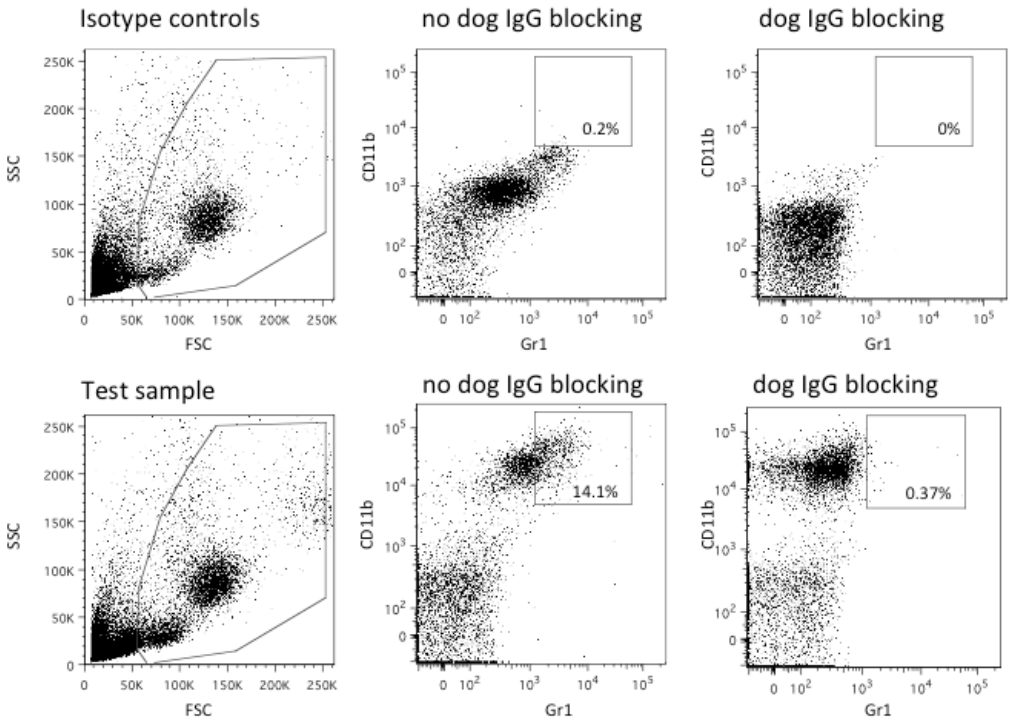
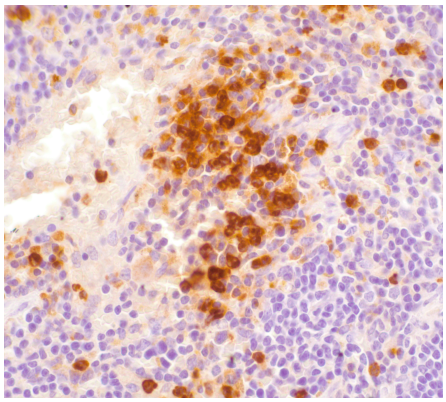
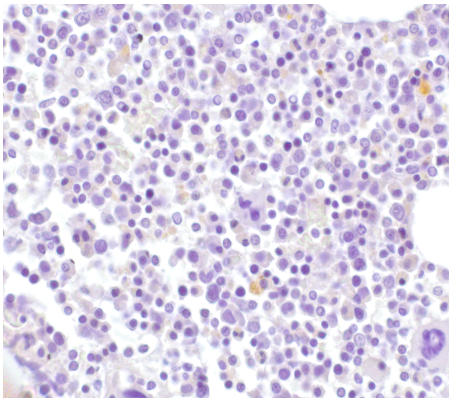


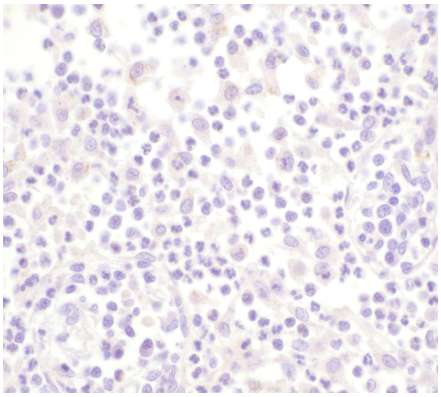
Fig 5. Anti-mouse Gr1 antibody binding of canine PBMCs is non-specific. Representative plots of canine glioma PBMCs after stained with CD11b and Gr1 antibodies or isotype controls with and without canine gamma globulin pre-treatment as antibody blocking. Top panel - cells stained with isotype control. Lower panel - test sample.



Mouse spleen – positive control



Canine bone marrow



Canine lymph

Fig 5. Anti-mouse Gr1 antibody failed to cross-react with canine tissues. IHC analysis of mouse bone marrow (top) canine bone marrow (lower left) and lymph node tissue samples (lower right) stained with anti-mouse Gr-1 antibody.

