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A functional comparison of canine and murine bone marrow derived cultured mast cells

Tzu-Yin Lin, Cheryl A. London *

Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210, United States

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Abstract

Disorders involving mast cells are extremely common in dogs, ranging from allergic diseases to neoplastic transformation resulting in malignant mast cell tumors. Relatively little is known regarding the basic biologic properties of normal canine mast cells, largely due to the difficulty in reliably purifying large numbers from canine skin. *In vitro* generated bone marrow derived cultured mast cells (BMCMCs) are routinely used in both human and murine studies as a ready source of material for *in vitro* and *in vivo* studies. We previously developed a technique to generate canine BMCMCs from bone marrow derived CD34+ cells and demonstrated that these cells exhibit the phenotypic properties characteristic of mast cells and release histamine in response to IgE cross-linking. The purpose of the following study was to characterize the functional properties of these canine BMCMCs and contrast these with the functional properties of murine BMCMCs. Our work demonstrates that both IL-4 and IL-10 promote canine BMCMC proliferation, possibly through upregulation of Kit expression, while TGFβ inhibits proliferation. The canine BMCMCs produce a variety of cytokines and chemokines in response to IgE cross-linking and chemical stimulation including IL-3, IL-4, IL-13, GM-CSF, RANTES, and MIP1α. Interestingly, the canine BMCMCs released significantly larger amounts of MCP-1 and tryptase and significantly smaller amounts of IL-6 following chemical stimulation and IgE cross-linking when compared to murine BMCMCs. Lastly, the canine BMCMCs produced larger amounts of active MMP9 than their murine counterparts. In summary, canine BMCMCs exhibit unique functional properties that distinguish them from murine BMCMCs and provide insight into the contribution of these cells to mast cell disorders in the dog.

Keywords: Mast cell; Dog; Mouse; Cytokine; Chemokine

1. Introduction

Disorders involving mast cells are common in dogs and include allergic diseases such as atopy as well as mast cell tumors (de Mora et al., 1993; Misdorp, 2004; von Ruedorffer et al., 2003), one of the most prevalent neoplasms found in this species. Furthermore, there is substantial evidence that many of these disorders exhibit particular breed predispositions suggesting that genetics plays a role in mast cell diseases (London and Seguin, 2003). Despite the abundance of mast cell disorders in dogs, relatively little is known regarding the biology of normal canine mast cells, particularly with respect to their responses to a variety of stimuli.

Historically, canine mast cell biology has been studied by purifying mast cells from canine skin (Brazis et al., 1998), or by evaluating cell lines derived from malignant mast cell tumors (Fang et al., 1996; Takahashi et al., 2001). With respect to canine skin derived mast cells,

* Corresponding author. Tel.: +1 614 292 9554; fax: +1 614 292 7559.
E-mail address: london.20@osu.edu (C.A. London).

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they have been shown to release histamine and TNFα through both IgE-dependent and -independent mechanisms which were enhanced by SCF stimulation (Brazis et al., 2000). Other studies have demonstrated that canine mast cells can respond to C-reactive protein (Fujimoto et al., 2003). Unfortunately, it is very difficult to obtain large numbers of mast cells from skin and in many cases, these populations are not pure. As such, detailed studies on the biologic function of skin derived canine mast cells are not possible.

Due to the historical difficulty in obtaining a large number of normal canine mast cells, the characterization of canine mast cell biology has relied up on evaluation of the functional properties of canine malignant mast cell lines. For example, both the BR and CM-MC canine mast cell lines failed to respond to IgE cross-linking (Garcia et al., 1998), but the CM-MC line released histamine in response to cross-linking of either IgG1 or IgG4 (Sato et al., 2004; Takahashi et al., 2001). These cell lines also produced TGFβ which inhibited their proliferation (Pennington et al., 1992). Furthermore, functional Kit, estrogen, and adenosine receptors were shown to be present on the cell lines (Auchampach et al., 1997; Larsen and Grier, 1989; Liao et al., 2002) and production of metalloproteinases MMP2, MMP9, IL-4, IL-5, FGFβ, PDGF, and PGD_2 could be demonstrated (Fang et al., 1999). However, the cell lines did not exhibit consistent cytokine expression profiles. Lastly, some receptors believed to be expressed on normal mast cells were not identified on the canine mast cell lines (Lin et al., 2006). These data suggest that while the malignant mast cell lines exhibit some functional properties that are seen in normal mast cells, they are probably not reliable for detailed studies aimed at dissecting the biology of these cells.

Human and mouse mast cells differentiated *in vitro* from bone marrow, cord blood or other peripheral blood have been used for several years to characterize basic aspects of mast cell biology (Dahl et al., 2002; Kinoshita et al., 1999; Mekori et al., 1993; Shimizu et al., 2002). These cell populations are considered a valuable resource as generating sufficient numbers of normal mast cells from either human or mouse tissues is extremely difficult, time consuming, and precludes certain studies, such as those that involve reconstitution of mast cell deficient mice. *In vitro* generated mast cells have been used to define the role of these cells in asthma (Stassen et al., 2000), automimmunity (Robbie-Ryan et al., 2003), as well as innate immune responses (Bidri et al., 1997). Furthermore, they have been instrumental in dissecting the regulation of mast cell mediator production such as proteases (chymases, tryptases), prostaglandins, and a variety of cytokines/chemokines. Recent work employing *in vitro* derived mast cells has defined their involvement in angiogenesis as it relates to both normal processes such as wound healing (Noli and Miolo, 2001), as well as pathologic processes such as neoplasia and fibrotic diseases (Theoharides and Conti, 2004).

Given the frequency of mast cell disease in the dog, it is important that the biology of normal canine mast cells be further explored to begin to identify those factors that ultimately lead to pathologic processes involving these cells. In a previous study, we developed a technique to generate canine mast cells from bone marrow derived CD34+ cells (Lin et al., 2006). These bone marrow derived cultured mast cells (BMCMCs) were found to contain chymase and tryptase and expressed typical cell surface markers including Kit, FcεRI, and integrins. The canine BMCMCs were dependent on canine stem cell factor (SCF) for survival and proliferated and migrated in response to SCF. Lastly, cross-linking of cell surface bound IgE induced histamine and TNFα release. Therefore, the canine BMMCs possess phenotypic and functional properties similar to those described of mast cells directly isolated from canine skin. The purpose of the following study was to expand on this initial work and begin a detailed characterization of the functional properties of canine BMCMCs, employing murine BMCMCs for comparison.

2. Material and methods

2.1. Bone marrow collection and BMCMC generation

Bone marrow was collected from canine patients undergoing routine ovariohysterectomy or castration at the Veterinary Medical Teaching Hospital (VTH) at The Ohio State University following appropriate client consent. The protocol for bone marrow collection was approved by the VTH Clinical Trials Committee. Approximately 10–15 ml of bone marrow was obtained from the proximal humerus and collected into 3.8% sodium citrate/PBS solution. Canine BMCMCs were differentiated from purified CD34+ cells derived from canine bone marrow as previously described. Briefly, cells were cultured in Stemline® II serum free medium (Sigma, St. Louis, MO) in the presence of 100 ng/ml rcSCF (R&D Systems, Minneapolis, MN) for 5–8 weeks. Canine BMCMC populations were determined to be greater than 90% pure by Wright-Giemsa and/or toluidine blue staining after cytospin preparation as previously described.

