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Characterization of myeloid-derived suppressor cells and cytokines GM-CSF, IL-10 and MCP-1 in dogs with malignant melanoma receiving a GD3-based immunotherapy

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ABSTRACT

Melanoma in humans and canines is an aggressive and highly metastatic cancer. The mucosal forms in both species share genetic and histopathologic features, making dogs a valuable spontaneous disease animal model. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells of myeloid origin with immunosuppressive capabilities, which are increased in many human cancers and contribute to tumor immune evasion. They are a possible target to improve immunotherapy outcomes. Current information regarding MDSCs in canines is minimal, limiting their use as translational model for the study of MDSCs. The objective of this study was to characterize major MDSCs subsets (monocytic and polymorphonuclear) and the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 10 (IL-10) and monocyte chemoattractant protein-1 (MCP-1) in canines with malignant melanoma and to evaluate changes in MDSCs and the cytokines over time in response to a GD3-based active immunotherapy. Whole blood and serum collected from 30 healthy controls and 33 patients enrolled in the University of Florida melanoma vaccine trial were analyzed by flow cytometry with canine specific CD11b, MHCII and anti-human CD14 antibodies to assess ostensibly polymorphonuclear-MDSC ($CD11b^+$ $MHCII^-$ $CD14^-$) and monocytic-MDSC ($CD11b^+$ $MHCII^-$ $CD14^+$) subsets. IL-10, MCP-1 and both MDSCs subsets were significantly elevated in melanoma dogs versus controls. Both MDSCs subsets decreased significantly following GD3-based immunotherapy administration but no significant changes in cytokines were seen over time. To our knowledge, this is the first report documenting increased monocytic-MDSCs in canine melanoma. This is consistent with human malignant melanoma data, supporting dogs as a valuable model for therapeutic intervention studies.

1. Introduction

In both humans and dogs, malignant melanoma is an aggressive cancer with a high metastatic rate, early in the disease course (Lindsay et al., 2015; Williams and Packer, 2003). While human mucosal and non-UV induced cutaneous melanoma have shared histopathologic and genetic features with canine mucosal and cutaneous malignant melanoma, a recent article by Wong et al (Wong et al., 2019) found similarities and differences in mutation profiles between canine (oral) and human mucosal melanoma. These differences in mutation profiles could influence tumor behavior and thus response to treatment. Nonetheless, canine mucosal melanoma can still serve a valuable role as a large

Abbreviations: ARG1, Arginase 1; CD11b, Cluster of differentiation 11b, Integrin alpha M; CD14, Cluster of differentiation 14, monocyte marker; CD15, Cluster of differentiation 15, glycan marker; CD66b, Cluster of differentiation 66b, granulocyte marker in humans; FACS, Flow assisted cell sorting; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GR1, Granulocytic marker; iNOS, Inducible nitric oxide synthase; MCP-1, Monocyte chemoattractant protein-1; MDSCs, Myeloid-derived suppressor cells; MM, Malignant melanoma; M-MDSCs, Monocytic myeloid-derived suppressor cells; MST, Median survival time; PMN-MDSCs, Polymorphonuclear myeloid-derived suppressor cells; TAMs, Tumor associated macrophages; Tregs, Regulatory T-cells

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animal translational model for the study of immunotherapeutic agents in naturally occurring cancers where immunotherapy targets are known to occur in both species e.g. GD3 (Gillard et al., 2014; Hernandez et al., 2018; Schiffman and Breen, 2015; Simpson et al., 2014; Wong et al., 2019). A key factor for the success of immunotherapy is overcoming tumor-induced host immune tolerance and evasion, which has led to increased research into tumor immunology and the suppressive tumor microenvironment (Banerjee et al., 2013; Tsai et al., 2014; Vasievich and Huang, 2011). Within this, myeloid-derived suppressor cells (MDSCs) have recently emerged as a key player in blocking immune effector cell function. (Tcyganov et al., 2018; Tsai et al., 2014).

Myeloid-derived suppressor cells (MDSCs) comprise a largely immature cell population with heterogeneous cell phenotypes, morphology, and functions which are unified by their strong immunosuppressive capabilities (Gabrilovich and Nagaraj, 2009). Originally identified due to their expansion associated with cancer they have since been shown to be expanded in other pathologic conditions, including inflammation, trauma and sepsis, but are present only in low numbers in healthy animals (Gabrilovich and Nagaraj, 2009; Goulart et al., 2012). MDSCs have a variety of mechanisms through which they can modulate the host immune response, including production of immunosuppressive mediators such as arginase 1 (ARG1), inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), IL-10, TGF-β and IFN-γ (Gabrilovich et al., 2012; Khaled et al., 2013). Multiple factors have been shown to be involved in MDSC upregulation in cancer (Gabrilovich et al., 2012; Khaled et al., 2013). Particularly, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been found to drive MDSC formation via bone marrow stimulation (Dolcetti et al., 2009; Lechner et al., 2010; Zhao et al., 2016) and monocyte chemoattractant protein (MCP-1, also known as chemokine (C-C motif) ligand 2 or CCL2) has been shown to have direct effects on cells within the tumor microenvironment, including MDSCs, which promotes tumorigenesis and metastasis (Zhang et al., 2010). Additionally, the MCP-1/CCR2 pathway has been found to have a key role in MDSCs migration to the tumor and on tumor growth (Huang et al., 2007).

In mice, MDSCs are defined by a characteristic co-expression of CD11b and GR1 (granulocyte marker), however human leukocytes have no analogous marker to GR1 and to date, no unique markers for MDSCs have been identified (Bronte et al., 2016; Khaled et al., 2013; Mandruzzato et al., 2016). Nonetheless, progress in analysis of morphology and phenotypic expression of cell surface markers has led to the identification at least two major MDSC subsets, monocytic (M-MDSCs) and polymorphonuclear (PMN-MDSCs) in both humans and mice and the pattern of major subset expansion has been shown to be variable among different human cancer types (Khaled et al., 2013; Marvel and Gabrilovich, 2015). To improve interpretation and comparison between human laboratories and studies minimum phenotype definitions of $CD11b^+CD14^-CD15^+$ (or $CD66b^+$) for PMN-MDSCs and $CD11b^+CD14^+HLA\text{-}DR^{low/-}CD15^-$ for M-MDSCs were recently proposed with functional demonstration of immunosuppressive capacity by T-cell suppression assay recommended to confirm MDSC identification (Bronte et al., 2016). MDSCs are significant contributors to the tumor immune evasion and host tumor tolerance, making them, along with cytokine mediators, attractive targets for therapeutic interventions (Gabrilovich and Nagaraj, 2009). However, until recently, only a few studies were published on MDSCs in dogs, which limited the ability to investigate these cells as a target in this model. Two previously published studies evaluating canine patients with various tumor types demonstrated MDSCs to be elevated in the cancer populations when compared to healthy controls with cancer but were insufficiently powered to establish significance among individual tumor types (Goulart et al., 2012; Sherger et al., 2012). Recently, a definitive paper by Goulart et al (Goulart et al., 2019) was published describing the phenotypic and transcriptomic characterization of canine MDSCs. This sentinel paper confirmed the existence of two phenotypical and functional subsets of MDSCs in the canine (Goulart et al., 2019).

Immunotherapy remains a crucial therapeutic option in the treatment of melanoma in humans and many different modalities have been used in the effort to improve outcomes (Lindsay et al., 2015). Similarly, the immunotherapy strategies used in canine patients with melanoma have been varied, with the majority using active specific immunity, through the administration of vaccines (Alexander et al., 2006; Bergman et al., 2003; Dow et al., 1998; Finocchiaro et al., 2015; Helfand et al., 1999, 1994; Hogge et al., 1999; MacEwen et al., 1999, 1986; von Euler et al., 2008; Watanabe et al., 2010). Previous work by Milner et al. (2006) has shown the melanoma cell surface disialoganglioside GD3 to be highly expressed in canine melanoma cell lines and that a disialoganglioside GD3-based vaccine was well tolerated and induced appropriate innate and adaptive immune responses in normal dogs. The tumor promoting capabilities of MDSCs and the MCP-1/CCR2 pathway make them attractive targets for therapeutic interventions and mitigating their effects may help improve responses immunotherapy (De Sanctis et al., 2016).

An ongoing clinical trial (IACUC # 201802334) at our institution investigating the use of the GD3-based vaccine in dogs with melanoma, presented a unique opportunity to evaluate MDSCs and serum cytokines before and during immunotherapeutic intervention in a relevant large animal translational model. The purpose of this study was to identify and characterize changes in MDSC subsets using a published flow cytometry protocol from Goulart et al., 2012 using commercially available antibodies in matched normal dogs and dogs with, cutaneous and digital melanoma. The first aim was a feasibility study to recapitulate the published protocol and to establish a baseline for future immunotherapeutic interventional studies. The second aim of this study was to compare changes in MDSC subsets overtime in response to the administration of the UF GD3-based vaccine in the absence of additional cytotoxic cancer therapy. Our third aim was to investigate the serum expression profiles of the chemokine MCP-1 and cytokines GM-CSF and IL-10, which have been implicated in recruiting MDSCs, over time and assess any correlation of their level in response to the administration of the GD3-based vaccine.

2. Materials and methods

2.1. Study populations

Canine patients presented at the University of Florida's Small Animal Veterinary Hospital were prospectively enrolled into two group populations between December 2015 and January 2018. Animal use was approved by the University of Florida's Institutional Animal Care and Use Committee (IACUC) under number 201802334 and by the University of Florida's Veterinary Hospital's Clinical Research and Review Committee. The first patient group consisted of dogs presenting to the Oncology Services with a cytologic or histopathologic diagnosis of oral, digital or cutaneous melanoma (melanoma population, abbreviated as MM). Additional inclusion criteria for the melanoma population were staging within two weeks of study entry with chemistry, CBC, urinalysis, three view thoracic radiographs, and regional lymph node aspirates when obtainable, and an expectation of a minimum of 4 months survival time. All patients were staged at study entry according to the World Health Organization (WHO) scheme for dogs with oral melanoma (Table 1) (Owen, 1980). Patients with stage I–IV disease were eligible for melanoma population enrollment.

