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Suppression of canine myeloid cells by soluble factors from cultured canine tumor cells

J. Wasserman^a, L. Diese^a, Z. VanGundy^a, C. London^a, W.E. Carson^b, and T.L. Papenfuss^{a,*}

^a Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210, United States

^b Department of Surgery, The Ohio State University Medical Center, Columbus, OH 43210, United States

Abstract

Background—Cancer profoundly affects immunity and causes immunosuppression that contributes to tumor escape, metastases and resistance to therapy. The mechanisms by which cancer cells influence immune cells are not fully known but both innate and adaptive immune cells can be altered by cancer. Myeloid cells are innate immune cells that comprise the mononuclear phagocytic system (MPS) and include monocytes, macrophages, dendritic cells (DCs) and their progenitors. Myeloid cells play important roles in both the promotion and regulation of immune responses. Dysregulated myeloid cells are increasingly being recognized as contributing to cancer-related immunosuppression. This study investigated whether soluble factors produced by canine tumor cells inhibited canine myeloid cell function.

Methods—These studies investigated the utility of using the canine DH82 cell line for assessment of canine myeloid responses to tumor-derived soluble factors (TDSFs). Phenotypic comparisons to canine bone marrow-derived DCs (BM-DCs) and bone marrow-derived macrophages (BM-MΦs) were performed and expression of myeloid cell markers CD11b, CD11c, CD80, and major histocompatibility complex (MHC) class II were evaluated by flow cytometry. Phenotypic and functional changes of DC populations were then determined following exposure to tumor-conditioned media (TCM) from canine osteosarcoma, melanoma and mammary carcinoma cell lines.

Results—We found that the canine BM-DCs and the DH82 cell line shared similar CD11b, CD11c and MHC II expression and morphologic characteristics that were distinct from canine BM-MΦs. Myeloid cells exposed to TDSFs showed decreased expression of MHC class II and CD80, had reduced phagocytic activity and suppressed the proliferation of responder immune cells.

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* Corresponding author at: Department of Veterinary Biosciences, College of Veterinary Medicine, 370 Veterinary Medical Academic Building, 1900 Coffey Road, Columbus, OH 43210, United States. Tel.: +1 614 292 7343; fax: +1 614 292 6473. papenfuss.1@osu.edu (T.L. Papenfuss)..

Conflict of interest statement

The authors declare no potential conflicts of interest.

Conclusion—These results show that soluble factors secreted from canine tumor cells suppress the activation and function of canine myeloid cells. Our results suggest that, similar to humans, dysregulated myeloid cells may contribute to immunosuppression in dogs with cancer.

Keywords

Canine; Myeloid; Dendritic cells; Macrophages; DH82; Cancer; Immunology

1. Introduction

Cancer is an important disease affecting all species. Within veterinary medicine, the National Cancer Institute estimates that nearly 6 million new cases are diagnosed in dogs each year (Shilling, 2010). Cancer has profound effects on immunity, resulting in tumor-related immunosuppression that contributes to tumor escape, metastases and resistance to therapy (Zou, 2005). In particular, immunosuppression limits the efficacy of cancer immunotherapy, a therapy designed to enhance anti-tumor immune responses (Zou, 2005). Numerous changes in both innate and adaptive immune responses occur in humans and mice with cancer. Alterations of T cell, B cell, NK cell and myeloid cell function, increased regulatory T cell activity, tumor-associated macrophages (TAMs) and recently, myeloid-derived suppressor cells (MDSCs) have all been described (Gabrilovich and Nagaraj, 2009; Mantovani et al., 2008; Pollard, 2004; Zou, 2005). Similarly, immune alterations in canine cancer patients have been reported including elevated levels of circulating regulatory T cells (Tregs), altered cytokine profiles, natural killer (NK) cells with a reduced proliferative and killing capacity and altered CD8+ (cytolytic) T cell to Treg ratios (Biller et al., 2010; Funk et al., 2005; Itoh et al., 2009; O'Neill et al., 2009) but at present, few studies have investigated altered myeloid cell function in canine cancer.