Studies involving murine mast cells were reviewed and approved by the OSU IACUC (Animal Protocol #1072). C57B/6 mice were sacrificed, intact femora were removed, and bone marrow cells were harvested by flushing the femora with medium. The isolated bone marrow cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM MEM non-essential amino acids (all GIBCO, Grand Island, NY) and 10 ng/ml murine IL-3 (PeproTech, Rocky Hill, NJ). Half the medium was replaced once per week. Non-adherent cells were transferred into new flasks to remove adherent fibroblasts and macrophages. After 8 weeks of culture, the cells were greater than 99% toluidine blue positive.

2.2. Assessment of cell proliferation

To assess the effects of cytokines on canine BMCMC proliferation, 1×10^4 five-to-seven-week-old cells were plated in 96-well plates in 150 μl Stemline® II medium supplemented with 50 ng/ml rcSCF. Cells were then treated with IL-4, IL-10 or TGFβ1 (R&D Systems and PeproTech) at varying concentrations, or left untreated. After 4 or 7 days in culture, the plates were collected and 10 μl working solution of WST-1 (Roche, Indianapolis, IN) was added to the wells and incubated for 4.5 h at 37 °C and 5% CO_2. Absorbance was then assessed using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). Identical quantities of medium and WST-1 were added to control wells to serve as blanks to subtract out background absorbance. Samples were analyzed in triplicate and experiments were repeated with three sets of BMCMCs from different dogs.

2.3. Flow cytometry

Kit and FcεRI expression were detected by flow cytometry as previously described. Briefly, cells were cultured with 50 ng/ml SCF alone or in the presence of 50 ng/ml cIL4, 50 ng/ml cIL10, or 10 ng/ml TGFβ1 for 2 days. Cells were then collected or loaded with 2 μg/ml canine IgE (TGFβ1 treated cells only, Bethyl Labs, Montgomery, TX) for 6 h prior to staining. Cells were then incubated with PE labeled anti-mouse Kit (AKC45, BD Biosciences, Franklin Lakes, NJ) or mouse anti-canine IgE (Serotec, Raleigh, NC) followed by FITC labeled goat anti-mouse IgG. To detect apoptosis associated with incubation of canine BMCMCs with cytokines, the cells were cultured in SCF alone, or SCF with IL-4, IL-10 or TGFβ1. After 48 h of culture, cells were collected, washed, and labeled with 5 μl of Annexin-V-FITC (BD Biosciences) for 15 min at room temperature. Before flow cytometric analysis, 5 μl of propidium iodide solution (50 μg/ml) was added to identify dead cells. Analysis of cell cycling was performed as previously described using propidium iodide staining following ethanol fixation (Lin et al., 2006). Experiments were repeated with three sets of BMCMCs from different dogs.

2.4. Activation of canine and mouse BMCMCs

Murine and canine BMCMCs at 5–10 weeks of age were collected, washed, and stimulated by IgE cross-linking or chemicals to induce activation. For IgE mediated activation, BMCMCs were loaded with 2 μg/ml of dog IgE (Bethyl Labs) or 2 μg/ml of mouse IgE (Sigma) overnight at 37 °C. Unbound IgE was removed by washing with PBS and 1×10^6 BMCMCs were then plated in 24-well plates containing 2 ml of complete medium. Goat anti-canine IgE (2 μg/ml) or goat anti-murine IgE (1 μg/ml) was then added, followed by 50 μl TrueBlot TM anti-goat Ig IP beads (eBioscience, San Diego, CA). After incubation for 2 or 16 h at 37 °C, the plates were then centrifuged and the supernatants were collected and stored at −20 °C for assessment; the remaining cell pellets were frozen at −80 °C in TRIzol for RNA extraction.

To stimulate chemical degranulation of the BMCMCs, 1×10^6 cells were washed and plated in 24-well plates containing 2 ml of complete medium. The cells were stimulated by adding 100 μl of a 100× solution of each stimulating agent: ConA (final concentration 3 μg/ml), or calcium ionophore A23187 (final concentration 100 ng/ml) and PMA (final concentration 50 ng/ml) (all from Sigma). After incubation at 37 °C for 2 or 16 h, the plates were centrifuged and the supernatants were collected and stored at −20 °C for assessment; the remaining cell pellets were frozen at −80 °C in TRIzol for RNA extraction.

2.5. β-Hexosaminidase assay

Degranulation of BMCMCs was measured by using the β-hexosaminidase assay as previously described. Briefly, 1×10^4 mature murine or canine BMCMCs were collected, washed and stimulated by A23187/PMA or ConA in 100 μl Tyrode's salt solution (Sigma). Supernatant (25 μl) was then collected and the cell pellets were lysed using 0.5% Tween-20/PBS following which another 25 μl supernatant was collected. Next, 75 μl of 1.2 mM 4-methylumbelliferyl *N*-acetyl-b-D-glucosaminide in 0.05 M sodium acetate buffer (pH 4.4)

was added to the supernatants followed by an incubation for 30 min at 37 °C. The reaction was quenched with cold 0.1 M glycine–carbonate buffer (pH 10.0) and absorbance was measured using an ELISA plate reader (Molecular Devices). Tyrode's salt solution served as the negative control and this absorbance value was subtracted from all the readings. The degranulation ratio was calculated using following formula: (OD in cell supernatants − OD in blank)/(OD in cell supernatants − OD in blank + OD in cell pellet − OD in blank) × 100. All experiments were repeated three times in triplicate.

2.6. RNA isolation and RT-PCR analysis

RNA was extracted from the cell pellets saved in TRIzol according to the manufacturer's instructions. To generate cDNA, 2 μg of total RNA and the Superscript III kit (Invitrogen, Carlsbad, CA) were used according to the manufacturer's instructions. Next, 1/20 of the resultant cDNA was used for each PCR reaction in a total volume of 50 μl. Nine pairs of primers designed for mouse or canine cytokines and chemokines were used. These, along with the necessary annealing temperatures, are summarized in Tables 1 and 2.

2.7. ELISAs for cytokines, chemokines, and growth factors

The cytokine/chemokine levels in the supernatants from activated mouse and canine BMCMCs were determined using commercially available ELISA kits. Canine MCP-1 and IL-6 and mouse MCP-1, IL-6, and VEGF concentrations in the tissue culture medium were measured by DuoSet® ELISA kits (R&D Systems), while canine and mouse TNFα and canine IL10 and IFNγ were measured by Quantikine® ELISA kits (R&D Systems). Mouse IFNγ and IL-10 were measured using the Ready-Set-Go ELISA Kits (eBioscience). Mouse and canine TGFβ1 were quantified using the

Table 1
Primer pairs for RT-PCR of canine cytokines, chemokines, and TLRs

Target gene	Forward/reverse sequence	Annealing temperature (°C)	Product size (bp)	Source
cIL-3	GTCCCACAGGCACAGGGGA/GGGTTTGTTCTTGAA ATTCAGAAAGTTA	53	373	Unpublished
cIL-4	ACTCACCAGCACCTTTGTCC/TGCTGCTGAGGTTCCTGTAG	51	248	Primer 3
cIL-13	ATCAATGTCTCCGACTGCAG/ATGAAGTGCAGACGGAGGAG	51	420	Yang et al. (2000)
cGM-CSF	GCAGAACCTGCTTTTCTTGG/AAGGGATTCTTGAGGCTGGT	51	273	Primer 3
cMIP-1a	CAATAGCCTGCTGCTTCTCC/CAGATCGGCCACATATTCCT	57	176	Primer 3
cRANTES	AGGTCTCCGCAGCTACCTTT/GCACTTGCTGCTGGTGTAGA	55	167	Primer 3
cTLR2	GGCTGTAACCAACCTCTCCA/AGGTTCACACAATCCCGAAG	55	201	Primer 3
cTLR 4	CTCTCCTGGAAGGACTGTGC/CCGTTGCCATCTGAGATTTT	55	179	Primer 3
GAPDH	ACCACAGTTCCATGCCATCAC/TCCACCACCCTGTTGCTGTA	55	451	Unpublished

