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...
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, 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 chemotactrant 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 of at least two major MDSC subsets, monocytic (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-DRlow/−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; Sherg er 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.
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 TCG TTT GGT GCT T-3’) and GDS (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 aliquoted into two samples and initially frozen at -80 °C for long term storage until cytokine kit analysis was performed. Heparinized whole blood was used for flow cytometric analysis with all samples processed within 24 h of collection.

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− cell population, and the monocyctic myeloid-derived suppressor cell (M-MDSCs) subset as the CD11b+ MHCII+ 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 467-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., 1993; Lana et al., 2006). Cells were then washed twice before erythrocytes were lysed with BD Pharmedye 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 parafomaldehyde 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 (CCYTMAG-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 × 106) 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).

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
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.

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<th>Cytokine</th>
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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 \((8.23 \times 10^3/\mu l)\) with IQR \(6.405-11.77\), neutrophil \((5.99 \times 10^3/\mu l)\) with IQR \(4.33-7.519\) and platelet counts \((295 \times 10^3/\mu l)\) with IQR \(225-391.5\) were all within reference ranges. Similarly, for the control population medians for WBC \((7.139 \times 10^6/\mu l)\) with IQR \(7.165-11.77\), neutrophil \((5.99 \times 10^3/\mu l)\) with IQR \(4.33-7.519\) and platelet counts \((230.5 \times 10^3/\mu l)\) with IQR \(225-391.5\) were also within reference range. Five dogs \((15\%)\) in the melanoma population had a leukocytosis \((ranging from 13.4 to 225 \times 10^3/\mu l)\); reference interval \(7.165 \times 10^3/\mu l\). Eight dogs \((24\%)\) in the melanoma population had a normocytic and normochromic anemia \((ranging from 4.69 to 5.64 \times 10^6/\mu l)\); reference interval \(4.69-5.64 \times 10^6/\mu l\). 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 \((6.556 \times 10^6/\mu l)\) with SD \(\pm 0.879\) and for the control population \((7.139 \times 10^6/\mu l)\) with SD \(\pm 0.777\) 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 \((ranging from 4.69 to 5.64 \times 10^6/\mu l)\); reference interval \(5.7-8.3 \times 10^6/\mu l)\) 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 Haas, 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 inflammatory 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 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.
increased capability to quantify M-MDSCs. We believe that our study is separated fresh and frozen PBMC technique) could have led to alterations in antibody combinations to define the major subsets of MDSCs demonstrated the presence of both subsets in a variety of canine cancers. That 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 in dogs 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 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; Shirato 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 Shirato et al. (2012) found that M-MDSCs were reduced with no change in PMN-MDSCs. We postulate that the CpG-ODNs contained in the GDI3-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; Sotnik 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 counts 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 in a 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