Chapter IV

DISSERTATION DISCUSSION AND FUTURE DIRECTIONS

Rationale

The study of naturally cancer in pet animals with the comparison to its human counterpart to identify promising treatments that can benefit both humans and animals has greatly increased in the past few years. Due to several similarities with human cancers and the ability to employ comparable therapeutic modalities, spontaneous cancers in companion animals are suited to uniquely contribute to the understanding of cancer pathogenesis, tumor biology and progression, and evaluation of new drugs and therapies (1, 3, 5). Therefore pet dogs with spontaneous cancers constitute a meaningful preclinical model for testing novel immunotherapeutic approaches and are considered outstanding platform for translational research.

The exceptional power of the immune system to identify and destroy specific targets has led to the development of a wide variety of immune-based approaches to treat cancer. However, multiple immunosuppressive mechanisms exist that considerably dampen antitumor responses and weaken the activity of current immunotherapeutic regimens (25, 105, 111). Abnormal accumulations of myeloid derived suppressor cells in cancer patients produce a profound, global immune suppression that significantly interferes with antitumor immunity and consequently impairs the efficacy of immune-based therapies. Therefore, development of effective immunotherapy for both humans and dogs will benefit from further understanding of the mechanisms that drive MDSC expansion and function in these patients.

Although an extensive amount of research has been done to identify and

target MDSC in human cancer patients and rodent model of disease (40, 49, 50, 57, 59-61, 63, 74, 81, 82, 84, 95, 124), research for clinical veterinary patients is still in its infancy. To date, in addition to our publication there are only two other studies describing potential markers to identify MDSC in dogs. Sherger et al. showed that the $CD11b^{low}$ $CADO48A^{low}$ cell population was significantly increased in the peripheral blood of dogs with a variety of tumors, including sarcomas, carcinomas, and melanomas, and that these cells were able to suppress lymphocyte proliferation in vitro (125). Krol et al. identified myeloid precursor cells by immunohistochemistry using the anti-mouse Gr-1 cell surface marker in metastatic and non-metastatic mammary carcinomas tissue samples from dogs (117).

Primary studies in humans documented that MDSC could be identified by double negative labeling for MHCII molecules and any other surface markers of mature lymphoid or myeloid cells, such as CD3, CD19, CD56, CD14 or $Lin^{-/low}$. Further analysis revealed that the double negative population, $Lin^{-/low}$ $HDLA\text{-}DR^{-}$ also express the myeloid markers, CD33 and CD11b (52). Based on this knowledge from human studies, as well as the lack of availability of canine-specific reagents, we decided to use CD11b, CD14 and MHCII surface markers to identify circulating MDSC in canine cancer patients.

Summary of Critical Findings

1. **MDSC accumulate in tumor-bearing hosts.** This finding has been extensively reported by several others in mouse tumor models, as well as

in human patients, where an increased frequency of heterogeneous populations of myeloid cells with variable phenotypes have been detected. We have found significantly elevated percentages of $CD11b^{+}CD14^{-}MHCII^{-}$ cells of the total PBMCs of canine patients with advanced cancer compared to those with early stage, non-metastatic tumors or healthy controls without cancer. Thus we demonstrated that this myeloid cell population accumulates in the peripheral blood of dogs with cancer, and also positively correlates with tumor burden. Our results corroborate published human studies that correlate increased numbers of circulating MDSC correlate with cancer stage and metastatic tumor burden (52). Moreover, this elevated population was consistent among the all tumor types analyzed, sarcomas, carcinomas, and mast cell tumors.

2. **$CD11b^{+}CD14^{-}MHCII^{-}$ cells exhibit granulocytic morphology and co-purify with PBMCs.** Granulocytic MDSCs in humans were originally defined as granulocytic cells that are enriched in the PBMC fraction through density gradient separation, and commonly express CD15 and CD66b markers. Cytospin analysis of sorted $CD11b^{+}CD14^{-}MHCII^{-}$ cells isolated from PBMC layer of blood sample from dogs with cancer showed granulocytic morphology that resemble neutrophils, which corroborates human studies. In renal cell carcinoma patients, Rodriguez et al. (59) demonstrated that MDSC share $CD15^{+}$ marker expression with normal neutrophils, but are less dense, which allows them to co-purify with

lymphocytes. Others have identified this population as having the phenotype $CD33^{-}MHCII^{-}CD14^{-}CD15^{+}$ (60) or $CD11b^{+}CD14^{-}$ (40). Despite the unavailability of canine-specific CD33, CD15 and CD66b antibodies to phenotype our canine MDSC population, we hypothesized that the granulocytic MDSC subset could be identified by the combination of $CD11b^{+}CD14^{-}MHCII^{-}$ markers. We confirmed that these cells had a granulocytic morphology by microscopic analysis.