Table 2
Primer pairs for RT-PCR of mouse cytokines, chemokines, and TLRs

Target gene	Forward/reverse sequence	Annealing temperature (°C)	Product size (bp)	Source
mIL-3	GAAGTGGATCCTGAGGACAGATACG/GACCAT GGGCCATGAGGAACATTC	55	550	Primer 3
mIL-4	TCAACCCCCAGCTAGTTGTC/TGTTCTTCGTTGCTGTGAGG	55	177	Primer 3
mIL-13	GGAGCTGAGCAACATCACACA/GGTCCTGTAGATGG	60	142	Overbergh et al. (2003)
mGM-CSF	GCCATCAAAGAAGCCCCTGAA/GCGGGTCTGCACACATGTTA	60	114	Overbergh et al. (2003)
mMIP-1a	GGTCTCCACCACTGCCCTTCG/GGTGGCAGGAATGTTCGGCTC	51	357	Cho et al. (2000)
mRANTES	CCTCACCATCATCCTCACTGCA/TCTTCTCTGGGTTGGCACACAC	55	215	Cho et al. (2000)
mTLR2	CTCCCACTTCAGGCTCTTG/ACCCAAAACACTTCCTGCTG	51	171	Primer 3
mTLR4	GCTTTCACCTCTGCCTTCAC/AGGCGATACAATTCCACCTG	51	259	Primer 3
GAPDH	ACCACAGTTCCATGCCATCAC/TCCACCACCCTGTTGCTGTA	55	451	Unpublished

TGFβ1 Emax ImmunoAssay System (Promega, Madison, WI). All procedures were performed according to the manufacturer's instructions.

Canine VEGF was detected using a formulated ELISA kindly provided by Dr. D. Thamm (Colorado State University, personal communication). ELISA plates were coated with 50 μl of mouse anti-canine VEGF (R&D Systems) at the concentration of 0.5 μg/ml at 4 °C overnight, washed, then 100 μl of Stabil-Coat® (Surmodics, Eden Prairie, MN) was added for 1 h at room temperature to block the plates; the plates were then dried overnight. The supernatants and canine VEGF standards (R&D system) were then added and incubated for 90 min at room temperature. Following washing, plates were incubated with 50 μl of biotinylated goat anti-human VEGF antibody (R&D system) at the concentration of 100 ng/ml in 1% BSA/PBS for 1 h at room temperature. The plates were again washed and incubated with peroxidase conjugated streptavidin for 2 h at room temperature. After washing the plates five times, the color was developed by adding 100 μl of substrate; the reaction was stopped after 30 min using 100 μl of H_2SO_4. For all ELISAs, absorbance was assessed using an ELISA plate reader (Molecular Devices). The negative control for each ELISA consisted of Stemline® II instead of supernatants. The concentrations of cytokines/chemokines were calculated according to the generated standard curve after subtracting out background.

2.8. Tryptase activity assay

The tryptase levels in the supernatants from activated mouse and canine BMCMCs were determined using a commercially available mast cell degranulation assay kit (Chemicon, Temecula, CA). The supernatants collected from active canine BMCMCs were diluted four times by assay buffer while the supernatants collected from active murine BMCMCs were not diluted. Supernatant (180 μl) and tryptase standards were incubated with 20 μl of 2.5 nM tryptase substrate solution (tosyl-gly-pro-lys-*p*NA) for 90 min at 37 °C. Absorbance was assessed using an ELISA plate reader (Molecular Devices). The negative control consisted of assay buffer instead of supernatants. The tryptase concentrations were calculated according to the generated standard curve after subtracting out background.

2.9. MMP gel zymography

The activity and expression of MMPs was detected using traditional MMP gel zymography. The supernatants collected from activated canine BMCMCs detailed above were used; however, supernatants collected from activated murine BMCMCs employing larger numbers of cells (5×10^6 cells) were used after culture in 500 μl Stemline® II (conditions for activation of BMCMCs were identical). Samples were centrifuged and 30 μl of each supernatant sample was incubated with 10 μl zymogram sample buffer (Bio-Rad, Hercules, CA) for 10 min at room temperature. Next, 10% SDS-PAGE gels containing 0.1% gelatin (Invitrogen, Carlsbad, CA) were used for electrophoresis. After electrophoresis, the gels were washed in renaturation buffer (Bio-Rad) for 30 min and transferred to development buffer (Bio-Rad) for another 30 min. The fresh development buffer was replaced and the reaction was incubated at 37 °C overnight. Gels were then stained with Coomasie Brilliant Blue (Bio-Rad) for 30–60 min and destained with 50% methanol/40% acetic acid in water.

2.10. Statistical analysis

All experiments were repeated three times and data was presented as mean values ± S.D.; significance was estimated using the Student's *t*-test. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. IL4 and IL10 promote, but TGFβ1 inhibits, canine BMCMC proliferation

Evidence suggests that a variety of cytokines can influence mast cell biology. For example, TGFβ1 is known to inhibit murine and human mast cell proliferation and survival, while IL-4 and IL-10 have variable effects (Bischoff and Sellge, 2002). To evaluate the effects of these cytokines on canine BMCMCs, we cultured these cells with IL-4, IL-10, or TGFβ1 in the absence of other cytokines. These cytokines all failed to support BMCMC survival on their own (data not shown) and therefore SCF was added to all cultures prior to the analysis. As shown in Fig. 1, canine BMCMCs cultured in rcSCF plus either IL-4 or IL-10 demonstrated a statistically significant increase in proliferation at days 4 and 7 of culture when compared to those cultured in rcSCF alone. In contrast, canine BMCMCs cultured in rcSCF and TGFβ1 exhibited a dose-dependent inhibition of cell proliferation.