Myeloid cells are innate immune cells including monocytes, macrophages, dendritic cells (DCs) and their progenitors (Gabrilovich and Nagaraj, 2009; Papenfuss, 2010). These potent immune cells play pivotal roles in initiating and regulating inflammation and shaping adaptive immune responses. Acting both on innate (e.g. NK cells, neutrophils and other myeloid cells) and adaptive (e.g. CD4+ T cells and CD8+ T cells) immune cells, myeloid cells play important roles in host defense and homeostasis. In cancer, dysregulated and suppressive myeloid cells are now recognized as contributing to immunosuppression, metastatic spread and therapeutic failures in humans and mice (Mantovani et al., 2010; Schmid and Varner, 2010; Yang and Carbone, 2004). Recent work in dogs suggests that peripheral blood myeloid cell populations (e.g. monocytes and neutrophils) may be a prognostic indicator in canine cancer and that canine myeloid cells may be useful for canine cancer vaccines but, at present, the effects of cancer on canine myeloid cell responses has not been studied (Bird et al., 2008; Perry et al., 2011; Sottnik et al., 2010).

Tumors are able to alter myeloid cell differentiation, maturation and function to promote systemic immunosuppression. Specifically, tumors are able to generate TAMs, dysregulated or tolerogenic DCs (Tol-DCs) and MDSCs, which can contribute to the immunosuppression seen during cancer (Chioda et al., 2011; Huang et al., 2011; Mantovani et al., 2009; Pollard, 2004). Tumor-derived soluble factors (TDSFs), such as cytokines, growth factors or tumor

exosomes secreted by tumor cells can have direct effects on myeloid cells (Finn, 2008; Mantovani et al., 2008; Pollard, 2004; Zou, 2005). Human and mouse studies have highlighted the extensive interactions between the tumor microenvironment and myeloid cells but no studies have evaluated these interactions in the dog (Chioda et al., 2011; Mantovani et al., 2009; Pollard, 2004; Schmid and Varner, 2010; Yang and Carbone, 2004). With the increased use of dogs for the development and application of cancer treatments in humans (i.e. comparative oncology), this study sought to investigate whether soluble factors produced by cultured tumor cells inhibited canine myeloid cell function (Gordon et al., 2009; Khanna et al., 2006; Paoloni and Khanna, 2008; Rowell et al., 2011).

2. Materials and methods

2.1. Canine myeloid cells: DH82 cell line and bone marrow-derived DCs and macrophages

The DH82 canine myeloid cell line (kindly provided by M. Wellman) were cultured in supplemented RPMI 1640 (R-10) containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 5×10^{-5} M beta-2-mercaptoethanol (Bird et al., 2008; Wellman et al., 1988). Media was replaced as necessary and the loosely adherent DH82 cells were collected for analyses (Barnes et al., 2000; Wellman et al., 1988). For BM-DCs and BM-MΦs, bone marrow cells were collected from both femurs of research dogs following humane euthanasia. Studies were approved by the Institutional Animal Care and Use Committee (IACUC) and all dogs were maintained in university laboratory animal resources (ULAR) facilities, housed according to all IACUC guidelines. Cells were collected from bone marrow with sterile PBS containing 50 U/ml penicillin and 50 mg/ml streptomycin. The cells were concentrated by centrifugation, erythrocytes lysed with ammonium chloride lysis buffer and cells washed twice with sterile 1× PBS. The cells were then resuspended and plated in RPMI supplemented with 10% FBS and either 20 ng/ml recombinant canine granulocyte-monocyte colony stimulating factor (GM-CSF) or 20 ng/ml recombinant human GM-CSF (R&D Systems, Minneapolis, MN). Cells were incubated at 37 °C for 6–10 days (Isotani et al., 2006). Adherent cells (mΦ-enriched) were removed from culture via trypsinization (0.25% trypsin) followed by two washes in sterile PBS and non-adherent (DC-enriched) were collected and washed twice with sterile PBS for phenotypic and functional assays (Inaba et al., 2009).