Additionally, tumors were coded for mitotic index (MI), defined as the number of mitotic figures per 10 high power fields, as either high MI or low MI. Based on previously published information, cutoffs of MI ≥ 4 for oral/mucocutaneous tumors and MI ≥ 3 for cutaneous/digit tumors used to define high MI tumors (Smedley et al., 2011). Exclusion criteria included the use of any immune modifying drug (e.g. prednisolone, nutraceutical e.g. *Aloe vera*, preexisting endocrine disease e.g. hyperadrenocorticism), the presence of any other preexisting cancer other than melanoma or of any active infection.

Table 1
Clinical staging system for oral and digital melanomas.
Sources: World Health Organization (Owen, 1980), (Manley et al., 2011).

Stage	Tumor size	Regional node status	Distant metastasis
I	< 2 cm	Negative	Negative
II	2-4 cm	Negative	Negative
III	2-4 cm	Positive	Negative
	> 4 cm	Negative	Negative
IV	Any	Any	Positive

The second group consisted of age, weight and sex matched dogs belonging to faculty and staff of the hospital and determined to be clinically healthy based on physical exam, CBC and no reported history of neoplasia (control population). Exclusion criteria included known systemic disease, including but not limited to preexisting endocrine disease or active infection and current or recent (within 1 month) use of any prescribed medications or nutraceuticals, including NSAIDS but excluding preventatives (e.g flea, tick and heartworm prevention).

2.2. Vaccine preparation and administration

The vaccine was prepared from the commercially available reagents monophosphoryl lipid A from *Salmonella enterica* serotype minnesota Re 595 (MPL) adjuvant (Sigma, MS, USA [Product no. L6895]), oligodeoxynucleotides containing cytosine-phosphate-guanine (CpG) oligodeoxynucleotide sequences (CpG-ODNs) (Alpha Diagnostic International, TX, USA [Catalog number ODN2007] CpG-ODN sequence 5´-TCG TCG TTG TCG TTT TGT CGT T-3´) and GD3 (Matreya LLC, Pleasant Gap, PA, USA [Catalog number 1504]) as previously describe (Milner et al., 2006). Vaccine preparation was performed just prior to administration.

The vaccine was administered intradermally to the melanoma population every 4 weeks for a series of four injections. Three separate vaccination sites were used in rotation as follows: the left shoulder at week 0, right shoulder at week 4, right gluteal region at week 8 and left shoulder at week 12. Separate vaccination sites were chosen to allow for monitoring of any acute cutaneous reaction secondary to the vaccine administration or any subsequent skin complications should they occur.

2.3. Sample collection

Blood was collected at each vaccine time point and at a restage visit 1 month after the 4th vaccine in the melanoma population. For the normal control population blood samples were collected at a single time point. All blood samples were collected by jugular venipuncture and divided into a serum separator tube and heparin tube. Serum was allowed to clot for at least 30 min before centrifugation at 1640 *g* for 8 min, then aliquoted into two samples and initially frozen at −20 °C before transfer to -80 °C for long term storage until batched use for cytokine analysis. Heparinized whole blood was used for flow cytometry analysis with all samples processed within 24 h of collection. Heparinized blood samples were stored at 4 °C until processing if processing was delayed for greater than 1 h.

2.4. Flow cytometric analysis

The flow cytometric analysis was based on a published protocol (Goulart et al., 2012) which defined the polymorphonuclear myeloid-derived suppressor cell (PMN-MDSCs) subset as the CD11b + MHCII-CD14- cell population, and the monocytic myeloid-derived suppressor cell (M-MDSCs) subset as the CD11b + MHCII-CD14 + cell population. For the MDSC subset evaluation, whole blood samples were incubated with primary unconjugated mouse anti-dog CD11b antibody (AbD Serotec, clone CA16.3E10) or mouse IgG1 isotype control (AbD Serotec) and then RPE-conjugated Rabbit F(ab') anti-mouse IgG (AbD Serotec) secondary antibody for 30 min per incubation. All incubations were performed at 4 °C in the dark, unless otherwise stated. Following indirect staining, cells were washed and stained with FITC-conjugated rat anti-dog MHCII (AbD Serotec, clone YKIX334.2) and Alexa fluor 647-conjugated mouse anti-human CD14 antibody (AbD Serotec, clone TÜK4) or isotypes controls (AbD Serotec) for 30 min according to manufacturer's protocol. The anti-CD11b and anti- MHCII clones are marketed as canine targeted by their manufacturer, as well as having shown reactivity in several previous studies (Brodersen et al., 1998; Goulart et al., 2012; Lana et al., 2006; Rao et al., 2011). The anti-CD14 clone has shown canine cross reactivity in several previous studies (Goulart et al., 2012; Jacobsen et al., 1993; Lana et al., 2006). Cells were then washed twice before erythrocytes were lysed with BD Pharmlyse red cell lysis buffer (BD, Franklin Lakes, NJ USA) and incubated at room temperature for 25 min in the dark. Antibody-labeled cells were then washed twice, fixed with 4% paraformaldehyde at room temperature for 20 min before a final wash was performed to remove excess paraformaldehyde prior to being re-suspended in the FACS buffer for flow cytometry analysis. Unstained and single stained samples were prepared for each patient for negative and compensation controls, respectively.

Samples were analyzed on a Becton Dickinson Canto three-laser flow cytometer (BD, Franklin Lakes, NJ USA) and 100,000 events were collected per sample. Quality control was performed daily at initial startup using manufacturer's setup beads (BD, Franklin Lakes, NJ, USA [Catalog number 642412]). Acquisition gate was set to exclude RBC/dead cells. Compensation was set based on single stained samples and unstained control. Post collection analysis was performed with FlowJo Single Cell analysis software (Tree Star, Ashland, OR, USA). Analysis gates were set based on unstained controls. For flow cytometry gating of populations forward scatter (FSC) versus side scatter (SSC) acquisition gate was first set to exclude dead cells. FSC-Area versus FSC-Height was used to exclude doublets and identify the single cell population. Next, CD11b versus MHCII was used to identify the $CD11b^+MHCII^-$ population, which was then gated as CD11b versus CD14 to identify the $CD11b^+MHCII^-CD14^+$ (M-MDSC) and $CD11b^+MHCII^-$CD14- (PMN-MDSC) populations (Goulart et al., 2012). The numbers of PMN-MDSCs and M-MDSCs were calculated as percentages of the single cell population.

2.5. Cytokine measurement

Serial serum concentrations of GMCSF, IL-10 and MCP-1 were measured using a customized Milliplex MAP Canine cytokine/chemokine kit (CCYTOMAG-90 K, EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions, with internal quality control. Overnight incubation at 4 °C and a magnetic plate washer were used. All samples, standards and quality controls were analyzed in duplicate. Plates were read using Bio-Plex® MAGPIX™ multiplex reader and accompanying Bio-Plex Manager Software version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA). Intra-assay and inter-assay coefficients of variation as well as minimum detectable concentrations can be viewed on line at http://www.millipore.com.

A single canine melanoma cell line (Remi) was trypsinized from T75 flasks and counted using trypan blue exclusion dye. Live cells (1×10^6) were spun down at 250 x gravity in Falcon #2052 tubes (BD, Franklin Lakes, NJ, USA). The supernatant was removed and partitioned into four aliquots, frozen initially at −20 °C and then transferred to -80 °C for long term storage until cytokine kit analysis was performed.

2.6. Statistical analysis

Quantitative differences between the two groups were analyzed using unpaired, two-tailed Student's *t*-test or the non-parametric Wilcoxon rank-sum (Mann–Whitney) test, if data was not normally

distributed. Differences between > 2 groups were analyzed using ANOVA on ranks. For comparing percent MDSCs at vaccine time points a one-way ANOVA on repeated measures was used. Chi- squared analysis was used for comparison of categorical data. For statistical analysis of cytokines the Mann-Whitney Rank Sum Test was used, the concentration was adjusted to the minimum detectable concentration listed by the manufacturer when sample concentration results produced values 0.0 pg/mL. All analyses were performed with Sigma-Plot software (SigmaPlot for Windows, version 13; Systat Software, Erkrath, Germany) and a p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Patient characteristics

Thirty-three dogs were enrolled in the melanoma population including 19 neutered males and 14 spayed females with a mean population age of 10.56 years (SD ± 3.43) and median weight of 27.3 kg (IQR 12.25–35.75). Thirty dogs were enrolled in the control population including 13 neutered males and 17 spayed females with a mean population age of 8.98 years (SD ± 2.75) and median weight of 20.6 kg (IQR 12.25–29.43). Characteristics of the control (n = 30) and melanoma populations are summarized in Table 2. Represented breeds were mixed breed, Labrador retriever, Golden retriever, Beagle, Yorkshire terrier, Miniature Schnauzer and other breeds. There was no statistical significance between the melanoma and control populations with regards to sex, weight, gender or age (p > 0.05).