To identify a potential mechanism responsible for the observed enhancement of proliferation, we evaluated both the percent of cells cycling as well as Kit

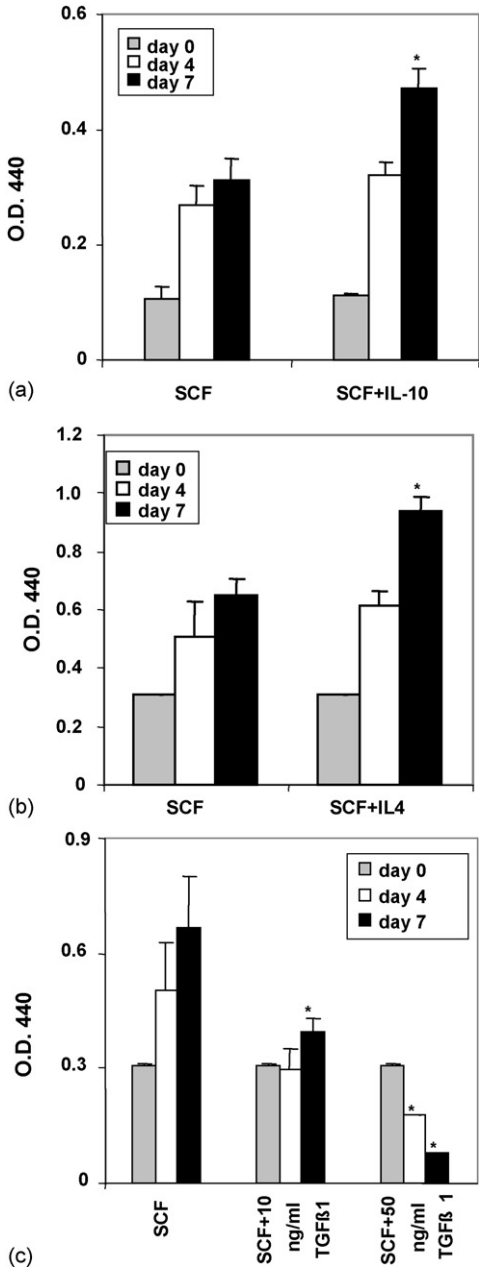

Fig. 1. Effect of IL-4, IL-10 and TGFβ on canine BMCMC proliferation. (a) Canine BMCMCs were cultured in Stemline plus SCF (50 ng/ml) or SCF (50 ng/ml) and IL-4 (50 ng/ml). (b) Canine BMCMCs were cultured in Stemline plus SCF (50 ng/ml) or SCF (50 ng/ml) and IL-10 (50 ng/ml). (c) Canine BMCMCs were cultured in Stemline plus SCF (50 ng/ml) or SCF (50 ng/ml) and TGFβ (10 or 50 ng/ml). Plates were collected at days 0, 4, and 7 of culture and proliferation was assessed using the Wst-1 assay. All experiments were repeated three times; $^{*}p < 0.05$.

expression in BMCMCs treated with the various cytokines. Fig. 2a demonstrates that IL-4 and IL-10 induce an increase in cell cycling when compared to rcSCF alone. Furthermore, Kit expression was increased in the presence of either IL-4 or IL-10, suggesting that enhanced Kit signaling may be responsible for the observed differences in proliferation (Fig. 2b). In contrast, TGFβ1 inhibited proliferation and induced downregulation of Kit expression, as well as FcεRI expression (Fig. 2b). However, this was not secondary to cell apoptosis, as canine BMCMCs cultured in SCF with TGFβ1 did not exhibit a significantly greater percentage of Annexin-V/propidium iodide positive cells when compared to those cultured with SCF alone or in the presence of IL-4 or IL-10 (Fig. 2a). It is interesting to note that IL-4 also enhanced the BMCMC granule content, as assessed by side scatter on flow cytometric analysis (data not shown); this effect was not noted following culture with IL-10.

3.2. cBMCMCs exhibit greater sensitivity to chemical degranulation than mBMCMCs

BMCMCs have been shown to degranulate following stimulation with a variety of different chemicals. To compare the sensitivities of canine and murine BMCMCs they were stimulated with different concentrations of ConA and A23187/PMA. As shown in Fig. 3, both mouse and canine BMCMCs released β-hexosaminidase (an indicator of mast cell degranulation) following stimulation with ConA and A23187/PMA. Interestingly, the canine BMCMCs exhibited significantly greater degranulation when compared to their murine counterparts under identical conditions of stimulation, suggesting that canine BMCMCs exhibit higher baseline sensitivity.

3.3. Cytokine, chemokine and growth factor expression and release following stimulation

Mast cells release several cytokines, chemokines and growth factors known to participate in allergic reactions as well as the innate immune response. To begin to characterize the functional responses of canine BMCMCs, they were stimulated by IgE cross-linking or chemicals and message/protein levels for several cytokines, chemokines and growth factors were assessed and compared directly to those of murine BMCMCs treated similarly. Protein levels were quantified when appropriate assays were available for both the murine and canine proteins. Fig. 4a and b

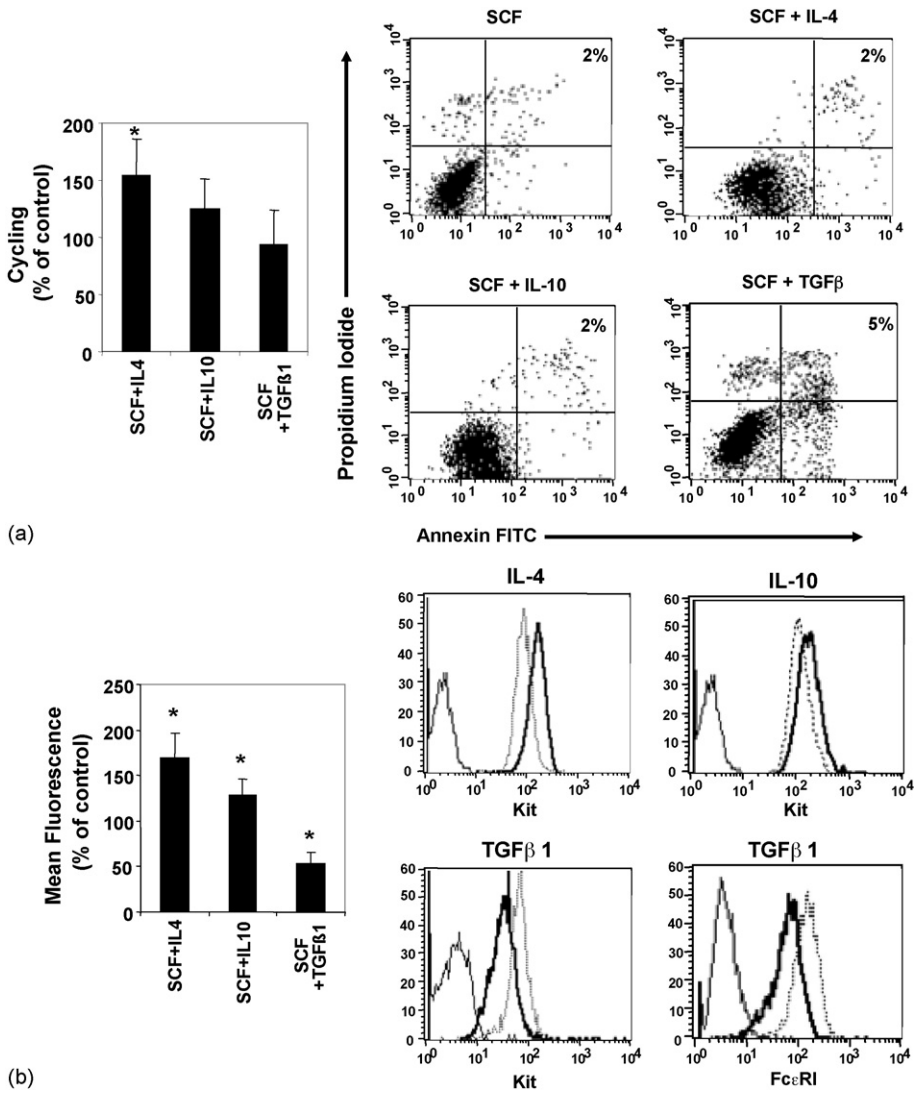

Fig. 2. Effect of IL-4, IL-10 and TGFβ on cell cycling and Kit expression. Canine BMCMCs were cultured in Stemline plus SCF alone, or with SCF and IL-4, IL-10, or TGFβ for 2 days. Cells were collected and analyzed for percent of cells cycling using propidium iodide (PI) staining (a) or Kit and FcεRI expression (b) using flow cytometry. Cells were also labeled with Annexin-V-FITC and PI to ensure that no significant apoptosis was evident at the time of analysis (a). All experiments were repeated three times; $^{*}p < 0.05$.