2.2. Canine tumor cells and generation of tumor-conditioned media

Canine tumor cell lines were kindly provided by Dr. Cheryl London (The Ohio State University, College of Veterinary Medicine) or generated in-house from canine oncology patients. Two canine osteosarcoma lines (OSA8 and OSA16), three canine melanoma cell lines (MEL3: 323610-3, MEL4: 325086-4, and MEL5: 326437-5) and canine mammary carcinoma (MCA) were grown in supplemented RPMI 1640 containing 10% FBS, 1% HEPES (Gibco), 1% sodium pyruvate, 1% non-essential amino acids, 100 Units penicillin, 100 µg streptomycin and 0.25 µg/ml amphotericin B and 1% Glutamax (Gibco, Carlsbad, CA). Cells lines were maintained at 37 °C and split twice weekly with 0.125% trypsin/EDTA and the tumor-conditioned media (TCM) containing TDSFs was collected, sterile filtered and stored at –20 °C prior to use. TCM was then used for in vitro treatment of canine myeloid cells at 20% or 50% concentration with R-10 media. Unless otherwise

indicated, a single osteosarcoma (OSA16), mammary carcinoma and MEL (MEL-14) cell lines were used for experiments. Representative tumor cell lines were chosen based on the degree of change seen in MHC class II expression on BM-DCs during initial screening experiments. Canine myeloid cells were plated at a concentration of $0.5\text{--}1.0 \times 10^6$ cells/ml and incubated for 24–72 h at 37 °C in the presence of media, 20% or 50% OSA, MEL or MCA TCM. Cells were collected, counted and prepared for phenotypic or functional analyses.

2.3. Stimulation of myeloid cells

A Falcon six-well plate was plated with canine myeloid cells at a concentration of 0.5×10^6 cells/ml per well and cells were rested for 24 h at 37 °C and 5% CO₂. After 24 h, the media was removed and replaced with fresh R-10 media containing the following concentrations of *Salmonella enterica* serotype *typhimurium* lipopolysaccharide (LPS; Sigma, St. Louis, MO): 0 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml, and 10 µg/ml. The cells were incubated for 24 h at 37 °C. Following incubation, the cells were collected from each well and prepared for flow cytometry; the supernatant collected for determination of nitric oxide (NO) production by the Greiss Reagent System (Promega, Madison, WI).

2.4. Phagocytosis assay

Myeloid cells were plated into a 12-well plate at a concentration of 0.5×10^6 cells/well and rested overnight at 37 °C and 5% CO₂. Media was removed and replaced with fresh R-10 media containing 20 or 50% TCM. Cells were cultured for 24–48 h and the media was gently aspirated from the wells. Approximately 200 µl of fresh media containing 2.5% fluorescent 1.0 µm diameter microspheres (Invitrogen, Carlsbad, CA) were added to wells and cells cultured for 1–2 h. Cells were visualized and imaged with an Olympus IX51 inverted fluorescent microscope and an Olympus Q-Color3 digital camera and subsequently collected and processed for evaluation by flow cytometry.

2.5. Proliferation assay

Canine myeloid cells were cultured in media or 20% TCM for 24 h. Following culture, cells were harvested, counted and washed with sterile PBS (or media) and used as stimulator cells. Spleens were collected and a single cell suspension of splenocytes, devoid of erythrocytes, was used as responder cells. Cells were cultured in round-bottom 96-well plates with 100,000 responder cells/well (responder cells kept constant) and stimulator myeloid cells plated at ratios of either 1:5 or 1:10 (responder:stimulator). Cells were cultured in triplicate in R-10 with or without the following stimuli: with medium, 0.5 µg/ml LPS, 10 µg/ml PHA and 3 µg/ml conA. Cellular proliferation was evaluated by culture of cells for 48–72 h, with incorporation of [³H] thymidine for the last 18 h of culture. Cells were harvested and counted using Perkin Elmer Top Count NXT, with Packard's Top Count NXT software. Results are expressed as the total cpm (mean cpm of cultures with Ag/mean cpm of cultures with medium alone) ± SEM for all treatments and the experiment repeated three times.