3. **$CD11b^{+}CD14^{-}MHCII^{-}$ cells suppress T cell proliferation and cytokine secretion.** Consistent with human and mice literature, our putative canine MDSC population, characterized by the three-marker phenotype are endowed with high immunosuppressive activity. The phenotype of the granulocytic subset is $CD11b^{+}CD14^{-}MHCII^{-}$ and of the monocytic subset is $CD11b^{+}CD14^{+}MHCII^{-}$. Our in vitro results demonstrated that the $CD11b^{+}CD14^{-}MHCII^{-}$ subset of cells inhibited the proliferation of T lymphocytes from autologous responders in a dose-dependent manner. It is well known that cancer patients have an impaired ability to respond to mitogen stimulation. Therefore we further evaluated the ability of these cells to suppress the proliferation of, and cytokine secretion by PBMCs collected from control dogs without cancer. Again, addition of $CD11b^{+}CD14^{-}MHCII^{-}$cells impaired T cell function that substantiates the immunosuppressive capacity of these cells. Moreover, although we have focused on the characterization of the granulocytic MDSC subset, we had

also conducted functional experiments with the putative monocytic MDSC subset, $CD11b^{+}CD14^{+}MHCII^{-}$. We demonstrated a robust ability of these cells to suppress T cell proliferative responses even though this is not the most prevalent subset in peripheral blood.

4. **$CD11b^{+}CD14^{-}MHCII^{-}$ cells express hallmark MDSC-derived factors.** Many immunosuppressive strategies by which MDSC inhibit the effectiveness of immune responses have been established. Up-regulation of ARG1, iNOS and ROS have been implicated as a major mechanism responsible for the immune suppressive activity of MDSC (40, 41, 59, 62, 92, 95, 119). Increased secretion of immunosuppressive cytokines TGF-β and IL-10, and induction of Treg development are indirect mechanisms of MDSC immune suppression (39, 54, 73, 80). In support of these findings we herein reported that the expression of ARG-1 and iNOS2, as well as the immunosuppressive cytokines, TGF- β and IL-10, were detected by PCR analysis in $CD11b+CD14^{-}MHCII^{-}$ cells sorted from peripheral blood of canine cancer patient. Due to the small percentage of monocytic $CD11b^{+}CD14^{+}MHCII^{-}$ cells, we were not able to sort sufficient numbers of cells for RNA isolation, therefore quantitative PCR analysis of this population could not be carried out.

5. **$CD11b^{+}CD14^{+}MHCII^{-}$ cells are prevalent in the peripheral blood of dogs with glioma.** Despite observing the accumulation of G-MDSC,

$CD11b^{+}CD14^{-}MHCII^{-}$ cells, in the peripheral blood of dogs with glioma, the monocytic ($CD11b^{+}CD14^{+}MHCII^{-}$) subset was also significantly elevated. Moreover, the percentage of $CD11b^{+}CD14^{+}MHCII^{-}$ cells was consistently high in all of subtypes of glioma analyzed. Besides that the data for M-MDSC in dogs with glioma differs from that observed in other canine tumor types, accumulations of monocytic $CD14^{+}$ (87), $CD14^{+}HLA\text{-}DR^{-}$ (80, 118) and $CD14^{+}IL4R\alpha^{+}$ (63, 84, 120) have been described in several human cancers including melanoma, glioblastoma, colon carcinoma and HNSCC. Although a glioma-specific phenotype of MDSC has not been clearly defined, there is strong evidence that supports increased numbers of the monocytic population. Kohanbash et al. have reported $CD14^{+}IL4R\alpha^{+}$ as the predominant MDSC population in human GBM tissues (63) and Gustafson et al. identified an expanded population of MDSC with the phenotype $CD14^{+}HLA\text{-}DR^{-/low}$ in steroid-treated GBM patients (126). Intriguingly, in our study all of canine patients from whom we collected blood for MDSC analysis had been previously treated with corticosteroids. Conflicting results with higher percentages of granulocytic MDSC subsets with the respective phenotypes $CD11b^{+}CD33^{lo}CD14^{-}HLA\text{-}DR^{-}$ and $CD33^{+}CD15^{+}CD14^{-}HLA\text{-}DR^{-}$ have been demonstrated in some GBM patients (50, 114). This data shows that phenotype of MDSC in human glioma may not be constant and needs to be further clarified. It is also possible that disregulated secretion of certain tumor-derived factors by gliomas induce the accumulation of different MDSC subsets.

In our analysis of canine glioma patients, elevated but not significantly different, percentages of the granulocytic subset were observed. Of note, the canine glioma patients' PBMC samples we used had been frozen, and the viability of the granulocytic subset may be more susceptible to freezing resulting in an underestimation of their numbers (122, 123). In our previous study, we analyzed both fresh and frozen samples from canine cancer patients and healthy control dogs, and .we were able to detect elevated percentages of G-MDSC in the cancer patients compared to health controls in both fresh and frozen samples. However, we did observe a slight, but insignificant, decrease in the percentage of G-MDSC from frozen samples compared to fresh samples. Importantly, samples in our published canine MDSC study were frozen no more than 7 days before flow cytometry, while in our canine glioma study, samples were frozen for months, or in some cases, years before thawing and analysis which may have a greater impact on the viability of certain cell subsets. Therefore, in order to avoid conflicting results and unexpected losses, future canine MDSC studies should be done with fresh blood samples.