demonstrates that murine and canine IL3, IL4, IL13, GM-CSF RANTES, and MIP1α were induced or upregulated in response to IgE cross-linking or chemical stimuli. In several instances, this upregulation was noted early following stimulation (2 h) then subsequently decreased at a later time point (16 h). Murine BMCMCs expressed higher levels of IL-13 and RANTES, while canine BMCMCs expressed higher levels of IL-3 following stimulation. The combination of A23187 and PMA has been suggested to induce the broadest production of cytokines/chemokines from mast cells. However, our results indicate that ConA is a far more potent stimulus than A23187/PMA for both canine and murine BMCMCs.

As previously mentioned, experiments in mice have suggested that mast cells participate in innate immune responses through direct recognition of bacterial products via TLR2 and TLR4 (Marshall and Jawdat, 2004). Fig. 4c demonstrates that both canine and murine BMCMCs express message for TLR2 and TLR4, although TLR2 expression appears to be low on resting canine BMCMCs. These data suggest that similar to mouse mast cells, canine mast cells exhibit the capacity to respond to bacterial challenge.

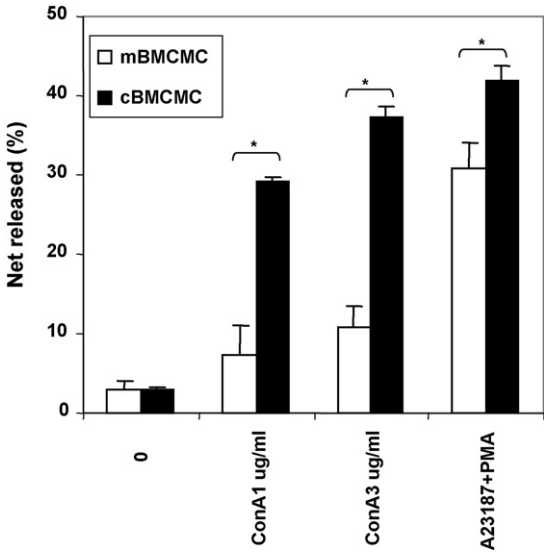

Fig. 3. Degranulation of murine and canine BMCMCs following chemical stimulation. Mature mouse and canine BMCMCs were washed, collected and plated at 1×10^4/well in 96-well plates. After stimulation with ConA or A23187/PMA, the supernatant and the pellets were collected for analysis of β-hexosaminidase content. The degranulation rate was calculated as percentage of β-hexosaminidase released. $^*p < 0.05$.

Because of limitation of canine specific reagents, cytokine/chemokine release following stimulation of BMCMCs was measured when reagents were available for both species. As shown in Fig. 5, both murine and canine BMCMCs released TNFα, TGFβ-1, IL6 and MCP-1 following stimulation with ConA, A23187/PMA and IgE cross-linking at both 2 and 16 h. Neither IL-10 nor INF-γ was detected following BMCMC stimulation with chemicals or IgE cross-linking (data not shown). Of note, unstimulated canine BMCMCs released MCP-1 spontaneously into the tissue culture medium while murine BMCMCs did not (data not shown). Furthermore, the canine BMCMCs released significantly more MCP-1 following chemical stimulation and IgE cross-linking. In contrast, murine BMCMCs released significantly more IL-6 following stimulation than canine BMCMCs.

Mast cells are known to produce and release proteases following activation. These serve to both modulate the extracellular matrix as well as cleave other substrates such as matrix metalloproteinases (MMPs) (Fang et al., 1997). Murine BMCMCs released tryptase following chemical stimulation, but not IgE cross-linking (Fig. 6). In contrast, canine BMCMCs released significantly larger quantities of tryptase following both chemical stimulation and IgE cross-linking.

There is now evidence that mast cells play a role in angiogenesis associated with both tissue repair and tumor growth. Vascular endothelial growth factor (VEGF) is known to be an important mediator of this process and mast cells have been reported to be a source of VEGF. As shown in Fig. 6, both murine and canine BMCMCs produce significant amount of VEGF after A23187/PMA stimulation, but release lower quantities of VEGF after IgE cross-linking and ConA stimulation. In summary, canine BMCMCs produce a similar array of cytokines, chemokines and growth factors compared to their murine counterparts. However, they do exhibit unique differences including significantly higher MCP-1 and tryptase and lower IL-6, demonstrating that canine BMCMCs exhibit unique functional characteristics.

3.4. Release of MMP2 and MMP9 by canine and murine BMCMCs

As previously discussed, mast cells participate in tissue remodeling in and both human and mouse BMCMCs have been reported to release MMP2 and MMP9 which are gelatinases (A and B, respectively). Previous reports have shown that malignant canine mast cell lines release both MMP2 and MMP9 (Fang et al., 1999), although given the neoplastic nature of these cells, it is not clear how accurately this reflects normal mast cell biology. To investigate whether canine BMCMCs are capable of producing MMP2 and MM9 and demonstrate possible role for these cells in tissue remodeling, canine and murine BMCMCs were left untreated or stimulated with A23187/PMA, ConA or IgE cross-linking. As shown in Fig. 7, inactive canine and murine BMCMCs spontaneously released MMP9 which was enhanced following activation. The level of MMP9, especially the active form, was much higher from canine BMCMCs when compared to their murine counterparts. In contrast, MMP2 release was barely detectable from canine BMCMCs compared to the murine cells which proteins small amounts of both inactive and inactive compounds.

4. Discussion

Mast cells disorders are extremely common in dogs, yet relatively little is known regarding the basic biological properties of these cells. This is due, in large part, to the relative difficulty in obtaining pure populations of mast cells from canine skin. As a result, most work has been performed using canine mastocytoma cells lines, not normal mast cells. Given that these