2.6. Flow cytometry

Approximately 0.5×10^6 myeloid cells from culture were aliquoted into flow tubes and stained with the following antibodies (Table 1): anti-canine CD11b (AbD Serotec, Raleigh, 208 NC) and anti-canine CD11c (AbD Serotec) were used as primary antibodies at the recommended concentrations from the supplier. Secondary goat anti-mouse FITC and rabbit anti-mouse RPE antibodies (AbD Serotec) were used at a concentration of 1:250 (FITC) and 1:20 (RPE) working dilution for detection of anti-CD11b and anti-CD11c. FITC-labeled anti-mouse CD80 (BD Pharmingen, Franklin Lakes, NJ) and MHC class II (AbD Serotec) were used to directly stain cells. Cells were allowed to incubate for 20 min for each primary and secondary staining procedure and dual FITC-PE staining was accomplished through a total of four separate staining steps. Cells were resuspended in 300 μ l FACS buffer and analyzed by flow cytometry. Fluorescently stained microspheres were detected on the FL-2 channel. Percent positive and mean fluorescence intensity was acquired using the Accuri C6 flow cytometer and analyzed using the Accuri Cflow Acquisition and Analysis software.

2.7. Nitric oxide (Griess Reagent) determination

The nitric oxide assay was performed using the Griess Reagent System (Promega, Madison, WI). Briefly, equal volumes (50 μ l) of experimental sample and sulfanil-amide solution (1% sulfanilamide in 5% phosphoric acid) were added to wells and samples were incubated for 5–10 min at room temperature. An equal (100 μ l) of 0.1% N-1-naphthylethylenediamine dihydrochloride solution was then added to all wells and samples incubated for another 5–10 min until color development. Absorbance was measured in a SpectraMax M2 plate reader at 535 nm to detect the colored azo compound and compared against standard nitrite reference curves.

2.8. Statistical analysis

Experimental groups were compared using analysis of variance (ANOVA) followed by Fisher's protected least significance post hoc analysis (PLSD) using X software. To compare two parameters against each other, Student's *t*-test was used. The significance level was set at $p < 0.05$.

3. Results

3.1. Characterization of canine myeloid cell populations

Phenotypic and morphologic characteristics of the DH82 cell lines were compared to canine BM-DCs and BM-M Φ s. In cell culture, the majority of DH82 cells and BM-DCs were non-adherent or loosely adherent and had a round to slightly irregular outline while BM-M Φ s were strongly adherent and more stellate in appearance in culture (Fig. 1A). As expected, both CD11c and CD11b were expressed by all three cell lines (Fig. 1B) with DH82 and BM-DCs sharing similar expression patterns in contrast to BM-M Φ s. Surprisingly, CD11c was higher in BM-M Φ s compared to DH82 and BM-DCs (Table 2) but the ratio of CD11c to CD11b was higher in DH82 and BM-DCs compared to BM-M Φ s (2.5, 1.4 and 1, respectively). Consistent with an immature DC phenotype, the expression of MHC class II was significantly lower (p -value < 0.05) in both DH82 and BMDCs (13.7 ± 1.2 and $18.0 \pm$

1.0 percent (average $\pm$ SEMs)) compared to BM-M Φ s (71.1 ± 0.5 percent). Additionally, consistent with DC-associated characteristics, DH82 cells were able to up-regulate CD80 and MHC class II expression (Fig. 2) and were unable to upregulate nitric oxide (NO), an inflammatory soluble mediator rapidly and typically produced by macrophages, in response to LPS (data not shown, Fig. 7). Taken together, these results show that DH82 cells are phenotypically and morphologically similar to BM-DCs and may be useful to evaluate the effects of the tumor microenvironment on canine myeloid DCs.

3.2. TCMs decrease activation of canine myeloid cells

Soluble factors from the tumor microenvironment have the capacity to alter immune cells. Using TCM, the effects of tumor-derived soluble factors (i.e. TDSFs) on the activation status of myeloid cells was assessed by detection of activation markers CD80 and MHC class II. Both BM-DCs and the DC cell line DH82 cultured in TCM (OSA or MEL) for 24–72 h had a significant decrease (p value < 0.05) in the percentage of MHC class II positive cells and reduced relative expression levels (as indicated by mean fluorescence intensity; MFI) (Fig. 3). MHC class II expression was consistently decreased in BM-DCs regardless of the TCM (i.e. both MEL and OSA TCM). MHC class II expression was variable in BM-M Φ s, although MEL TCM consistently resulted in decreased MHC class II expression while OSA TCM-exposed m occasionally showed increased MHC class II expression (data not shown, Figs. 8 and 9). BM-DCs exposed to TCM showed decreased percent positive CD80 (B7-H1) cells and reduced relative expression levels (as indicated by MFI) than control cells (Table 3). The CD80 expression of cells following TCM exposure was similar between three independent experiments. Exposure of cells to OSA TCM resulted in a dramatic decrease in the MFI of CD80 than did exposure to MEL TCM (19% versus 5% decrease, respectively) and similar decreases were seen in three independent experiments. CD80 expression was routinely high ($>90\%$) in untreated DH82 cells and BM-M Φ s with little to no effect of TCM on either of these cell populations.