3.2. Flow cytometry characterization of MDSC subsets in dogs

The percentages of MDSCs in peripheral blood of melanoma and control populations were evaluated by flow cytometry to characterize the MDSC subsets present in each population as characterized by Goulard et al 2012. Based on commercially available antibodies we defined the polymorphonuclear myeloid-derived suppressor cell (PMN-MDSCs) subset as the $CD11b^+MHCII^-CD14^-$ cell population, and the monocytic myeloid-derived suppressor cell (M-MDSCs) subset as the $CD11b^+MHCII^-CD14^+$ cell population, using the gating strategy as outlined in Fig. 1. These MDSC subsets in the melanoma population at baseline prior to the first vaccination were compared to the control population. In both the melanoma and control groups, M-MDSCs comprised a relatively small fraction of the overall single cell population but were found to be significantly increased in the melanoma population versus the control population (p < 0.001) (Fig. 2A). In the melanoma population, the PMN-MDSCs comprised the majority of the single cell population and were significantly increased compared to the control population (p < 0.001) (Fig. 2B).

Table 2
Patient characteristics for control and melanoma populations.

	Melanoma (n = 33)	Control (n = 30)
Breed		
Mixed Breed	14	20
Labrador Retriever	5	0
Golden Retriever	2	1
Beagle	2	1
Yorkie	2	0
Miniature Schnauzer	2	0
Other (1 each)	6	8
Age (years)		
Mean (± SD)	10.56 (± 3.425)	8.98 (± 2.75)
Sex		
Male, neutered	19	13
Female, spayed	14	17
Weight (kg)		
Median (range)	27.3 (2.8-48.8)	20.6 (4.3-40.0)
Anatomic Location		
Oral	25	
Digit	4	
Cutaneous	4	
Stage		
I	15	
II	8	
III	6	
IV	4	
Coded Mitotic Index (n = 32)		
High	23	
Low	9	

3.3. MDSC subsets compared to stage, anatomic location and mitotic index

We categorized the melanoma population according to stage, anatomic location and mitotic index (Table 2), which are known prognostic factors for melanoma (Smedley et al., 2011), and then evaluated these in relation to PMN-MDSCs and M-MDSCs populations. Within the melanoma population, there were 15 stage I dogs, 8 stage II dogs, 6 stage III dogs and 4 stage IV dogs. There were no significant differences in MDSC subsets between any stage or between grouped stage I/II versus grouped stage III/IV. Twenty-five of the dogs had oral tumors, 4 had digital tumors and 4 had cutaneous tumors. No significant differences in MDSC subsets of patients with oral/mucocutaneous versus digit versus cutaneous locations were found.

Histopathology was available for 32 patients in the melanoma population for assessment of mitotic index. One patient had a cytologic diagnosis only and was excluded from this portion of the analysis. Tumors were categorized as high-MI if the MI was ≥ 4 for oral/mucocutaneous tumors or if the MI was ≥ 3 for cutaneous/digit tumors, tumors below these cutoffs were categorized as low-MI. Twenty-three dogs had high- MI tumors and 9 had low- MI. There was no significant difference in the percentage of PMN-MDSCs and M-MDSCs for high MI tumors versus low MI tumors.

3.4. MDSC subset changes with GD3-based immunotherapy vaccine administration

We sought to investigate if there were any changes in the MDSC subsets in dogs with melanoma over time following four GD3-based vaccine administrations. Blood samples were collected prior to vaccination at each of the four vaccine visits and 1 month following the fourth vaccine. The samples were then analyzed using flow cytometry for identification and quantification of the PMN-MDSCs and M-MDSCs. The percentage of both PMN-MDSCs and M-MDSCs decreased significantly each time point, from the second vaccine visit through the 1 month post vaccine visit when compared to the baseline first vaccine visit ((p < 0.001 for all PMN-MDSC time points, p = 0.003 for M-MDSC second vaccine time point and p < 0.001 for all other M-MDSC time points), Fig. 2C and 2D).

3.5. Cytokines and chemokine profiles in the melanoma and control populations

To monitor changes in cytokines and chemokines in dogs with melanoma serum samples collected prior to vaccination at each of the four vaccine visits and 1 month following the fourth vaccine were analyzed for IL-10, GM-CSF and MCP-1. The median cytokine concentration for the melanoma and control populations at baseline are summarized in Table 3.

Serum levels of both IL-10 and MCP-1 were significantly higher at baseline in the melanoma population compared to the control population (p = 0.046 and p = 0.035 respectively) but not GM-CSF (p = 0.354) (Fig. 3). Within the melanoma population there were no significant differences in serum levels of GM-CSF, IL-10 and MCP-1 among the five evaluated time points.

We performed a pilot assessment for MCP-1 using a canine

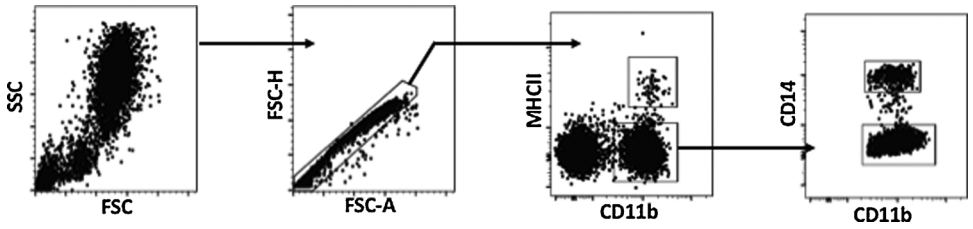

Fig. 1. Gating strategy for analysis of M-MDSC and PMN-MDSC in canine peripheral blood. Representative flow cytometric analysis of cells from a patient with melanoma. Whole blood was stained with CD11b, MHCII and CD14 antibodies. FSC vs. SSC acquisition gate was set to exclude dead cells. FSC-A vs. FSC-H was used to exclude doublets. CD11b vs. MHCII was used to identify the $CD11b^{+}MHCII^{-}$ population, which was then gated as CD11b vs. CD14 to identify the $CD11b^{+}MHCII^{-}CD14^{+}$ (M-MDSC) and $CD11b^{+}MHCII^{-}$CD14- (PMN-MDSC) populations.

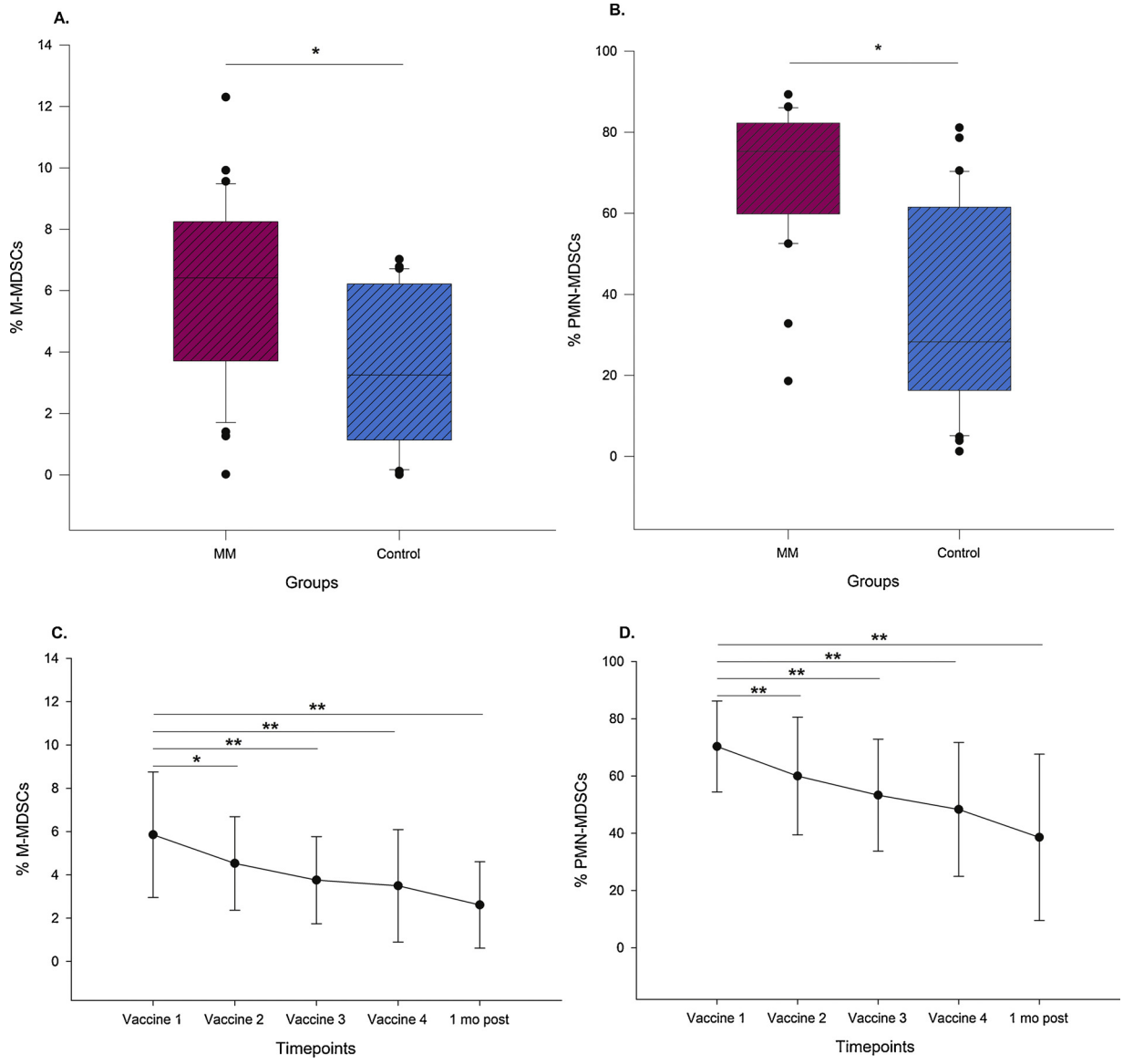

Fig. 2. Comparison of percent M-MDSCs and PMN-MDSCs between melanoma (MM) and control groups and across vaccine time points. Measurements in A and B were at baseline which is equivalent to the vaccine 1 time point in C and D. Box plot, whiskers and dots in A and B represent IQR, 10^{th} and 90^{th} percentile and outliers respectively. Error bars represent standard deviation in the line-scatter plot for C and D. Percent M-MDSCs (A) and PMN-MDSCs (B) were significantly higher in the MM group were compared to the control group (Mann-Whitney Rank Sum Test $p = 0.001$ and $p < 0.001$ respectively). Percent MDSCs decreased from baseline (designated vaccine 1) at each vaccine time point for both PMN-MDSCs (C) and M-MDSCs (D). (ANOVA * $p = 0.003$, ** $p < 0.001$).

metastatic melanoma cell line (Remi) to see if tumor cells were capable of MCP-1 production and to what concentration. The Remi cell line had a MCP-1 concentration of 577.735 pg/mL, which is similar to the median serum MCP-1 concentration of 529.32 pg/mL seen in the melanoma population.