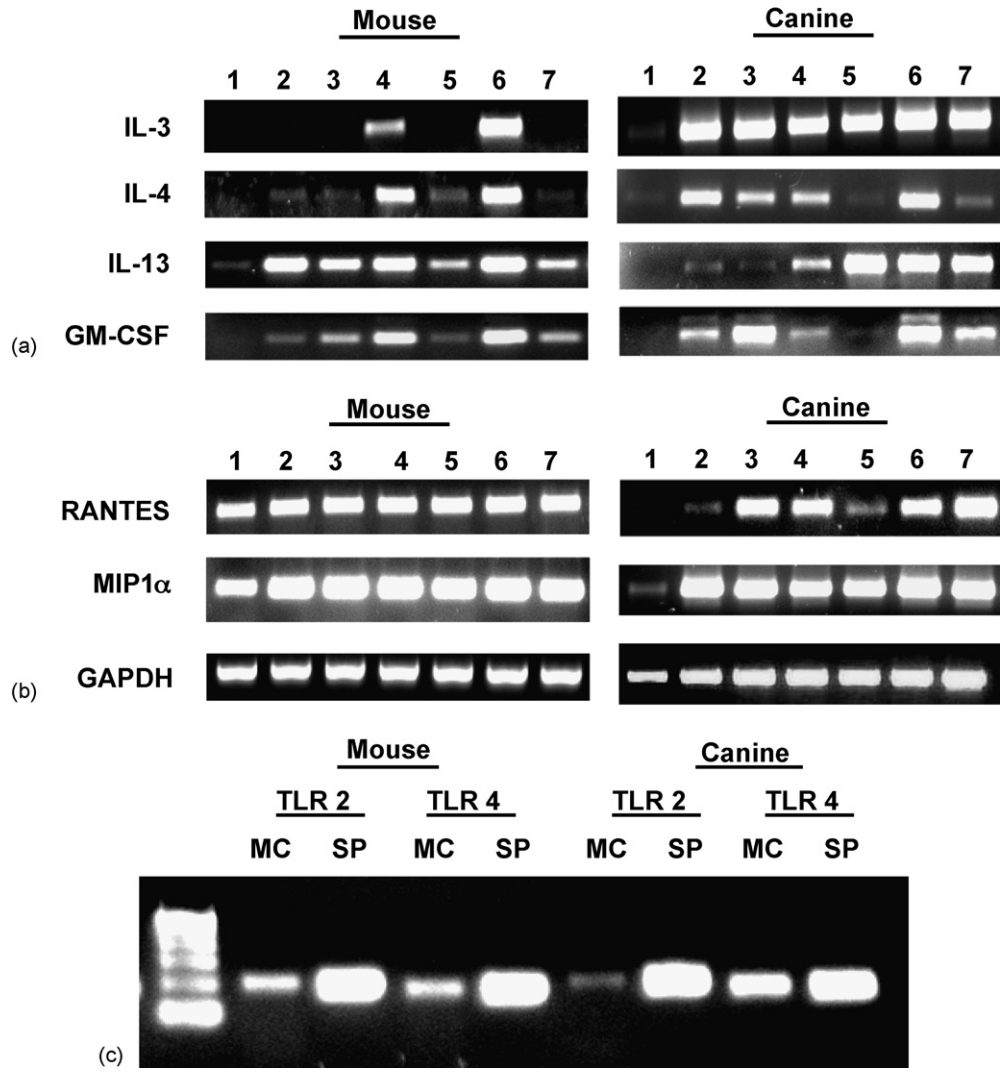

Fig. 4. Modulation of cytokine, chemokine and TLR expression following activation of canine and murine BMCMCs. Mouse and canine BMCMCs were cultured at a concentration of 5×10^5 ml^{-1} in Stemline® II with mIL-3 (10 ng/ml) or rcSCF (50 ng/ml), respectively. Cells were left untreated or treated for 2 or 16 h with A23187/PMA (lanes 2 and 3), ConA (lanes 6 and 7) or cross-linked using anti-canine or anti-murine IgE after IgE sensitization overnight (lanes 4 and 5). Cells were collected and RNA was extracted at 2 or 16 h after activation and RT-PCR was performed to detect cytokines including IL-3, IL-4, IL-10, IL-13 and GM-CSF (a), and chemokines including RANTES and MIP1α (b). RT-PCR for GAPDH was used as a positive control. RNA from untreated canine and murine BMCMCs was used for RT-PCR of TLR2 and TLR4 (c). Positive controls consisted of RNA generated from canine and murine spleen cells.

cells are neoplastic in nature, it is likely that at least some of these findings are not reflective of true mast cell biology. Indeed, many of the neoplastic mast cell lines have lost expression of the FcεRI, a key receptor that defines the mast cell.

In both murine and human studies of mast cell biology, bone marrow derived cultured mast cells (BMCMCs) are routinely employed for experimental work and a variety of studies have demonstrated that these cells are representative of mast cell populations *in vivo*. To facilitate the study of canine mast cell biology, we developed a technique to generate large numbers of canine mast cells from purified bone marrow derived CD34+ cells. We demonstrated that these cells exhibit the typical phenotypic properties of mast cells including the presence of tryptase, chymase, MCP-1, and IL-8 in granules, the expression of Kit, and FcεRI, and release of histamine and TNFα following IgE cross-linking. The purpose of this study was to investigate the functional characteristics of these cells and compare them to what is know about human and murine mast cells.