6. **Canine glioma patients have elevated levels of arginase activity**. One of the most important mechanisms by which MDSC disrupt T-cell function is by reducing the availability of L- arginine, which impairs T-cell proliferation and cytokine production, and reduces the expression of TCR

CD3ζ chain (40, 59, 95). Several groups have found high arginase activity in plasma from cancer patients (40, 50, 59, 114). We also observed significantly elevated levels of arginase activity in the samples from canine glioma patients relative to healthy control dogs. We speculate that this increase in arginase activity could be due to an increased number of MDSC in the peripheral blood of these dogs with cancer. However, we were unable to correlate the arginase activity with the capacity of MDSC to suppress T cells since we did not use fresh blood samples on which functional studies can be performed. Therefore, further studies should be done with fresh blood samples to provide evidence of this association.

7. **Mouse CD11b but not Gr-1 antibody cross-react with canine cells**. The mouse MDSC phenotype has been established as positive expression of the myeloid markers, $CD11b^+$ and GR^+, and can be further characterized as $Ly6G^+$ or $Ly6C^+$ for the granulocytic and monocytic subsets, respectively. Although there has been an increase in the availability of canine specific reagents, they are still limited. Therefore identification of antibodies that cross-react with canine species can greatly aid comparative research. Krol et al. describe the use of mouse Gr-1 antibody to identify of myeloid precursors in canine mammary tissues using immunohistochemical staining (117). In our previously published study, the Gr-1 antibody clone (RB6-8C5) used by Krol, and the anti-mouse CD11b clone (M1/70), were assess cross-reactive double labeling

of myeloid cells and staining was observed with both antibodies. In the current study, we performed additional tests to evaluate the specificity of these antibodies for the MDSC subset of myeloid cells. Unfortunately our current data demonstrated that the anti-mouse CD11b stained canine myeloid cells, but the anti-mouse Gr-1 clone did not. Flow cytometry analysis of cells stained with Gr-1 and CD11b antibodies revealed that Gr-1 stain was only detected when canine gamma globulin blocking was not performed, while CD11b stain was positive with and without blocking. In addition, we performed IHC analysis of Gr-1 antibody stain on canine bone marrow and lymph nodes samples using mouse splenic tissue as the positive control. Again, mouse Gr-1 antibody failed to stain the canine tissues but did stain the mouse splenic tissue. Therefore, our data using anti-mouse Gr-1 conflicts with that of Krol et al. (117). Overall, this data suggests that anti-mouse Gr-1 antibody binding to canine cells may be nonspecific and this marker should not be used to identify MDSC in dogs. However isolation of the cells nonspecifically stained with Gr-1 antibodies and co-culture with responder PBMCs confirmed that highly suppressive cells are indeed present in the peripheral blood of dogs with cancer.

Future Directions:

Although somewhat limited by the lack of available reagents for canine research, our studies validated the presence of MDSC in canine cancer patients and underlined the potential role of these cells in the immunosuppression in dogs with cancer. This was the first set of studies investigating MDSC in this species and future research to better define the phenotype and function of this cell population in dogs with cancer should be conducted.

From a translational perspective based on the human literature, future studies on canine MDSC should include the use of markers for mature lymphocytes, such as CD3, CD4, CD8, CD5, CD21, for gating analysis of a lineage negative population. Importantly, all of these surface markers are commercially available for canines. A single specific marker for NK cells has not yet been identified in dogs, however recent publications demonstrate that these cells are characterized as a $CD5^-$ phenotype within a population of cells expressing the MHCII marker (127), therefore these cells would be excluded from our $MHCII^-$ MDSC pool. It was recently demonstrated that CD124 or IL4Rα expression on monocytic MDSC correlates with highly immunosuppressive function (84, 120), and a cross-reactive polyclonal IL4R-α antibody is commercially available. Studies should be carried out to evaluate whether this cell surface marker is present on canine monocytic MDSC and could be used to identify and isolate this population. Another potential marker that could be tested for further immunophenotyping of the granulocytic MDSC subset in canine blood is CD11/18. CD11/CD18 are β2 integrins that play a key role in the activation,

adhesion, migration, and phagocytosis of neutrophils (128). Knowing that granulocytes upregulate the expression of CD11/CD18 upon activation, we propose that this marker could be used in dogs as an analogue to CD66b in humans