3.6. Blood cell counts at study entry in the melanoma and control populations

We evaluated complete blood cell count (CBC) data in both populations to look at the overall cell distribution for identification of

Table 3
Summary of GM-CSF, IL-10 and MCP-1 concentrations (pg/mL) at baseline for the melanoma and control populations.

Cytokine	Melanoma			Control			
	Median	IQR	Range	Median	IQR	Range	*p* value
GM-CSF	141.36	9.2-1194.63	9.2-326546.78	35.31	9.2-209.52	9.2-433,897.32	0.354
IL-10	**8.5**	**8.5-28.32**	**0.99-207.96**	**8.5**	**8.5-8.5**	**8.5-96.33**	**0.046**
MCP-1	**529.32**	**352.35-655.07**	**216.30-3,774.48**	**334.85**	**263.42-599.76**	**21.0-8,886.72**	**0.035**

Significant differences between cytokine groups are indicated in bold.

possible extreme outliers that might need to be excluded from flow cytometry analysis. No extreme outliers were identified. We were then able to compare the baseline CBC data collected at time of study entry for both groups and prior to vaccination for the melanoma population, for any significant differences between the populations. Median, mean and range for both groups are summarized in Table 4.

The median white blood cell count (WBC), neutrophil and platelet counts were significantly higher in the melanoma population compared to the control population ($p = 0.036$, $p = 0.002$ and $p = 0.025$, respectively). The clinical significance of this finding is unknown as the medians in the melanoma population for WBC (8.23 x 10^3/μl with IQR 6.405–11.77), neutrophil (5.99 × 10^3/μl with IQR 4.33–7.519) and platelet counts (295 × 10^3/μl with IQR 225–391.5) were all within reference ranges. Similarly, for the control population medians for WBC (7.165 × 10^3/μl with IQR 6.405–11.77), neutrophil (4.58 × 10^3/μl with IQR 4.33–7.519) and platelet counts (230.5 × 10^3/μl with IQR 225–391.5) were also within reference range. Five dogs (15%) in the melanoma population had a leukocytosis (ranging from 13.4 to 30.4 × 10^3/μl; reference interval 5–13 × 10^3/μl), characterized by a mature neutrophilia (ranging from 9.24 to 26 × 10^3/μl; reference interval 2.7–8.9 × 10^3/μl). Eight dogs (24%) in the melanoma population has mild to moderate thrombocytosis (ranging from 402 to 709 × 10^3/μl; reference interval 134–396 × 10^6/μl). There were no significant differences in the lymphocyte, monocyte, eosinophil or basophil counts between both group populations.

The mean red blood cell count (RBC) and median hematocrit were significantly lower in the melanoma population when compared to the control population ($p = 0.007$ and $p = 0.014$, respectively). The mean RBC for the melanoma population (6.556 x 10^6/μl with SD ± 0.879) and for the control population (7.139 × 10^6/μl with SD ± 0.777) remained within the reference interval. Similarly, the median hematocrit in the melanoma (44.4% with IQR 40.3–49.9) and control populations (48.25% with IQR 43.9–52.675) was also within the reference intervals. Four dogs (12%) in the melanoma population did have RBC counts mildly below reference interval (ranging from 4.69 to 5.64 × 10^6/μl; reference interval 5.7–8.3 × 10^6/μl) with concurrent mildly decreased hematocrit (ranging from 33.4 to 39.8%; reference interval 40–56%). All of the anemias were found to be non-regenerative with two being mildly microcytic and normochromic and two being normocytic and normochromic.

4. Discussion

Immunotherapy is currently a major field of research in the treatment of cancer, with the goal of using the immune system to prevent tumor development or reject a tumor once development has occurred. The greatest challenge of immunotherapy is that tumors develop in spite of the immune system, which is thought to be, in part, due to an immunosuppressive tumor microenvironment that prevents effective infiltration of immune cells (Tikoo and Haass, 2015; Vesely et al., 2011). Counteracting the immunosuppressive tumor microenvironment is a potential way to hinder mechanisms of tumor evasion and to improve the efficacy of immunotherapy. MDSCs have recently emerged as one of these key players (Tcyganov et al., 2018; Tsai et al., 2014). The goal of this study was to recapitulate the flow cytometry protocol by Goulard et al., 2012 in whole blood samples from dogs with melanoma and to compare changes in MDSC subsets and potential influential cytokines overtime in response to the administration of the UF GD3-based vaccine in the absence of additional cytotoxic cancer therapy.

As part of our flow cytometric analysis, we purposefully chose to use whole blood as opposed to PBMC technique, which is in contrast to the majority of published protocols described for isolation of MDSCs

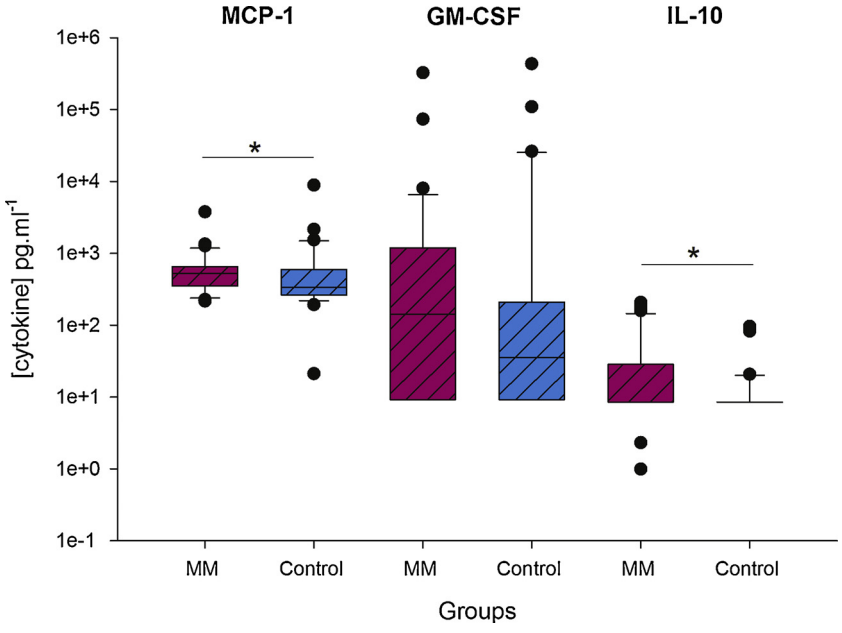

Fig. 3. Differences in serum concentrations of MCP-1, GM-CSF and IL-10 between melanoma (MM) and control groups. A log10 scale was used for the y-axis for best visual display of results. MCP-1 and IL-10 were significantly higher in the MM group compared to control group (Mann-Whitney Rank Sum Test * $p = 0.035$ and $p = 0.046$ respectively). There was no significant difference in GM-CSF concentration between groups (Mann-Whitney Rank Sum Test $p > 0.05$).

Table 4
Summary of Complete Blood Count (CBC) data and relevant statistics for the melanoma and control populations at baseline.

Parameter	Reference interval	Melanoma			Control			*p* value
		Median	Mean	Range	Median	Mean	Range	
WBC	5-13 (x 10^3/μL)	**8.230**	9.558	5.34-30.4	**7.165**	7.379	2.930-13.42	**0.036**
Neutrophils	2.7-8.9 (x 10^3/μL)	**5.99**	6.979	2.790-26.0	**4.58**	4.676	1.8-9.24	**0.002**
Lymphocytes	0.9-3.4 (x 10^3/μL)	1.41	1.556	0.63-3.46	1.73	0.606	0.38-3.0	0.372
Monocytes	0.1-0.8 (x 10^3/μL)	0.45	0.565	0.0-2.8	0.33	0.218	0.07-0.81	0.196
Platelets	134-396 (x 10^3/μL)	**295**	330.121	174-739	**230.5**	257.533	136-562	**0.025**
RBC	5.7-8.3 (x 10^6/μL)	6.64	**6.556**	4.66-8.18	7.085	**7.139**	5.82-9.07	**0.007**
HCT	40-56%	**44.4**	44.398	32.2-54.7	**48.846**	47.513	41.6-61.6	**0.014**

Data presented as mean, median and range. RBC = red blood cell, WBC = white blood cell, HCT = hematocrit.
Significantly different between the medians or means of the groups is indicated in bold.