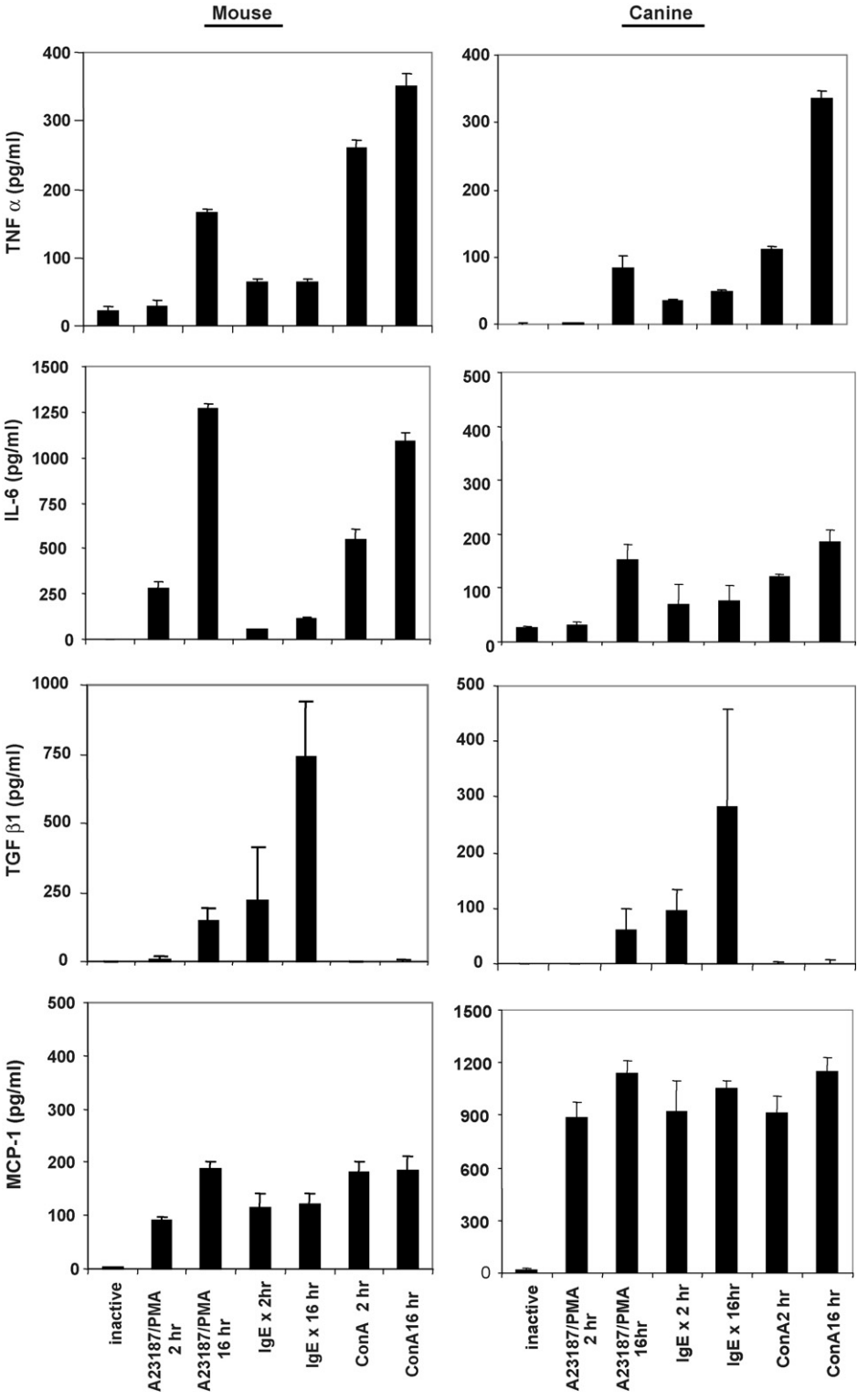

Fig. 5. Cytokine and chemokine release following BMCMC stimulation. Mouse and canine BMCMCs were cultured at a concentration of 5×10^5 ml^{-1} in Stemline® II with mIL-3 (10 ng/ml) or rcSCF (50 ng/ml), respectively. Cells were left untreated or treated with A23187/PMA, ConA or cross-linked using anti-canine or anti-murine IgE after IgE sensitization overnight. Supernatant was collected at 2 and 16 h following stimulation and used for measurement of TNFα (a), IL6 (b), TGFβ1 (c) and MCP-1 (d) by ELISA. Data shown are representative of three independent experiments.

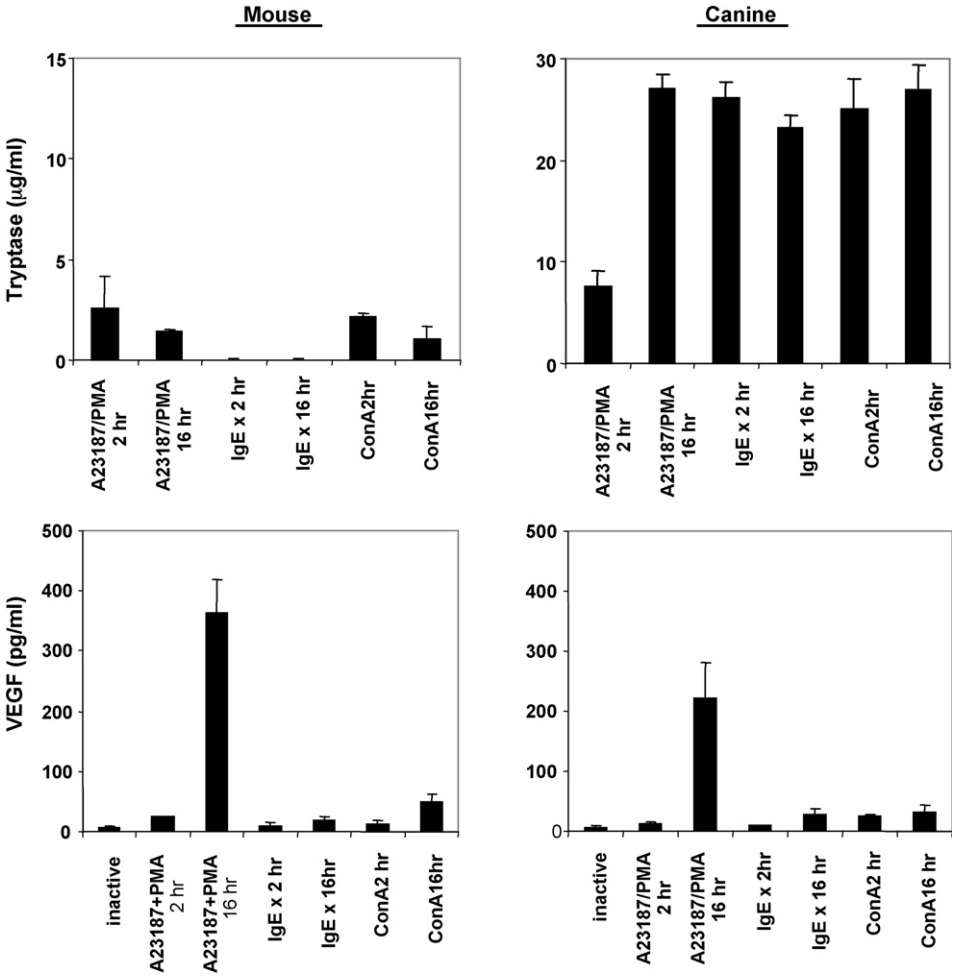

Fig. 6. Effect of IgE cross-linking and chemical stimulation on VEGF and tryptase release by BMCMCs. Mouse and canine BMCMCs were cultured at a concentration of 5×10^5 ml^{-1} in Stemline® II with mIL-3 (10 ng/ml) or rcSCF (50 ng/ml), respectively. Cells were left untreated or treated with A23187/PMA, ConA or cross-linked using anti-canine or anti-murine IgE after IgE sensitization overnight. Supernatant was collected at 2 and 16 h following stimulation and used for measurement of tryptase (a), determined by an enzyme activity assay and VEGF (b) determined by ELISA. Data shown are representative of three independent experiments.

While it is known that IL-3 and stem cell factor (SCF) are important for mast cell differentiation in most species, the effects of additional cytokines on mast cell proliferation and survival tend to vary. For example, both IL-4 and IL-10 have been reported to induce apoptosis or inhibit growth of immature mouse mast cells and downregulate both FcεRI and Kit expression (Bouton et al., 2004; Mirmonsef et al., 1999; Ryan et al., 1998). However, these cytokines promote proliferation of human mast cells and a rodent mast cell line without affecting Kit expression (Imlach et al., 2002; Kulka and Metcalfe, 2005). If immature human mast cells are cultured with IL-4, they suppress maturation, most likely through downregulation of Kit expression (Kulka and Metcalfe, 2005).

Our data demonstrated that IL-4 and IL-10, when used in combination with rcSCF, promote canine BMCMC proliferation when compared to the effects of rcSCF alone. Interestingly, we found that Kit expression was upregulated on canine BMCMCs cultured in IL-4 or IL-10 when compared to SCF alone. It is therefore possible that the increased level of Kit resulted in enhanced SCF induced signaling, leading to the observed differences in proliferation. This is supported by our previous data demonstrating that SCF is required for canine BMCMC survival, proliferation and migration. In contrast to IL-4 and IL-10, TGFβ1 has been reported to suppress mast cell proliferation, inducing downregulation of both Kit and FcεRI resulting in eventual apoptosis (Gebhardt et al., 2005;

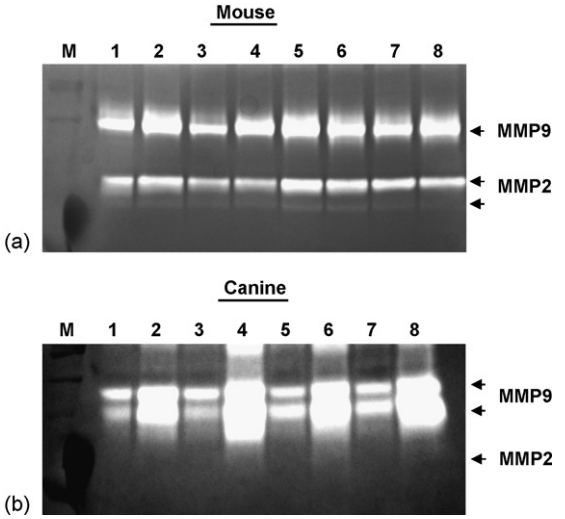

Fig. 7. Active MMP2 and MMP9 release following BMCMCs stimulation. (a) Mouse BMCMCs were cultured at a concentration of 5×10^6 ml^{-1} in Stemline® II and mIL-3 (10 ng/ml). (b) Canine BMCMCs were cultured at a concentration of 2.5×10^6 ml^{-1} in Stemline® II with rcSCF (50 ng/ml). Cells were left untreated or treated with A23187/PMA, ConA or cross-linked using anti-canine or anti-murine IgE after IgE sensitization overnight. Supernatant was collected at 2 and 16 h following stimulation and used for standard gelatin zymography. Bands representing MMP2 and MMP9 are indicated.