Cryopreservation reduces the viability of MDSC and certain sample handling and PBMC isolation protocols can cause variability in the PBMC fraction. Therefore to prevent inaccurate analyses, future studies of canine MDSC should be performed using fresh whole blood samples. Moreover, while there is accumulating evidence implicating ARG-1, iNOS2, TGF-β and IL10 in the mechanisms of inhibition, quantitative studies are needed to confirm the roles each of these candidate molecules are play in mediating the immunosuppression in the various tumors. Similarly, quantitative studies to assess whether potential tumor-derived factors, such as GM-CSF, VEGF, S100A8/ S100A9 proteins, PGE2 and COX-2 are involved in MDSC accumulation in dogs. Tumor cell supernatant has been used to show direct involvement of cancer cells in MDSC generation and accumulation; normal neutrophils and/or monocytes can be differentiated into MDSC by in vitro culture with conditioned media from malignant tumors (69, 70, 83, 121). These experiments are also feasible in veterinary medicine and may offer meaningful results. Recent canine study has shown that myeloid cells exposed to soluble tumor-derived factors decreased expression of MHC class II and CD80, reduced phagocytic activity, and suppressed the proliferation of responder immune cells. (129)

Finally, new therapeutic approaches, such as the use of PDE-5 inhibitors, COX-2 inhibitors, and kinase inhibitors, to decrease the number of MDSC and thereby improve T cell function in canine cancer patients should be tested. COX-2 inhibitors, commonly know as NSAIDs, are widely used in both human and veterinary medicine to relieve pain and inflammation, and could easily be incorporated into immunotherapeutic protocols. Sunitinib, a tyrosine kinase inhibitor approved by the FDA to treat renal cell carcinoma patients, significantly decreased circulating MDSC and Tregs in renal cell cancer patients resulting in improved T cell function (60). Recently, the FDA approved Palladia, a tyrosine kinase inhibitor, for the treatment of mast cell tumors in dogs. This drug could be employed to target MDSC to enhance the response to immunotherapy (130, 131). Pharmacologic agents that regulate MDSC recruitment, differentiation or expansion, and inhibit their suppressive function may be useful to control cancer growth and progression and provide more effective responses to immunotherapy.

Altogether the results of our research demonstrate for the first time that similar to mice and humans, MDSC accumulate in the peripheral blood of canine cancer patients and have a potent ability to suppress T cell responses. Our studies were completed using peripheral blood of dogs with various tumor types including sarcomas, carcinomas, mast cell tumors, and glioma. Elevated numbers of suppressive MDSC were detected in the peripheral blood of all canine cancer patients regardless of tumor type compared of healthy control dogs with evidence of cancer. Importantly, this study characterized a three surface marker immunophenotype that can be used for future studies in dogs to

identify MDSC, paving a way to monitor MDSC burden in canine cancer clinical trials as a potential biomarker to offer insights into the efficacy of the new treatments that may benefit human cancer patients as well.

Bibliography:

1. Khanna C, Lindblad-Toh K, Vail D*, et al.* The dog as a cancer model. Nat Biotechnol 2006;24: 1065-6.
2. Pinho SS, Carvalho S, Cabral J, Reis CA, Gartner F. Canine tumors: a spontaneous animal model of human carcinogenesis. Transl Res;159: 165-72.
3. Paoloni M, Khanna C. Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer 2008;8: 147-56.
4. Vail DM, MacEwen EG. Spontaneously occurring tumors of companion animals as models for human cancer. Cancer Invest 2000;18: 781-92.
5. Paoloni MC, Khanna C. Comparative oncology today. Vet Clin North Am Small Anim Pract 2007;37: 1023-32; v.
6. Khanna C, London C, Vail D, Mazcko C, Hirschfeld S. Guiding the optimal translation of new cancer treatments from canine to human cancer patients. Clin Cancer Res 2009;15: 5671-7.
7. Mack GS. Clinical trials going to the dogs: canine program to study tumor treatment, biology. J Natl Cancer Inst 2006;98: 161-2.
8. Hoption Cann SA, van Netten JP, van Netten C, Glover DW. Spontaneous regression: a hidden treasure buried in time. Med Hypotheses 2002;58: 115-9.
9. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5: 263-74.
10. Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest 2007;117: 1130-6.
11. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity 2004;21: 137-48.
12. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007;117: 1137-46.
13. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol 2004;22: 329-60.
14. Moser M, Leo O. Key concepts in immunology. Vaccine;28 Suppl 3: C2-13.
15. Lu B, Finn OJ. T-cell death and cancer immune tolerance. Cell Death Differ 2008;15: 70-9.
16. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007;25: 267-96.
17. Vergati M, Schlom J, Tsang KY. The consequence of immune suppressive cells in the use of therapeutic cancer vaccines and their importance in immune monitoring. J Biomed Biotechnol;2011: 182413.
18. Topfer K, Kempe S, Muller N*, et al.* Tumor evasion from T cell surveillance. J Biomed Biotechnol;2011: 918471.
19. Whiteside TL, Gulley JL, Clay TM, Tsang KY. Immunologic monitoring of cellular immune responses in cancer vaccine therapy. J Biomed Biotechnol;2011: 370374.
20. Finn OJ. Cancer immunology. N Engl J Med 2008;358: 2704-15.