(Bronte et al., 2016; Damuzzo et al., 2015; Khaled et al., 2013). Although a PBMC technique offers the convenience of cryopreservation, allowing samples to be stored or batched for future analysis, previously published reports indicate that gradient separation and cryopreservation may cause alterations in the MDSC subsets (Draxler et al., 2017; Duffy et al., 2013; Flörcken et al., 2015). One study found that the proportion of M-MDSCs were increased in PBMCs that were frozen overnight when compared to whole blood from the same patients (Draxler et al., 2017), while another study found that, although the relative frequencies of M-MDSCs were not significantly different between whole blood, gradient separated fresh PBMCs and gradient separated frozen PBMCs from the same patients, the absolute count of M-MDSCs was significantly greater in whole blood compared to fresh PBMCs (Duffy et al., 2013). Since M-MDSCs were of particular interest in our study due to their consistently reported elevation in human melanoma patients (Jiang et al., 2014; Khaled et al., 2013), we chose a whole blood protocol in order to minimize any potential influence from the processing method on their assessment and to maximize absolute numbers per volume of blood.

Using whole blood and the Goulard et al. (2012) flow cytometry protocol, we were able to demonstrate consistently the presence of two distinct subsets of cells, $CD11b^+MHCII^-CD14^-$ defined as PMN-MDSCs and $CD11b^+MHCII^-CD14^+$ defined as M-MDSCs, in both the healthy control dogs and melanoma patients. Both subsets were significantly increased in the melanoma patients compared to the control group. Previous work published by Goulart et al. (2012) using the same antibody combinations to define the major subsets of MDSCs demonstrated the presence of both subsets in a variety of canine cancers. That group found only the PMN-MDSC subset to be significantly increased in dogs with advanced stage or metastatic cancer compared to healthy control dogs. The type and degree of MDSC subset expansion is known to vary in humans among different cancer types patients (Jiang et al., 2014; Khaled et al., 2013) and Goulart et al. (2012) did not have any melanoma patients in their study, which may have been a reason they did not find significant increases in the M-MDSCs. Additionally, it is possible that the protocol used by Goulart et al. (2012) (gradient separated fresh and frozen PBMC technique) could have led to alterations in quantities of MDSCs, especially an increase in PMN-MDSCs and decreased capability to quantify M-MDSCs. We believe that our study is the first to describe an increased percentage of M-MDSCs in tumor-bearing dogs. This finding is consistent with the human data, as M-MDSCs are frequently reported to be increased in human advanced stage cutaneous melanoma (Jiang et al., 2015; Meyer et al., 2014; Rudolph et al., 2014) and elevation in both major MDSC subsets has been occasionally reported (Jordan et al., 2013; Schilling et al., 2013).

Interestingly, our study did not find any statistical difference associated with the stage of melanoma when individual stages were evaluated or when stages were grouped into those that included metastasis versus those that did not. Increasing MDSC levels have been reported with increasing stage in multiple human cancers, including melanoma, as well as in dogs with mammary tumors (Jiang et al., 2014; Khaled et al., 2013; Mucha et al., 2014), however, the finding of MDSCs increasing as stage increases is not a universally reported in humans. Rudolph et al. (2014) reported that M-MDSCs became elevated early in the disease course of human cutaneous melanoma and did not significantly change with increasing disease stage. This is in contrast to other reports which found that MDSCs increased as the stage of cutaneous melanoma increased (Jiang et al., 2015; Jordan et al., 2013). The available literature for MDSCs and stage in humans is only for cutaneous melanoma and there is not information regarding MDSCs and stage for the mucosal form, which canine oral melanoma most may closely resembles (Gillard et al., 2014; Schiffman and Breen, 2015; Simpson et al., 2014). It is possible that the difference seen between our canine melanoma patient and the human data described could be due to inherent differences between the tumor forms studied. Indeed, a recent article by Wong et al (Wong et al., 2019) found differences in mutation profiles between canine (oral) and human mucosal melanoma which may cast some doubt on the true value of canine melanoma as a model for human mucosal melanoma. Additionally, the small group size in each stage category in our study population could have led to a type II error.

No significant difference in levels of either MDSC subsets between high MI and low MI tumors was identified in our patient population although mitotic index has been associated with prognosis and used as a predictor for more aggressive biologic behavior in dogs diagnosed with melanoma (Smedley et al., 2011). This lack of significance could be due to a type II error as only 9 patients in our study population had a low MI tumor. While additional patients with low MI tumors would help to further investigate this, accrual may be difficult at our referral institution as these patients do not typically need any additional therapy beyond local treatment.

With regard to the GD3-based immunotherapy, both MDSC subsets were found to significantly decrease after a single administration of the GD3-based vaccine and they continued to decrease after additional vaccinations. The initial pilot safety study of the GD3-based vaccine showed that maximal GD3-specific IgG and IgM titers were achieved after the second and third vaccines, respectively (Milner et al., 2006). An immune response causing a decrease in tumor burden and subsequent decrease in MDSCs could explain our results. However, we suspect that this is unlikely as the majority of patients in this study had no evidence of disease on staging at the time of study entry, with only 7 having measurable disease at the time of the first vaccine and multiple vaccines are needed for a significant immune response to arise. Approximately 90% (n = 30) of the melanoma patients had surgical removal of the primary tumor prior to enrollment with only 3 patients receiving the first vaccine ≤ 7 days post-operatively making surgical intervention alone unlikely to be the cause of the observed decrease in MDSCs.

An alternative explanation for the early onset of MDSCs decline could be linked to the vaccine itself, which contains CpG motifs. CpG

motifs have been shown to lead to a reduction in MDSCs in multiple murine studies, often through causing MDSC maturation and differentiation (James et al., 2014; Shirota et al., 2012; Zoglmeier et al., 2011). In these studies, this phenomenon was accompanied by a reduction of the immunosuppressive capacity of the remaining MDSCs. There is contradictory evidence as to which MDSC subset is most affected. Both James et al. (2014) and Zoglmeier et al. (2011) found that following CpG treatment, PMN-MDSCs were significantly reduced with a corresponding slight increase in M-MDSCs, whereas Shirota et al. (2012) found that M-MDSCs were reduced with no change in PMN-MDSCs. We postulate that the CpG-ODNs contained in the GD3-based vaccine may be the cause of the early significant reduction in both MDSC subsets in our population and that it also significantly contributes to the continued MDSC reductions seen with each subsequent vaccine, although the possibility of decreasing tumor burden as a contributing factor that cannot be completely excluded.

We found serum IL-10 and MCP-1 concentrations were significantly higher in the melanoma group than the control group, with no significant changes in those serum concentrations in melanoma patients over time during therapy, while serum GM-CSF was not significantly different between the groups. While MDSC levels decreased significantly with each vaccination, this decline was not associated with a corresponding decrease in IL-10 or MCP-1 serum concentration. IL-10 is an inhibitory cytokine that inhibits the actions of activated macrophages and dendritic cells, thereby controlling innate immune reactions and cell mediated immunity (Yao et al., 2013). Increased serum IL-10 levels have been previously observed in other canine cancers as well, including lymphoma and inflammatory mammary cancer (Calvalido et al., 2016; de Andrés et al., 2013) and to our knowledge, this has not been reported yet in canine melanoma. While the relative stability of IL-10 levels in face of the immunotherapy provided in this study is an interesting finding, its significance remains unclear. MDSCs can produce IL-10 and this has been implicated as one of the mechanisms by which they cause immune suppression (Marvel and Gabrilovich, 2015). However, other cells such as subsets of lymphocytes and mast cells can also produce this interleukin (Yao et al., 2013), and this could, in part, play a role in the results obtained in this study.

We speculate that the tumor cells in our patients could be responsible for producing the high levels of MCP-1 circulating in their serum, which is supported by our in vitro assessment of a metastatic melanoma cell line which showed that this tumor cell line was able to produce MCP-1 at a concentration comparable to the median serum value observed in the melanoma population. However, the MCP-1 serum concentration in the melanoma population did not significantly change over five months of observation despite decreases in MDSCs and low population tumor burdens. Increased MCP-1 has also been reported in dogs with inflammatory disease and endocrine disease and may not be a specific marker for cancer in dogs (Ishioka et al., 2013; O'Neill et al., 2009) and the specific cause of the persistent MCP-1 elevation seen in our study is not currently clear. Although MCP-1 has been shown to be elevated in multiple human cancers, the nature of its role still remains unclear as it has been reported to have both pro and antitumor effects (Conti and Rollins, 2004). In recent years, the signaling axis between MCP-1 and its receptor CCR2 has been implicated in having a key role in tumorigenesis and metastasis (Lim et al., 2016; Zhang et al., 2010). Particularly, MCP-1 has been shown to play a major role in MDSC recruitment to the tumors sites and enhance their immunosuppressive function (Chang et al., 2016; Chun et al., 2015; Huang et al., 2007; Lim et al., 2016). MCP-1 has previously been reported to be elevated in other canine cancers, including lymphoma and histiocytic sarcoma, and was found to be an independent poor prognostic factor for lymphoma (Calvalido et al., 2016; Nikolic Nielsen et al., 2013; Perry et al., 2011). To our knowledge this is the first report of elevated serum MCP-1 concentrations in canine melanoma patients. Further investigation is needed to elucidate if MCP-1 has any prognostic significance in canine melanoma.

We evaluated CBC data at baseline in the melanoma and control groups, as elevated pre-treatment neutrophil and monocyte counts have been reported to be associated with worse outcomes in human melanoma and some canine cancers (Perry et al., 2011; Schmidt et al., 2005; Sottnik et al., 2010). Both WBC and neutrophil counts were both significantly higher in melanoma dogs compared to the controls. A similar finding has been previously reported in dogs diagnosed with lymphoma (Calvalido et al., 2016), but to our knowledge, this has not yet been reported in dogs diagnosed with melanoma. No significant difference in monocyte count between the two groups was observed, which is in contrast to the data reported in canine patients diagnosed with lymphoma, in which monocyte counts were elevated compared to controls (Perry et al., 2011). We also found that both RBC counts, and hematocrit were significantly lower in the melanoma group. This did not translate to clinical significance as the 4 anemic patients in this study had only a very mild anemia which was consistent with anemia of inflammatory disease. The platelet counts in the melanoma group were significantly higher than the control group, which is consistent with previous studies reporting inflammation and neoplasia to be the most common causes of thrombocytosis in dogs (Athanasiou et al., 2017; Neel et al., 2012).