3.3. Canine DCs have decreased phagocytic function in the presence of TCMs

To evaluate the effects of TDSFs on myeloid cell function, DCs exposed to TCMs were evaluated for their ability to phagocytose fluorescently labeled microsphere beads. Treatment of DCs with OSA and MEL TCMs resulted in a decreased percentage of the cells that phagocytosed beads (Fig. 4). Additionally, the MFI was decreased in TCM-exposed DCs, demonstrating that TCMs could cause decreased DC phagocytic efficiency as shown by the reduced number of beads taken up by individual canine DCs (Fig. 4). Similar effects on phagocytosis and MFI were seen in two additional independent experiments.

3.4. TCM-exposed DCs inhibit proliferation of responder immune cells

To evaluate whether TCM-exposed myeloid cells could alter adaptive immune responses, TCM-exposed DCs were cultured with responder immune cells in a standard proliferation assay. TCM-exposed DCs cultured with MEL TCM were significantly able to suppress proliferation of responder cells ($p < 0.05$) at a 1:5 ratio regardless of the proliferation stimulus (Fig. 5A). This suppressive ability was lost at a 1:10 ratio (Fig. 5B). Similarly, decreased proliferation of responder immune cells was seen with OSA and MCA TCM-

exposed DCs (data not shown) at a 1:5 ratio and lost at a 1:10 ratio; however, suppression was most marked and significant in MEL TCM-exposed DCs (p -value < 0.05). These results show that soluble factors secreted by tumor cells induce DCs (myeloid cells) capable of suppressing the proliferation of responder immune cells (Figs. 6–9).

4. Discussion

In this work, we have investigated the effects of soluble factors from cultured tumor cells (i.e. TCM) on canine myeloid cells. We found that soluble factors from tumor cells suppressed the activation and function of canine myeloid cells. Specifically, myeloid cells cultured in TCM had decreased expression of the activation markers MHC class II and CD80, suppressed functional phagocytic activity and could inhibit proliferation of responder immune cells. Additionally, our data show that the canine DH82 cell line may be useful for cancer immunology studies.

Cancer profoundly affects immunity and causes immunosuppression, which contributes to tumor escape, metastases and resistant to therapy. Both innate and adaptive immune cells can be altered during cancer and the contribution of dysregulated myeloid cells is increasingly being recognized in humans and mice with cancer (Mantovani et al., 2010; Schmid and Varner, 2010; Yang and Carbone, 2004). This study showed that canine tumor cells affect canine myeloid cell function, which may explain the findings that increased myeloid cells or myeloid cell markers are negative prognostic indicators (Perry et al., 2011; Sottnik et al., 2010). It is possible that these dogs with cancer were less able to generate anti-tumor immunity, even in the presence of elevated myeloid cell numbers, due to suppressive or dysfunctional myeloid cells. The elevated myeloid cells described in these studies may represent immunosuppressive MDSCs (Perry et al., 2011; Sottnik et al., 2010). At present, MDSCs have not been described in dogs but we have found elevated numbers of putative MDSCs both in vitro and in vivo in canine cancer (manuscript in preparation). Findings from this study show that cancer in dogs can also affect mature myeloid cell populations (i.e. DCs and M s) similar to what is seen in humans and mice (Chioda et al., 2011; Finn, 2008; Huang et al., 2011; Mantovani et al., 2009; Pollard, 2004).