21. Andersen BM, Ohlfest JR. Increasing the efficacy of tumor cell vaccines by enhancing cross priming. Cancer Lett;325: 155-64.
22. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007;7: 41-51.
23. Bui JD, Schreiber RD. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? Curr Opin Immunol 2007;19: 203-8.
24. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. Immune-mediated dormancy: an equilibrium with cancer. J Leukoc Biol 2008;84: 988-93.
25. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol 2001;2: 293-9.
26. Peggs KS, Quezada SA, Allison JP. Cancer immunotherapy: co-stimulatory agonists and co-inhibitory antagonists. Clin Exp Immunol 2009;157: 9-19.
27. Murdoch C, Muthana M, Coffelt SB, Lewis CE. The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 2008;8: 618-31.
28. Kaplan RN, Riba RD, Zacharoulis S*, et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005;438: 820-7.
29. Curiel TJ. Regulatory T cells and treatment of cancer. Curr Opin Immunol 2008;20: 241-6.
30. Kim JH, Hur JH, Lee SM, Im KS, Kim NH, Sur JH. Correlation of Foxp3 positive regulatory T cells with prognostic factors in canine mammary carcinomas. Vet J;193: 222-7.
31. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133: 775-87.
32. Lindau D, Gielen P, Kroesen M, Wesseling P, Adema GJ. The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology;138: 105-15.
33. Biller BJ, Guth A, Burton JH, Dow SW. Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. J Vet Intern Med;24: 1118-23.
34. Fazekas de St Groth B, Zhu E, Asad S, Lee L. Flow cytometric detection of human regulatory T cells. Methods Mol Biol;707: 263-79.
35. Sakaguchi S, Ono M, Setoguchi R*, et al.* Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 2006;212: 8-27.
36. Tominaga M, Horiuchi Y, Ichikawa M*, et al.* Flow cytometric analysis of peripheral blood and tumor-infiltrating regulatory T cells in dogs with oral malignant melanoma. J Vet Diagn Invest;22: 438-41.
37. Biller BJ, Elmslie RE, Burnett RC, Avery AC, Dow SW. Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. Vet Immunol Immunopathol 2007;116: 69-78.

38. Garden OA, Pinheiro D, Cunningham F. All creatures great and small: regulatory T cells in mice, humans, dogs and other domestic animal species. Int Immunopharmacol;11: 576-88.
39. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9: 162-74.
40. Zea AH, Rodriguez PC, Atkins MB*, et al.* Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion. Cancer Res 2005;65: 3044-8.
41. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin Cancer Biol 2006;16: 53-65.
42. Serafini P. Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly. Immunol Res;57: 172-84.
43. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol;12: 253-68.
44. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother 2002;51: 293-8.
45. Kusmartsev S, Gabrilovich DI. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol Immunother 2006;55: 237-45.
46. Sevko A, Umansky V. Myeloid-derived suppressor cells interact with tumors in terms of myelopoiesis, tumorigenesis and immunosuppression: thick as thieves. J Cancer;4: 3-11.
47. Nagaraj S, Gabrilovich DI. Myeloid-derived suppressor cells. Adv Exp Med Biol 2007;601: 213-23.
48. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol;32: 19-25.
49. Markowitz J, Wesolowski R, Papenfuss T, Brooks TR, Carson WE, 3rd. Myeloid-derived suppressor cells in breast cancer. Breast Cancer Res Treat;140: 13-21.
50. Raychaudhuri B, Rayman P, Ireland J*, et al.* Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. Neuro Oncol;13: 591-9.
51. Zhang B, Wang Z, Wu L*, et al.* Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma. PLoS One;8: e57114.
52. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 2009;58: 49-59.
53. 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: 678-89.

54. Marigo I, Dolcetti L, Serafini P, Zanovello P, Bronte V. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev 2008;222: 162-79.
55. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 2009;182: 4499-506.
56. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res 2004;64: 6337-43.
57. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. J Immunol 2008;181: 4666-75.
58. Fujita M, Kohanbash G, Fellows-Mayle W*, et al.* COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. Cancer Res;71: 2664-74.
59. Rodriguez PC, Ernstoff MS, Hernandez C*, et al.* Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 2009;69: 1553-60.
60. Ko JS, Zea AH, Rini BI*, et al.* Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res 2009;15: 2148-57.
61. Pan PY, Wang GX, Yin B*, et al.* Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. Blood 2008;111: 219-28.
62. Rodriguez PC, Hernandez CP, Quiceno D*, et al.* Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med 2005;202: 931-9.
63. Kohanbash G, McKaveney K, Sakaki M*, et al.* GM-CSF promotes the immunosuppressive activity of glioma-infiltrating myeloid cells through interleukin-4 receptor-alpha. Cancer Res;73: 6413-23.
64. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. Eur J Immunol;40: 2969-75.
65. Larrivee B, Pollet I, Karsan A. Activation of vascular endothelial growth factor receptor-2 in bone marrow leads to accumulation of myeloid cells: role of granulocyte-macrophage colony-stimulating factor. J Immunol 2005;175: 3015-24.
66. 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: 4150-66.
67. Serafini P. Editorial: PGE2-producing MDSC: a role in tumor progression? J Leukoc Biol;88: 827-9.
68. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res 2007;67: 4507-13.

69. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. Blood;118: 5498-505.
70. Obermajer N, Kalinski P. Generation of myeloid-derived suppressor cells using prostaglandin E2. Transplant Res;1: 15.
71. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J Immunol 2006;176: 284-90.
72. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 2007;67: 10019-26.
73. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. J Immunol;184: 3106-16.
74. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. J Leukoc Biol;91: 167-81.
75. Youn JI, Kumar V, Collazo M*, et al.* Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. Nat Immunol;14: 211-20.
76. Peranzoni E, Zilio S, Marigo I*, et al.* Myeloid-derived suppressor cell heterogeneity and subset definition. Curr Opin Immunol;22: 238-44.
77. Greten TF, Manns MP, Korangy F. Myeloid derived suppressor cells in human diseases. Int Immunopharmacol;11: 802-7.
78. Filipazzi P, Huber V, Rivoltini L. Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. Cancer Immunol Immunother;61: 255-63.
79. Khaled YS, Ammori BJ, Elkord E. Myeloid-derived suppressor cells in cancer: recent progress and prospects. Immunol Cell Biol;91: 493-502.
80. Hoechst B, Ormandy LA, Ballmaier M*, et al.* A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+)Foxp3(+) T cells. Gastroenterology 2008;135: 234-43.
81. Ko JS, Bukowski RM, Fincke JH. Myeloid-derived suppressor cells: a novel therapeutic target. Curr Oncol Rep 2009;11: 87-93.
82. Kusmartsev S, Su Z, Heiser A*, et al.* Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. Clin Cancer Res 2008;14: 8270-8.
83. Lechner MG, Megiel C, Russell SM*, et al.* Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. J Transl Med;9: 90.
84. Mandruzzato S, Solito S, Falisi E*, et al.* IL4Ralpha+ myeloid-derived suppressor cell expansion in cancer patients. J Immunol 2009;182: 6562-8.

85. Mirza N, Fishman M, Fricke I*, et al.* All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. Cancer Res 2006;66: 9299-307.
86. Payne KK, Zoon CK, Wan W*, et al.* Peripheral blood mononuclear cells of patients with breast cancer can be reprogrammed to enhance anti-HER-2/neu reactivity and overcome myeloid-derived suppressor cells. Breast Cancer Res Treat;142: 45-57.
87. Serafini P, Meckel K, Kelso M*, et al.* Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 2006;203: 2691-702.
88. Sevko A, Michels T, Vrohlings M*, et al.* Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. J Immunol;190: 2464-71.
89. Waldron TJ, Quatromoni JG, Karakasheva TA, Singhal S, Rustgi AK. Myeloid derived suppressor cells: Targets for therapy. Oncoimmunology;2: e24117.
90. Zoglmeier C, Bauer H, Norenberg D*, et al.* CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice. Clin Cancer Res;17: 1765-75.
91. 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: 2777-90.
92. Serafini P, De Santo C, Marigo I*, et al.* Derangement of immune responses by myeloid suppressor cells. Cancer Immunol Immunother 2004;53: 64-72.
93. Ye XZ, Yu SC, Bian XW. Contribution of myeloid-derived suppressor cells to tumor-induced immune suppression, angiogenesis, invasion and metastasis. J Genet Genomics;37: 423-30.
94. Nagaraj S, Gabrilovich DI. Tumor escape mechanism governed by myeloid-derived suppressor cells. Cancer Res 2008;68: 2561-3.
95. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. Immunol Rev 2008;222: 180-91.
96. Rosenberg SA, Sherry RM, Morton KE*, et al.* Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol 2005;175: 6169-76.
97. Roth F, De La Fuente AC, Vella JL, Zoso A, Inverardi L, Serafini P. Aptamer-mediated blockade of IL4Ralpha triggers apoptosis of MDSCs and limits tumor progression. Cancer Res;72: 1373-83.
98. Khanna C, Gordon I. Catching cancer by the tail: new perspectives on the use of kinase inhibitors. Clin Cancer Res 2009;15: 3645-7.
99. Molon B, Ugel S, Del Pozzo F*, et al.* Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med;208: 1949-62.
100. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. J Immunol 2009;183: 937-44.

101. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 2008;181: 5791-802.
102. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. Cancer Res 2001;61: 4756-60.
103. Bronte V, Apolloni E, Cabrelle A*, et al.* Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood 2000;96: 3838-46.
104. Louis DN. Molecular pathology of malignant gliomas. Annu Rev Pathol 2006;1: 97-117.
105. Mitchell DA, Sampson JH. Toward effective immunotherapy for the treatment of malignant brain tumors. Neurotherapeutics 2009;6: 527-38.
106. Mitchell DA, Fecci PE, Sampson JH. Immunotherapy of malignant brain tumors. Immunol Rev 2008;222: 70-100.
107. Khan-Farooqi HR, Prins RM, Liau LM. Tumor immunology, immunomics and targeted immunotherapy for central nervous system malignancies. Neurol Res 2005;27: 692-702.
108. Yang L, Ng KY, Lillehei KO. Cell-mediated immunotherapy: a new approach to the treatme
nt of malignant glioma. Cancer Control 2003;10: 138-47.
109. Wu A, Oh S, Gharagozlou S*, et al.* In vivo vaccination with tumor cell lysate plus CpG oligodeoxynucleotides eradicates murine glioblastoma. J Immunother 2007;30: 789-97.
110. Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin;60: 166-93.
111. Okada H, Kohanbash G, Zhu X*, et al.* Immunotherapeutic approaches for glioma. Crit Rev Immunol 2009;29: 1-42.
112. Rolle CE, Sengupta S, Lesniak MS. Challenges in clinical design of immunotherapy trials for malignant glioma. Neurosurg Clin N Am;21: 201-14.
113. Waziri A. Glioblastoma-derived mechanisms of systemic immunosuppression. Neurosurg Clin N Am;21: 31-42.
114. Sippel TR, White J, Nag K*, et al.* Neutrophil degranulation and immunosuppression in patients with GBM: restoration of cellular immune function by targeting arginase I. Clin Cancer Res;17: 6992-7002.
115. Kimmelman J, Nalbantoglu J. Faithful companions: a proposal for neurooncology trials in pet dogs. Cancer Res 2007;67: 4541-4.
116. Goulart MR, Pluhar GE, Ohlfest JR. Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. PLoS One;7: e33274.
117. Krol M, Pawlowski KM, Dolka I*, et al.* Density of Gr1-positive myeloid precursor cells, p-STAT3 expression and gene expression pattern in canine mammary cancer metastasis. Vet Res Commun;35: 409-23.
118. Filipazzi P, Valenti R, Huber V*, et al.* Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol 2007;25: 2546-53.

119. Vasquez-Dunddel D, Pan F, Zeng Q*, et al.* STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients. J Clin Invest;123: 1580-9.
120. Kohanbash G, Okada H. Myeloid-derived suppressor cells (MDSCs) in gliomas and glioma-development. Immunol Invest;41: 658-79.
121. Rodrigues JC, Gonzalez GC, Zhang L*, et al.* Normal human monocytes exposed to glioma cells acquire myeloid-derived suppressor cell-like properties. Neuro Oncol;12: 351-65.
122. Kotsakis A, Harasymczuk M, Schilling B, Georgoulias V, Argiris A, Whiteside TL. Myeloid-derived suppressor cell measurements in fresh and cryopreserved blood samples. J Immunol Methods;381: 14-22.
123. Trellakis S, Bruderek K, Hutte J*, et al.* Granulocytic myeloid-derived suppressor cells are cryosensitive and their frequency does not correlate with serum concentrations of colony-stimulating factors in head and neck cancer. Innate Immun;19: 328-36.
124. Wang L, Chang EW, Wong SC, Ong SM, Chong DQ, Ling KL. Increased myeloid-derived suppressor cells in gastric cancer correlate with cancer stage and plasma S100A8/A9 proinflammatory proteins. J Immunol;190: 794-804.
125. Sherger M, Kisseberth W, London C, Olivo-Marston S, Papenfuss TL. Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. BMC Vet Res;8: 209.
126. Gustafson MP, Lin Y, New KC*, et al.* Systemic immune suppression in glioblastoma: the interplay between CD14+HLA-DRlo/neg monocytes, tumor factors, and dexamethasone. Neuro Oncol;12: 631-44.
127. Michael HT, Ito D, McCullar V, Zhang B, Miller JS, Modiano JF. Isolation and characterization of canine natural killer cells. Vet Immunol Immunopathol;155: 211-7.
128. Mazzone A, Ricevuti G. Leukocyte CD11/CD18 integrins: biological and clinical relevance. Haematologica 1995;80: 161-75.
129. Wasserman J, Diese L, VanGundy Z, London C, Carson WE, Papenfuss TL. Suppression of canine myeloid cells by soluble factors from cultured canine tumor cells. Vet Immunol Immunopathol;145: 420-30.
130. Bernabe LF, Portela R, Nguyen S*, et al.* Evaluation of the adverse event profile and pharmacodynamics of toceranib phosphate administered to dogs with solid tumors at doses below the maximum tolerated dose. BMC Vet Res;9: 190.
131. Pan X, Tsimbas K, Kurzman ID, Vail DM. Safety evaluation of combination CCNU and continuous toceranib phosphate (Palladia) in tumour-bearing dogs: a phase I dose-finding study. Vet Comp Oncol.