This study has several limitations. First, the majority of dogs in this study had undergone excision of the primary tumor before enrollment, which may have affected baseline levels of MDSCs. Previous studies have shown that surgery can affect MDSC levels, with an initial increase, usually within seven days, and then decrease a in MDSCs post-surgery (Brusa et al., 2013; Yuan et al., 2015). The time between surgery and presentation to our institution varied widely in our population from 1 to 75 days, with only 3 out of 33 patients presenting for baseline enrollment at seven days or less post-operatively. Therefore, we suspect that the influence of surgery with regards to increasing the baseline numbers of MDSCs was likely minimal.

Second, we were unable to assess the function of the MDSCs identified in our study. Despite optimization of the technique, with the use of a whole blood protocol to maximize MDSC numbers, we were unable to sort adequate numbers of viable cells for co-culture experiments or RT-PCR assays. Goulart et al. (2012) were able to perform co-culture experiments for both PMN-MDSC and M-MDSC and demonstrated their T-cell suppression activity. Since we used the same antibody combination and clones to define our MDSC subsets, we speculate that the cells identified in our study would have had similar immune suppressive function. We speculate that the combination of advanced stage patients, which per their results had the highest levels of MDSCs, and macroscopic disease likely provided an overall greater quantity of MDSCs in their study than we were able to achieve.

Third, in this study, we chose to focus on circulating MDSCs and serum cytokine levels as opposed to evaluating tumor tissue. Previous evidence has shown that MDSC number and phenotype can shift in the tumor microenvironment, with M-MDSC being predominant and being driven into differentiating into tumor-associated macrophages (TAMs) (Tcyganov et al., 2018). Chemokine and cytokine concentrations have also shown to differ between paired serum and tumor samples, and studies have shown greater concentrations of MCP-1, IL-10 and GM-CSF, among others in tumor samples compared to serum (Kim et al., 2014; Yamauchi et al., 2018). Evaluation of both serum and tumor lysate MDSCs and cytokine levels would have allowed for a more comprehensive assessment of the immune response in our patients, and this will be a future focus of our group.

In conclusion, our group was able to identify both major MDSC subsets in two canine populations based on the Goulard et al. (2012) protocol. We observed differences in the serum cytokines IL-10 and MCP-1 levels, in the major MDSC subsets and in CBC profiles in dogs diagnosed with melanoma compared to healthy controls. This characterization can serve as a baseline data for the use of investigation of various therapeutics in a canine melanoma. The perfection and implementation of reliable, repeatable techniques for identification of

MDSCs in veterinary patients is an important tool in achieving this goal. Furthermore, we were able to show that MDSC subsets decreased over time in response to a GD3-CpG-ODN-based vaccine, and that serum concentrations of GM-CSF, MCP-1, and IL-10 were unchanged overtime in this population of dogs with melanoma. Data from this study will eventually be used to identify potential prognostic indicators related to the MDSCs and to the cytokines/chemokines GM-CSF, MCP-1, and IL-10 at baseline and over time, but further accrual of long term patient data is needed.

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Declaration of Competing Interest

None.

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References

Alexander, A.N., Huelsmeyer, M.K., Mitzey, A., Dubielzig, R.R., Kurzman, I.D., Macewen, E.G., Vail, D.M., 2006. Development of an allogeneic whole-cell tumor vaccine expressing xenogeneic gp100 and its implementation in a phase II clinical trial in canine patients with malignant melanoma. Cancer Immunol. Immunother. CII 55, 433–442. https://doi.org/10.1007/s00262-005-0025-6.

Athanasiou, L.V., Polizopoulou, Z.S., Papavasileiou, E.G., Mpairamoglou, E.L., Kantere, M.C., Rousou, X.A., 2017. Magnitude of reactive thrombocytosis and associated clinical conditions in dogs. Vet. Rec. 181, 267. https://doi.org/10.1136/vr.104042.

Banerjee, A., Vasanthakumar, A., Grigoriadis, G., 2013. Modulating T regulatory cells in cancer: how close are we? Immunol. Cell Biol. 91, 340–349.

Bergman, P.J., McKnight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., Wulderk, M., Jeffers, Y., Sadelain, M., Hohenhaus, A.E., Segal, N., Gregor, P., Engelhorn, M., Riviere, I., Houghton, A.N., Wolchok, J.D., 2003. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin. Cancer Res. 9, 1284–1290.

Brodersen, R., Bijlsma, F., Gori, K., Jensen, K., Chen, W., Dominguez, J., Haverson, K., Moore, P., Saalmüller, A., Sachs, D., Slierendrecht, W., Stokes, C., Vainio, O., Zuckermann, F., Aasted, B., 1998. Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against various leucocyte surface antigens of human and 11 animal species. Vet. Immunol. Immunopathol. 64, 1–13. https://doi.org/10.1016/S0165-2427(98)00117-2.

Bronte, V., Brandau, S., Chen, S.-H., Colombo, M.P., Frey, A.B., Greten, T.F., Mandruzzato, S., Murray, P.J., Ochoa, A., Ostrand-Rosenberg, S., Rodriguez, P.C., Sica, A., Umansky, V., Vonderheide, R.H., Gabrilovich, D.I., 2016. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat. Commun. 7, 12150. https://doi.org/10.1038/ncomms12150.

Brusa, D., Simone, M., Gontero, P., Spadi, R., Racca, P., Micari, J., Degiuli, M., Carletto, S., Tizzani, A., Matera, L., 2013. Circulating immunosuppressive cells of prostate cancer patients before and after radical prostatectomy: profile comparison. Int. J. Urol. 20, 971–978. https://doi.org/10.1111/iju.12086.

Calvalido, J., Wood, G.A., Mutsaers, A.J., Wood, D., Sears, W., Woods, J.P., 2016. Comparison of serum cytokine levels between dogs with multicentric lymphoma and healthy dogs. Vet. Immunol. Immunopathol. 182, 106–114. https://doi.org/10.1016/j.vetimm.2016.10.009.

Chang, A.L., Miska, J., Wainwright, D.A., Dey, M., Rivetta, C.V., Yu, D., Kanojia, D., Pituch, K.C., Qiao, J., Pytel, P., Han, Y., Wu, M., Zhang, L., Horbinski, C.M., Ahmed, A.U., Lesniak, M.S., 2016. CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory t cells and myeloid-derived suppressor cells. Cancer Res. 76, 5671–5682. https://doi.org/10.1158/0008-5472.CAN-16-0144.

Chun, E., Lavoie, S., Michaud, M., Gallini, C.A., Kim, J., Soucy, G., Odze, R., Glickman, J.N., Garrett, W.S., 2015. CCL2 Promotes Colorectal Carcinogenesis by Enhancing Polymorphonuclear Myeloid-Derived Suppressor Cell Population and Function. Cell Rep. 12, 244–257. https://doi.org/10.1016/j.celrep.2015.06.024.

Conti, I., Rollins, B.J., 2004. CCL2 (monocyte chemoattractant protein-1) and cancer. Semin. Cancer Biol. 14, 149–154. https://doi.org/10.1016/j.semcancer.2003.10.009.

Damuzzo, V., Pinton, L., Desantis, G., Solito, S., Marigo, I., Bronte, V., Mandruzzato, S., 2015. Complexity and challenges in defining myeloid-derived suppressor cells: defining Myeloid-Derived Suppressor Cells. Cytometry B Clin. Cytom. 88, 77–91. https://doi.org/10.1002/cyto.b.21206.

de Andrés, P.J., Illera, J.C., Cáceres, S., Díez, L., Pérez-Alenza, M.D., Peña, L., 2013. Increased levels of interleukins 8 and 10 as findings of canine inflammatory mammary cancer. Vet. Immunol. Immunopathol. 152, 245–251. https://doi.org/10.1016/j.vetimm.2012.12.010.

De Sanctis, F., Solito, S., Ugel, S., Molon, B., Bronte, V., Marigo, I., 2016. MDSCs in cancer: conceiving new prognostic and therapeutic targets. Biochim. Biophys. Acta BBA - Rev. Cancer, The role of the immune system in cancer: from mechanisms to clinical applications 1865, 35–48. https://doi.org/10.1016/j.bbcan.2015.08.001.

Dolcetti, L., Peranzoni, E., Ugel, S., Marigo, I., Fernandez Gomez, A., Mesa, C., Geilich, M., Winkels, G., Traggiai, E., Casati, A., Grassi, F., Bronte, V., 2009. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. Eur. J. Immunol. 40, 22–35. https://doi.org/10.1002/eji.200939903.

Dow, S.W., Elmslie, R.E., Willson, A.P., Roche, L., Gorman, C., Potter, T.A., 1998. In vivo tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. J. Clin. Invest. 101, 2406–2414. https://doi.org/10.1172/JCI510.

Draxler, D.F., Madondo, M.T., Hanafi, G., Plebanski, M., Medcalf, R.L., 2017. A flowcytometric analysis to efficiently quantify multiple innate immune cells and T Cell subsets in human blood. Cytom. Part J. Int. Soc. Anal. Cytol. 91, 336–350. https://doi.org/10.1002/cyto.a.23080.

Duffy, A., Zhao, F., Haile, L., Gamrekelashvili, J., Fioravanti, S., Ma, C., Kapanadze, T., Compton, K., Figg, W.D., Greten, T.F., 2013. Comparative analysis of monocytic and granulocytic myeloid-derived suppressor cell subsets in patients with gastrointestinal malignancies. Cancer Immunol. Immunother. 62, 299–307. https://doi.org/10.1007/s00262-012-1332-3.