In this study, soluble factors produced by canine tumor cells were able to down-regulate MHC class II and CD80 in canine DCs and M s. MHC class II and co-stimulatory molecule expression (e.g. CD80 and CD86) are typically up-regulated upon activation and maturation and play an important role during antigen presentation and in stimulation of T cell responses. Suppression of myeloid cell function contributes to the immunosuppression in mice with cancer and human cancer patients (Chioda et al., 2011; Finn, 2008; Huang et al., 2011; Mantovani et al., 2009; Pollard, 2004). Similarly, immunosuppressive compounds such as glucocorticoids and vitamin D also cause down-regulation of these activation markers in myeloid cells (Szatmari and Nagy, 2008). Our results show that down-regulation of MHC class II was more profound than CD80 whereby both the percentage of MHC class II and MFI were more dramatically decreased for MHC class II versus CD80. The reason for this is unknown but suggests that regulation of MHC class II expression may be more sensitive to suppressive microenvironmental changes or that cells may decrease the relative expression of CD80 (i.e. MFI) in response to microenvironmental factors rather than

decrease the number of overall CD80+ cells. It was somewhat surprising that the BM-MΦs had increased MHC class II expression upon exposure to OSA TCM. The cause of this is unknown but may be due to soluble factors produced by neoplastic osteoblasts. Bony remodeling often occurs in osteosarcomas and is a dynamic process where osteoblasts and stromal cells activate osteoclasts (i.e. multi-nucleate macrophage-like cell). It is possible that the osteoblasts in this osteosarcoma are producing factors that activate our BM-MΦs. Alternatively, down-regulation of CD80 by soluble factors from the tumor microenvironment may not be a primary mechanism by which TCMs alter myeloid cell function. Down-regulation of other activating co-stimulatory molecules (e.g. CD86) or up-regulation of inhibitory costimulatory molecules (e.g. PD-L1, PD-L2, B7-H3 or B7-H4) may also be influenced by the tumor microenvironment. Although anti-human CD86 clone IT2.2 (BioLegend) has been reported to be cross-reactive with canine myeloid cells, this antibody did not detect CD86 in our myeloid cells (data not shown). The reason for this is unknown but may be due to minimal cross-reactivity or distinct differences in cell culture conditions that prevented detection of CD86 (Bonnefont-Rebeix et al., 2006). Additionally, it was difficult to fully assess the impact of TCMs on CD80 expression in all canine myeloid cell populations due to high baseline CD80 expression in BM-MΦs and DH82 cells. The reason for this high baseline expression of CD80 is unknown but may represent an in vitro phenomenon due to plastic adherence for macrophages and the extended cell culture passages and culture adaptation of the DH82 cell lines.

The DH82 cell line has been described as a macrophage/monocytic cell line but recent work and our studies suggest that these cells are more similar to DCs than macrophages (Barnes et al., 2000; Bird et al., 2008; Wellman et al., 1988). Adherence and morphologic characteristics of DH82 cells in culture are similar to the DC cell line DC2.4, and GM-CSF treatment generated BM-DCs in mice and dogs (Lutz et al., 1999; Papenfuss, 2010, unpublished observations; Shen et al., 1997). Unlike in mice, a single individual marker such as CD11c or CD1c does not exclusively identify DC populations or differentiate these from monocytes or macrophages (Ricklin Gutzwiller et al., 2010). For our studies, we compared CD11c, CD11b and MHC class II expression between three myeloid cell populations and found that BM-DCs expressed similar CD11c, CD11b and MHC class II levels as DH82 cells, which differed from expression patterns in BM-MΦs. Interestingly, BM-MΦs expressed higher CD11c levels than both DC populations further highlighting that CD11c cannot be exclusively used to identify canine DCs (Ricklin Gutzwiller et al., 2010). The higher CD11c expression in BM-MΦs was somewhat surprising but may be due to the fact that BM-MΦs in these studies were identified exclusively based on adherence characteristics in culture conditions that are DC-promoting (i.e. GM-CSF) but do not necessarily inhibit macrophage differentiation (Lutz et al., 1999; Ricklin Gutzwiller et al., 2010). At present, definitive generation of canine BM-MΦs is limited by a lack of recombinant canine M-CSF, a growth factor that generates macrophages. Comparison of the BM-MΦs of this study to other described canine macrophage populations was beyond the scope of this study (Pinelli et al., 1999; Ricklin Gutzwiller et al., 2010; Tipold et al., 1998). Interestingly, our findings suggest that a higher CD11c to CD11b ratio in conjunction with low MHC class II expression may be an effective means to identify canine DC populations (Table 2). DH82 cells had the highest CD11c:CD11b ratio and were similar to BM-DCs in

phenotype and function, indicating that this DC myeloid cell line can be used to evaluate immune responses in cancer and for potential cancer immunotherapy. Indeed, their potential application for cancer immunotherapy (e.g. hybrid-fusion vaccine against canine mammary carcinoma) was recently described (Bird et al., 2008).