Gomez et al., 2005; Norozian et al., 2006). Our data demonstrated a similar effect of TGFβ on canine BMCMCs, supporting the notion that TGFβ may also be a regulator of mast cell proliferation in dogs. Lastly, we noted an increase in granule content in canine BMCMCs treated with SCF and IL-4 as assessed by side scatter. This morphologic change associated with IL-4 has been previously described to occur with human mast cells and is associated with an increase in mast cell histamine content (Nabeshima et al., 2005).

Dogs are known to exhibit significant sensitivity to a variety of chemicals known to induce mast cell degranulation. These include such things as Cremophor (Price and Castells, 2002) and polysorbate 80 (Masini et al., 1985), both of which are used as carrier agents for chemotherapy agents, as well as doxorubicin (Gerritsen et al., 1998). Our data demonstrate that canine BMCMCs exhibit greater degranulation as measured by β-hexosaminidase release in response to ConA, as well as A23187/PMA, when compared to their murine counterparts. This supports the notion that the biologic basis for chemotherapy associated anaphylaxis in dogs is secondary to enhanced sensitivity of canine mast cells to chemical compounds, highlighting the differences in functional properties of mast cells among different species.

Mast cells are known to produce a wide variety of cytokines, chemokines and lipid-based inflammatory mediators (Galli et al., 2005; Marshall and Jawdat, 2004; Puxeddu et al., 2003). Some of these are preformed and stored in granules, while others require activation through IgE cross-linking or complement to be produced and released. Our data demonstrate that both canine and murine BMCMCs do not express IL-3, IL-4, IL-13, or GM-CSF prior to stimulation, but do so in response to chemicals or IgE cross-linking. These cytokines have been associated with eosinophil recruitment, differentiation, survival, and activation (Shakoory et al., 2004). Interestingly, we found that in contrast to murine BMCMCs, the canine cells rapidly expressed IL-3 upon activation through IgE cross-linking, A23187/PMA and ConA at all time points. IL-3 is a critical growth factor for hematopoietic stem cells and likely plays a role in mast cell differentiation *in situ*.

In allergic responses, mast cells directly or indirectly contribute the recruitment of other effect cells such as eosinophils, basophils, neutrophils, monocytes/macrophages and lymphocytes, thereby modulating the local microenvironment (Puxeddu et al., 2003; Schramm and Thorlacius, 2004; Szalai et al., 2001; Teran et al., 1996). For example, MCP-1 has been reported to attract and activate basophils to secrete histamine, while RANTES and MIP-1α appear to have a similar effect, but at a lower potency (Kaplan, 2001). Recently, polymorphisms in the regulatory region of MCP-1 were found to be associated with levels of protein expression in mice (Szalai et al., 2001). MCP-1 is known to be a key player in the pathology of asthma and allergy by promoting leukocyte recruitment, Th2 polarization, TGFβ and procollagen production from fibroblasts, and leukotriene release (Rose et al., 2003). Both canine and murine BMCMCs expressed message for RANTES and MIP-1α and released MCP-1 after activation, although the level of MCP-1 was far greater from canine BMCMCs. This difference in MCP-1 release may contribute to the heightened sensitivity of dogs to a variety of chemical agents, such as Cremophor. That is, the release of MCP-1 from canine mast cells following stimulation may induce basophils to degranulate along with local mast cell populations, amplifying the subsequent allergic response. Furthermore, differences in mast cell mediator production may also exist among various breeds and provide at least some explanation for the breed predispositions known to be associated with allergic disorders.

IL-6 is known to be released from mast cells is reported to be associated with the induction of local inflammatory responses (Diaz et al., 2002; Noguchi

et al., 2005). In human mast cells cultures, IL-6 has been shown to promote mast cell proliferation and differentiation and enhance histamine release from mast cells (Conti et al., 2002; Kikuchi et al., 2002). With respect to allergic responses, IL-6 has been suggested to play a role in Th2 polarization and antibody production (Romagnani, 2002). While substantial quantities of IL-6 were released by murine BMCMCs following chemical stimulation, the amounts released by canine BMCMCs were lower under all conditions tested. The biological effect of this difference is not clear, but it is possible that the enhanced production of other cytokines and/or inflammatory mediators such as MCP-1 and IL-3 supplants the need for high local IL-6 concentrations.

In addition to cytokines and chemokines, tryptase released from mast cells participates in the innate immune response, tissue repair, and angiogenesis. Tryptase is a key mast cell protease that can prevent coagulation by inactivating fibrinogen and activating prostromelysin to cleave latent collagenase (D'Andrea et al., 2001; Schwartz, 1990). In dogs, it was found that tryptase promoted pulmonary smooth muscle contraction in response to histamine (Schwartz, 1990). Furthermore, tryptase has been linked to metastasis, primarily through modulation of the extracellular matrix. Our data demonstrate that canine BMCMCs release significantly greater quantities of tryptase than murine BMCMCs. It is possible that this feature of normal canine mast cells contributes to the ability of neoplastic mast cells to metastasize to local lymph nodes, as well as cutaneous sites distant from the primary tumor.

Matrix metalloproteinases (MMPs) are a family of enzymes involved in the degradation and remodeling of extracellular matrix and in angiogenesis, and as such, are known to participate in tumor invasion and metastasis (VanSaun and Matrisian, 2006). Neoplastic mast cells are known to produce both MMP2 and MMP9 (Leibman et al., 2000), and evidence suggests that stimulation of mast cell tumor cell lines with stem cell factor (SCF, Kit ligand) can enhance production (Fang et al., 1999). Furthermore, grade III mast cell tumors were found to express significantly higher levels of MMP9 in proactive and active forms, which was proposed to be associated with the high degree of malignant behavior of these tumors (Leibman et al., 2000). Our data demonstrate that canine BMCMCs, while producing large quantities of inactive and active MMP9 in response to various stimuli, released little detectable MMP2. This was in contrast to murine BMCMCs that released smaller quantities of both MMP2 and MMP9 that did not increase with either chemical stimulation or IgE cross-linking. It is possible that the ability to produce MMP2 is a feature acquired by malignant mast cells following neoplastic transformation. Alternatively, additional cytokines/chemokines may be necessary to promote MMP2 expression by canine BMCMCs.

In summary, these studies demonstrate that canine mast cells contain a wide variety of inflammatory mediators, some of which are produced in substantially larger quantities than those found in murine mast cells. These differences may provide a possible explanation for the high prevalence of inflammatory disorders involving mast cells in the dog population. Future work would involve identifying functional differences in mast cell populations among specific breeds predisposed to allergic disease.

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