Finocchiaro, L.M.E., Fondello, C., Gil-Cardeza, M.L., Rossi, Ú.A., Villaverde, M.S., Riveros, M.D., Glikin, G.C., 2015. Cytokine-enhanced vaccine and Interferon-β plus suicide gene therapy as surgery adjuvant treatments for spontaneous canine melanoma. Hum. Gene Ther. 26, 367–376. https://doi.org/10.1089/hum.2014.130.

Flörcken, A., Takvorian, A., Singh, A., Gerhardt, A., Ostendorf, B.N., Dörken, B., Pezzutto, A., Westermann, J., 2015. Myeloid-derived suppressor cells in human peripheral blood: optimized quantification in healthy donors and patients with metastatic renal cell carcinoma. Immunol. Lett. 168, 260–267. https://doi.org/10.1016/j.imlet.2015.10.001.

Gabrilovich, D.I., Nagaraj, S., 2009. Myeloid-derived suppressor cells as regulators of the immune system. Nat. Rev. Immunol. 9, 162–174. https://doi.org/10.1038/nri2506.

Gabrilovich, D.I., Ostrand-Rosenberg, S., Bronte, V., 2012. Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 12, 253–268. https://doi.org/10.1038/nri3175.

Gillard, M., Cadieu, E., De Brito, C., Abadie, J., Vergier, B., Devauchelle, P., Degorce, F., Dréano, S., Primot, A., Dorso, L., Lagadic, M., Galibert, F., Hédan, B., Galibert, M.-D., André, C., 2014. Naturally occurring melanomas in dogs as models for non-UV pathways of human melanomas. Pigment Cell Melanoma Res. 27, 90–102. https://doi.org/10.1111/pcmr.12170.

Goulart, M.R., Pluhar, G.E., Ohlfest, J.R., 2012. Identification of myeloid derived suppressor cells in dogs with naturally occurring Cancer. PLoS One 7, e33274. https://doi.org/10.1371/journal.pone.0033274.

Goulart, M.R., Hlavaty, S.I., Chang, Y.-M., Polton, G., Stell, A., Perry, J., Wu, Y., Sharma, E., Broxholme, J., Lee, A.C., Szladovits, B., Turmaine, M., Gribben, J., Xia, D., Garden, O.A., 2019. Phenotypic and transcriptomic characterization of canine myeloid-derived suppressor cells. Sci. Rep. 9, 3574. https://doi.org/10.1038/s41598-019-40285-3.

Helfand, S.C., Dickerson, E.B., Munson, K.L., Padilla, M.L., 1999. GD3 ganglioside antibody augments tumoricidal capacity of canine blood mononuclear cells by induction of interleukin 12. Cancer Res. 59, 3119–3127.

Helfand, S.C., Soergel, S.A., Modiano, J.F., Hank, J.A., Sondel, P.M., 1994. Induction of lymphokine-activated killer (LAK) activity in canine lymphocytes with low dose human recombinant interleukin-2 in vitro. Cancer Biother. 9, 237–244.

Hernandez, B., Adissu, H.A., Wei, B.-R., Michael, H.T., Merlino, G., Simpson, R.M., 2018. Naturally Occurring Canine Melanoma as a Predictive Comparative Oncology Model for Human Mucosal and Other Triple Wild-Type Melanomas. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19020394.

Hogge, G.S., Burkholder, J.K., Culp, J., Albertini, M.R., Dubielzig, R.R., Yang, N.S., MacEwen, E.G., 1999. Preclinical development of human granulocyte-macrophage colony-stimulating factor-transfected melanoma cell vaccine using established canine cell lines and normal dogs. Cancer Gene Ther. 6, 26–36. https://doi.org/10.1038/sj.cgt.7700015.

Huang, B., Lei, Z., Zhao, J., Gong, W., Liu, J., Chen, Z., Liu, Y., Li, D., Yuan, Y., Zhang, G.-M., Feng, Z.-H., 2007. CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers. Cancer Lett. 252, 86–92. https://doi.org/10.1016/j.canlet.2006.12.012.

Ishioka, K., Suzuki, Y., Tajima, K., Ohtaki, S., Miyabe, M., Takasaki, M., Mori, A., Momota, Y., Azakami, D., Sako, T., 2013. Monocyte chemoattractant protein-1 in dogs affected with neoplasia or inflammation. J. Vet. Med. Sci. 75, 173–177.

Jacobsen, C.N., Aasted, B., Broe, M.K., Petersen, J.L., 1993. Reactivities of 20 anti-human monoclonal antibodies with leucocytes from ten different animal species. Vet. Immunol. Immunopathol. 39, 461–466. https://doi.org/10.1016/0165-2427(93)90075-F.

James, B.R., Anderson, K.G., Brincks, E.L., Kucaba, T.A., Norian, L.A., Masopust, D., Griffith, T.S., 2014. CpG-mediated modulation of MDSC contributes to the efficacy of Ad5-TRAIL therapy against renal cell carcinoma. Cancer Immunol. Immunother. 63,