Importantly, besides simply decreased expression of activation markers, our results showed that the function of myeloid cells was altered following exposure to soluble factors from tumor cells. The ability of myeloid cells to sample the tumor environment by phagocytosis, process and present antigen to T cells and enhance innate and adaptive immune responses are necessary to induce an anti-tumor immune response. We found that canine myeloid cells exposed to TCM were less able to phagocytose microparticles from the environment and phagocytosed fewer microparticles than control myeloid cells (Fig. 4). These results suggest that anti-tumor immunity may be impaired, in part, through diminished phagocytic uptake and presentation of tumor antigens by myeloid cells following exposure to soluble factors produced by tumor cells. Our results also showed that DCs exposed to TCM suppressed proliferation of responder immune cells regardless of the proliferation stimulus, indicating that TCM-exposed DCs may be actively involved in suppressing immune responses.

Decreased tumor antigen uptake from reduced phagocytosis, decreased tumor antigenic presentation from decreased MHC class II expression, decreased stimulation of adaptive (e.g. T cell) responses from lower co-stimulatory molecule expression or active suppressive mechanisms are all potential mechanisms by which myeloid cells contribute to the immunosuppression in cancer. Our results show that all of these processes occur in canine myeloid cells following exposure to soluble factors produced by cultured tumor cells. Suppressive myeloid cells, such as mature DCs, macrophages or immature MDSCs are potent contributors to the immune dysregulation and immunosuppression seen in cancer. We report that soluble factors (TDSFs) produced by cultured tumor cells alter the phenotype and function of canine myeloid cells to contribute to immunosuppression. In combination with similar findings in human and mouse studies, these studies suggest that tumors use common mechanisms, regardless of species, to act on myeloid cells and contribute to immunosuppression in cancer. Findings from these studies will facilitate our understanding of cancer immunology and lead to development of translational target therapies both in human and veterinary cancer patients.

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Abbreviations

BM-DC	bone marrow-derived dendritic cell
BM-MΦ	bone marrow-derived macrophage
Treg	regulatory T cell
TAM	tumor-associated macrophage

MDSC	myeloid derived suppressor cell
DC	dendritic cell
Tol-DC	tolerogenic dendritic cell
TDSF	tumor-derived soluble factor
GM-CSF	granulocyte–monocyte colony stimulating factor
OSA	osteosarcoma
MEL	melanoma
TCM	tumor-conditioned media
CD	cluster of differentiation

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Fig. 1.

Phenotypic and morphological characteristics of canine myeloid cells. (A) The morphology of canine myeloid cell populations by inverted microscopy. (B) Cell surface expression of CD11b and CD11c of these canine myeloid cell populations.

Fig. 2.

MHC class II and CD80 expression in the canine DH82 myeloid cell line. Canine DH82 myeloid cells were fully capable of increasing expression of both (A) CD80 and (B) MHC class II in response to 24 h exposure to LPS. Increased expression levels of both CD80 and MHC class II are seen upon LPS (gray) stimulation compared to unstimulated (black) media controls.

Fig. 3. MHC class II expression in canine DCs. Both canine (A) BM-DCs or (B) DH82 cells had decreased numbers of cells expressing MHC class II.

Fig. 4.

CD80 expression in canine BM-DCs following 72-h exposure to TCMs. Both the percentage of CD80 positive cells and relative expression levels (as determined by mean fluorescence intensity; MFI) was decreased in DCs exposed to OSA and MEL TCMs.

Fig. 5.