1213–1227. https://doi.org/10.1007/s00262-014-1598-8.
Jiang, H., Gebhardt, C., Umansky, L., Beckhove, P., Schulze, T.J., Utikal, J., Umansky, V., 2015. Elevated chronic inflammatory factors and myeloid-derived suppressor cells indicate poor prognosis in advanced melanoma patients. Int. J. Cancer 136, 2352–2360. https://doi.org/10.1002/ijc.29297.
Jiang, J., Guo, W., Liang, X., 2014. Phenotypes, accumulation, and functions of myeloid-derived suppressor cells and associated treatment strategies in cancer patients. Hum. Immunol. 75, 1128–1137. https://doi.org/10.1016/j.humimm.2014.09.025.
Jordan, K.R., Amaria, R.N., Ramirez, O., Callihan, E.B., Gao, D., Borakove, M., Manthey, E., Borges, V.F., McCarter, M.D., 2013. Myeloid-derived suppressor cells are associated with disease progression and decreased overall survival in advanced-stage melanoma patients. Cancer Immunol. Immunother. 62, 1711–1722. https://doi.org/10.1007/s00262-013-1475-x.
Khaled, Y.S., Ammori, B.J., Elkord, E., 2013. Myeloid-derived suppressor cells in cancer: recent progress and prospects. Immunol. Cell Biol. 91, 493–502.
Kim, Y.W., Kim, S.-K., Kim, C.S., Kim, I.Y., Cho, M.Y., Kim, N.K., 2014. Association of serum and intratumoral cytokine profiles with tumor stage and neutrophil lymphocyte ratio in colorectal Cancer. Anticancer Res. 34, 3481–3487.
Lana, S., Plaza, S., Hampe, K., Burnett, R., Avery, A.C., 2006. Diagnosis of Mediastinal Masses in dogs by flow cytometry. J. Vet. Intern. Med. 20, 1161–1165. https://doi.org/10.1111/j.1939-1676.2006.tb00716.x.
Lechner, M.G., Liebertz, D.J., Epstein, A.L., 2010. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J. Immunol. 185, 2273–2284. https://doi.org/10.4049/jimmunol.1000901.
Lim, S.Y., Yuzhalin, A.E., Gordon-Weeks, A.N., Muschel, R.J., 2016. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget 7, 28697.
Lindsay, C.R., Spiliopoulou, P., Waterston, A., 2015. Blinded by the light: why the treatment of metastatic melanoma has created a new paradigm for the management of cancer. Ther. Adv. Med. Oncol. 7, 107–121.
MacEwen, E.G., Kurzman, I.D., Vail, D.M., Dubielzig, R.R., Everlith, K., Madewell, B.R., Rodriguez, C.O., Phillips, B., Zwahlen, C.H., Obradovich, J., Rosenthal, R.C., Fox, L.E., Rosenberg, M., Henry, C., Fidel, J., 1999. Adjuvant therapy for melanoma in dogs: results of randomized clinical trials using surgery, liposome-encapsulated muramyl tripeptide, and granulocyte macrophage colony-stimulating factor. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. 5, 4249–4258.
MacEwen, E.G., Patnaik, A.K., Harvey, H.J., Hayes, A.A., Matus, R., 1986. Canine oral melanoma: comparison of surgery versus surgery plus Corynebacterium parvum. Cancer Invest. 4, 397–402.
Mandruzzato, S., Brandau, S., Britten, C.M., Bronte, V., Damuzzo, V., Gouttefangeas, C., Maurer, D., Ottensmeier, C., van der Burg, S.H., Welters, M.J.P., Walter, S., 2016. Toward harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study. Cancer Immunol. Immunother. 65, 161–169. https://doi.org/10.1007/s00262-015-1782-5.
Manley, C.A., Leibman, N.F., Wolchok, J.D., Rivière, I.C., Bartido, S., Craft, D.M., Bergman, P.J., 2011. Xenogeneic murine tyrosinase DNA vaccine for malignant melanoma of the digit of dogs. J. Vet. Intern. Med. 25, 94–99. https://doi.org/10.1111/j.1939-1676.2010.0627.x.
Marvel, D., Gabrilovich, D.I., 2015. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. J. Clin. Invest. https://doi.org/10.1172/JCI80005.
Meyer, C., Cagnon, L., Costa-Nunes, C.M., Baumgaertner, P., Montandon, N., Leyvraz, L., Michielin, O., Romano, E., Speiser, D.E., 2014. Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. Cancer Immunol. Immunother. 63, 247–257. https://doi.org/10.1007/s00262-013-1508-5.
Milner, R.J., Salute, M., Crawford, C., Abbot, J.R., Farese, J., 2006. The immune response to disialoganglioside GD3 vaccination in normal dogs: a melanoma surface antigen vaccine. Vet. Immunol. Immunopathol. 114, 273–284. https://doi.org/10.1016/j.vetimm.2006.08.012.
Mucha, J., Majchrzak, K., Taciak, B., Hellmén, E., Król, M., 2014. MDSCs mediate angiogenesis and predispose canine mammary tumor cells for metastasis via IL-28/IL-28RA (IFN-λ) signaling. PLoS One 9, e103249. https://doi.org/10.1371/journal.pone.0103249.
Neel, J.A., Snyder, L., Grindem, C.B., 2012. Thrombocytosis: a retrospective study of 165 dogs. Vet. Clin. Pathol. 41, 216–222. https://doi.org/10.1111/j.1939-165X.2012.00416.x.
Nikolic Nielsen, L., Kjelgaard-Hansen, M., Kristensen, A.T., 2013. Monocyte chemotactic protein-1 and other inflammatory parameters in Bernese Mountain dogs with disseminated histiocytic sarcoma. Vet. J. 198, 424–428. https://doi.org/10.1016/j.tvjl.2013.07.030.
O'Neill, K., Guth, A., Biller, B., Elmslie, R., Dow, S., 2009. Changes in regulatory t cells in dogs with Cancer and associations with tumor type. J. Vet. Intern. Med. 23, 875–881. https://doi.org/10.1111/j.1939-1676.2009.0333.x.
Owen, L., 1980. TNM Classification of Tumours in Domestic Animals.
Perry, J.A., Thamm, D.H., Eickhoff, J., Avery, A.C., Dow, S.W., 2011. Increased monocyte chemotactic protein-1 concentration and monocyte count independently associate with a poor prognosis in dogs with lymphoma. Vet. Comp. Oncol. 9, 55–64. https://doi.org/10.1111/j.1476-5829.2010.00235.x.
Rao, S., Lana, S., Eickhoff, J., Marcus, E., Avery, P.R., Morley, P.S., Avery, A.C., 2011. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-Cell lymphoma. J. Vet. Intern. Med. 25, 1097–1105. https://doi.org/10.1111/j.1939-1676.2011.0767.x.
Rudolph, B.M., Loquai, C., Gerwe, A., Bacher, N., Steinbrink, K., Grabbe, S., Tuettenberg, A., 2014. Increased frequencies of CD11b(+) CD33(+) CD14(+) HLA-DR(low) myeloid-derived suppressor cells are an early event in melanoma patients. Exp. Dermatol. 23, 202–204. https://doi.org/10.1111/exd.12336.
Schiffman, J.D., Breen, M., 2015. Comparative oncology: what dogs and other species can teach us about humans with cancer. Philos. Trans. R. Soc. B Biol. Sci. 370, 20140231. https://doi.org/10.1098/rstb.2014.0231.
Schilling, B., Sucker, A., Griewank, K., Zhao, F., Weide, B., Görgens, A., Giebel, B., Schadendorf, D., Paschen, A., 2013. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells: vemurafenib and MDSCs in melanoma. Int. J. Cancer 133, 1653–1663. https://doi.org/10.1002/ijc.28168.
Schmidt, H., Bastholt, L., Geertsen, P., Christensen, I.J., Larsen, S., Gehl, J., Maase, Hvonder, 2005. Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: a prognostic model. Br. J. Cancer 93, 273–278. https://doi.org/10.1038/sj.bjc.6602702.
Sherger, M., Kisseberth, W., London, C., Olivo-Marston, S., Papenfuss, T.L., 2012. Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. BMC Vet. Res. 8, 209.
Shirota, Y., Shirota, H., Klinman, D.M., 2012. Intratumoral injection of CpG oligonucleotides induces the differentiation and reduces the immunosuppressive activity of myeloid-derived suppressor cells. J. Immunol. 188, 1592–1599. https://doi.org/10.4049/jimmunol.1101304.
Simpson, R.M., Bastian, B.C., Michael, H.T., Webster, J.D., Prasad, M.L., Conway, C.M., Prieto, V.M., Gary, J.M., Goldschmidt, M.H., Esplin, D.G., Smedley, R.C., Piris, A., Meuten, D.J., Kiupel, M., Lee, C.-C.R., Ward, J.M., Dwyer, J.E., Davis, B.J., Anver, M.R., Molinolo, A.A., Hoover, S.B., Rodriguez-Canales, J., Hewitt, S.M., 2014. Sporadic naturally occurring melanoma in dogs as a preclinical model for human melanoma. Pigment Cell Melanoma Res. 27, 37–47. https://doi.org/10.1111/pcmr.12185.
Smedley, R.C., Spangler, W.L., Esplin, D.G., Kitchell, B.E., Bergman, P.J., Ho, H.-Y., Bergin, I.L., Kiupel, M., 2011. Prognostic markers for canine melanocytic neoplasms: a comparative review of the literature and goals for future investigation. Vet. Pathol. 48, 54–72. https://doi.org/10.1177/0300985810390717.
Sottnik, Jl., Rao, S., Lafferty, Mh., Thamm, Dh., Morley, Ps., Withrow, Sj., Dow, Sw., 2010. Association of blood monocyte and lymphocyte count and disease-free interval in dogs with osteosarcoma. J. Vet. Intern. Med. 24, 1439–1444. https://doi.org/10.1111/j.1939-1676.2010.0591.x.
Tcyganov, E., Mastio, J., Chen, E., Gabrilovich, D.I., 2018. Plasticity of myeloid-derived suppressor cells in cancer. Curr. Opin. Immunol. 51, 76–82. https://doi.org/10.1016/j.coi.2018.03.009.
Tikoo, S., Haass, N.K., 2015. Friends or foes: IL-10 and TGF-β in melanoma. Exp. Dermatol. 24, 254–255. https://doi.org/10.1111/exd.12661.
Tsai, M.-J., Chang, W.-A., Huang, M.-S., Kuo, P.-L., 2014. Tumor microenvironment: a new treatment target for Cancer. ISRN Biochem. 2014, 1–8. https://doi.org/10.1155/2014/351959.
Vasievich, E.A., Huang, L., 2011. The suppressive tumor microenvironment: a challenge in cancer immunotherapy. Mol. Pharm. 8, 635–641.
Vesely, M.D., Kershaw, M.H., Schreiber, R.D., Smyth, M.J., 2011. Natural innate and adaptive immunity to Cancer. Annu. Rev. Immunol. 29, 235–271. https://doi.org/10.1146/annurev-immunol-031210-101324.
von Euler, H., Sadeghi, A., Carlsson, B., Rivera, P., Loskog, A., Segall, T., Korsgren, O., Tötterman, T.H., 2008. Efficient adenovector CD40 ligand immunotherapy of canine malignant melanoma. J. Immunother. 1997 (31), 377–384. https://doi.org/10.1097/CJI.0b013e31816a812d.
Watanabe, Y., Kano, R., Maruyama, H., Hasegawa, A., Kamata, H., 2010. Small interfering RNA (siRNA) against the Bcl-2 gene increases apoptosis in a canine melanoma cell line. J. Vet. Med. Sci. 72, 383–386.
Williams, L.E., Packer, R.A., 2003. Association between lymph node size and metastasis in dogs with oral malignant melanoma: 100 cases (1987-2001). J. Am. Vet. Med. Assoc. 222, 1234–1236.
Wong, K., van der Weyden, L., Schott, C.R., Foote, A., Constantino-Casas, F., Smith, S., Dobson, J.M., Murchison, E.P., Wu, H., Yeh, I., Fullen, D.R., Joseph, N., Bastian, B.C., Patel, R.M., Martincorena, I., Robles-Espinoza, C.D., Iyer, V., Kuijjer, M.L., Arends, M.J., Brenn, T., Harms, P.W., Wood, G.A., Adams, D.J., 2019. Cross-species genomic landscape comparison of human mucosal melanoma with canine oral and equine melanoma. Nat. Commun. 10. https://doi.org/10.1038/s41467-018-08081-1.
Yamauchi, Y., Safi, S., Blattner, C., Rathinasamy, A., Umansky, L., Juenger, S., Warth, A., Eichhorn, M., Muley, T., Herth, F.J.F., Dienemann, H., Platten, M., Beckhove, P., Utikal, J., Hoffmann, H., Umansky, V., 2018. Circulating and tumor myeloid-derived suppressor cells in resectable non-small-cell lung Cancer. Am. J. Respir. Crit. Care Med. https://doi.org/10.1164/rccm.201708-1707OC.
Yao, Y., Simard, A.R., Shi, F.-D., Hao, J., 2013. IL-10-Producing lymphocytes in inflammatory disease. Int. Rev. Immunol. 32, 324–336. https://doi.org/10.3109/08830185.2012.762361.
Yuan, L., Xu, B., Fan, H., Yuan, P., Zhao, P., Suo, Z., 2015. Pre- and post-operative evaluation: percentages of circulating myeloid-derived suppressor cells in rectal cancer patients. Neoplasma 62, 239–249. https://doi.org/10.4149/neo_2015_029.
Zhang, J., Patel, L., Pienta, K.J., 2010. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev. 21, 41–48. https://doi.org/10.1016/j.cytogfr.2009.11.009.
Zhao, Yang, Wu, T., Shao, S., Shi, B., Zhao, Yong, 2016. Phenotype, development, and biological function of myeloid-derived suppressor cells. OncoImmunology 5, e1004983. https://doi.org/10.1080/2162402X.2015.1004983.
Zoglmeier, C., Bauer, H., Norenberg, D., Wedekind, G., Bittner, P., Sandholzer, N., Rapp, M., Anz, D., Endres, S., Bourquin, C., 2011. CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice. Clin. Cancer Res. 17, 1765–1775. https://doi.org/10.1158/1078-0432.CCR-10-2672.