Phagocytosis of fluorescently labeled beads by canine myeloid cells exposed to tumor-derived soluble factors (TDSFs). The top panels show both the percentage of cells containing phagocytosed beads where the M2 gate represents the overall percentage of DH82 cells that have phagocytosed beads and are PE positive. Mean fluorescent intensity (MFI), determined from PE positive (M2-gated) DH82 cells, detects the relative PE intensity of individual cells and represents the relative number of fluorescent beads phagocytosed by the DH82 cells. The bottom panels are images of cultured DH82 cells containing intracellular phagocytosed particles taken by an inverted fluorescent microscope.

Fig. 6.

Stimulation of canine spleen cell proliferation by various antigens in the presence of DH82 canine myeloid cells cultured in normal media (DH82) and in the presence of canine melanoma TCM (MEL). Cells were cultured (in triplicate) in the presence of either media, *Salmonella* sub-type *typhimurium* lipopolysaccharide (LPS), phytohemagglutinin (PHA), and concanavalin A (ConA). Proliferation was suppressed at a 1:5 ratio (melanoma-conditioned myeloid cells versus spleen cells) while suppression was lost at a ratio of 1:10 ($p < 0.05$). There was significantly increased suppression ($17.3 \pm 7.9\%$; average $\pm$ SEM) for melanoma-exposed myeloid cells ($p < 0.01$; t-test where average $\pm$ SEM were each calculated using triplicate values). Similar results were obtained from three independent experiments, each having triplicates for each treatment group.

Fig. 7.
Failure of DH82 cells to upregulate nitric oxide production following LPS stimulation.
Nitric oxide levels remained low across a broad range of LPS concentrations.

Fig. 8.

MHC class II expression levels of BM-DCs differentiated in the presence or absence of TCM (OSA or MEL) from two individual (separate) experiments. There was consistent downregulation of MHC class II expression in BM-DCs differentiated in the presence of either OSA or MEL TCM.

Fig. 9.

MHC class II expression levels of BM-MΦs differentiated in the presence or absence of TCM (OSA or MEL) from two individual (separate) experiments. (A) BM-MΦs differentiated in the presence of OSA TCM had variable expression patterns of MHC class II between experiments while (B) BM-MΦs differentiated with MEL TCM showed consistently decreased MHC class II expression between experiments.

Table 1

Antibodies used for surface expression analysis of DH82 canine myeloid cells, bone marrow-derived dendritic cells, and bone marrow-derived macrophages.

Specificity	Clone	Immunoglobulin subclass
Mouse anti dog CD11b	CA16.3E10 ^a	IgG1
Mouse anti dog CD11c	CA11.6A1 ^a	IgG1
Hamster anti mouse CD80	16-10A1 ^b	IgG2κ
Rat anti dog MHC class II	YKIX334.2 ^a	IgG2a

^aSource: AbD Serotec.

^bSource: BD Pharmingen.

Table 2

Percent expression ($\pm$ standard error) of common canine myeloid cell markers in DH82 myeloid cells, bone marrow-derived dendritic cells (BM-DC), and bone marrow-derived macrophages (BM-MFs).

Cell line	CD11c	CD11b	MHC class II	CD11c:CD11b
DH82	90.4 ($\pm$ 2.2)	35.6 ($\pm$ 2.7)	13.7 ($\pm$ 1.2)	2.5
BM-DC	66.5 ($\pm$ 4.1)	48.7 ($\pm$ 3.3)	18.0 ($\pm$ 1.0)	1.4
BM-MF	88.9 ($\pm$ 1.9)	88.6 ($\pm$ 2.5)	71.1 ($\pm$ 0.5)	1

Table 3

CD80 expression of bone marrow-derived dendritic cells cultured in osteosarcoma (OSA16) and melanoma (MEL4) tumor-conditioned media and in unconditioned media (RPMI) in terms of percent of cells expressing CD80 and mean fluorescence intensity (MFI). Percent change in CD80 expression is in terms of MFI. The CD80 expression of cells following TCM exposure was similar between three independent experiments.

	% Positive	MFI	% Change
IgG2 isotype	7.1	109,146.5	
BM-DC RPMI CD80	67.6	277,554.0	
BM-DC OSA16 CD80	62.6	225,810.8	−19%
BM-DC MEL4 CD80	65.0	261,692.8	−6%