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Generation and characterization of bone marrow-derived cultured canine mast cells

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Abstract

Disorders of mast cells, particularly mast cell tumors (MCTs), are common in dogs. There now is evidence that many of these disorders exhibit breed predilections, suggesting an underlying heritable component. In comparison to humans and mice, little is known regarding the biology of canine mast cells. To facilitate the study of mast cell biology in other species, bone marrow-derived cultured mast cells (BMCMCs) often are used because these represent a ready source of large numbers of cells. We have developed a protocol to successfully generate canine BMCMCs from purified $CD34^+$ cells. After 5–7 weeks of culture with recombinant canine stem cell factor (rcSCF), greater than 90% of the cell population consisted of mast cells as evidenced by staining with Wright's-Giemsa, as well as production of chymase, tryptase, IL-8 and MCP-1. These cells expressed cell surface markers typical of mast cells including Kit, FcεRI, CD44, CD45 and CD18/CD11b. The canine BMCMCs were dependent on rcSCF for survival and proliferation, and migrated in response to rcSCF gradients. Cross-linking of cell surface-bound IgE induced the release of histamine and TNFα. Histamine release could also be stimulated by ConA, compound 48/80, and calcium ionophore. In summary, canine BMCMCs possess phenotypic and functional properties similar to mast cells found *in vivo*. These cells represent a novel, valuable resource for investigating normal canine mast cell biology as well as for identifying factors that lead to mast cell dysregulation in the dog.

Keywords: Mast cell; Dog; Bone marrow; In vitro differentiation

1. Introduction

Disorders involving mast cells are becoming increasingly more common in dogs, including such diverse diseases as atopy and mast cell tumors (MCTs) (Misdorp, 2004). Evidence suggests that many of

Abbreviations: BMCMC, bone marrow-derived cultured mast cells; MCTs, mast cell tumors; PI, propidium iodide; rcSCF, recombinant canine stem cell factor; SCF, stem cell factor

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these occur in a breed-specific manner, indicating a possible heritable component (London and Seguin, 2003). Despite the fact that mast cell diseases are widespread in the canine population, little is known regarding the biology of canine mast cells. This, in part, is due to the difficulty in obtaining large numbers of purified mast cells for study. Historically, mast cells have been isolated from canine skin, although these generally are mixed with other cell types resulting in impure populations and a less-than-optimal analysis of biological properties. Furthermore, the small numbers of cells typically obtained limits the number and extent of analyses that can be performed (de Mora et al., 1993).

Mast cell lines derived from malignant MCTs have been used to characterize certain aspects of canine mast cell biology, such as chymase and gelatinase expression (Fang et al., 1996; Caughey et al., 1997; Fang et al., 1997; Fang et al., 1999). However, these cells are not normal and often do not exhibit classical mast cell properties. For example, although the C2 and BR canine mast cell lines have been used to investigate the properties of mast cells, they do not release histamine after IgE cross-linking and in some instances have lost expression of FcεRI (Garcia et al., 1998; Brazis et al., 2002a, 2002b). Therefore, information obtained from studies of cell lines derived from malignant tumors may not accurately represent normal mast cells *in vivo*.

In both humans and rodents, the study of mast cell biology has been facilitated greatly by the generation of mast cells *in vitro* from $CD34^+$ precursors. In mice and rats, the process is relatively simple because bone marrow from these species contains a high percentage of hematopoietic stem cells, and thus no purification is required (Spangrude and Brooks, 1993). Culture of rodent bone marrow with recombinant IL-3 results in the generation of a relatively homogenous population of mast cells by 4–6 weeks of culture, with full maturity attained at 8 weeks of culture (Levi-Schaffer and Shalit, 1993).

The process of generating human mast cells *in vitro* is more difficult and generally requires a population of purified $CD34^+$ cells and serum-free medium (Ahn et al., 2000; Mwamtemi et al., 2001; Tachimoto et al., 2001; Kulka and Metcalfe, 2005). Moreover, depending on the culture conditions, differentiation into mature mast cells requires recombinant stem cell factor (SCF, the ligand for Kit) in addition to either IL-6 or IL-9 (Kinoshita et al., 1999; Matsuzawa et al., 2003). Human mast cells can be generated from $CD34^+$ cells isolated from bone marrow (Shimizu et al., 2002), cord blood (Dahl et al., 2002) or peripheral blood, although cell numbers are limiting in this situation (Inomata et al., 2005). Bone marrow-derived cultured mast cells (BMCMCs) generated from humans and rodents have been extremely useful for defining the role of mast cells in innate immune responses (Varadaradjalou et al., 2003) and autoimmunity (Supajatura et al., 2001; Kraneveld et al., 2002; Robbie-Ryan et al., 2003; Woolley, 2003; Marshall and Jawdat, 2004) as well as for investigating differences in gene expression (Nakajima et al., 2001). Recently, the generation of pig mast cells from fetal hematopoietic progenitors was also reported; these cells required IL-3 and SCF for survival and possessed morphologic and biochemical properties similar to pig mast cells found *in vivo* (Femenia et al., 2005).

Given the prevalence of mast cell diseases in dogs, the availability of large populations of normal mast cells would be extremely useful not only for dissecting the biological properties of canine mast cells, but also for the identification of molecular mechanisms that promote malignant transformation. As such, the purpose of the following study was to identify the optimal conditions to reliably generate mature and functional canine BMCMCs from purified $CD34^+$ cells. Our data demonstrate that recombinant canine SCF is required for mast cell development, proliferation and survival *in vitro*. Canine BMCMCs were found to contain abundant metachromatic granules positive for chymase and tryptase. Furthermore, these cells expressed phenotypic markers characteristic of mast cells including FcεRI, Kit and integrins. Lastly, the canine BMCMCs released histamine and TNFα following IgE cross-linking. In summary, functional canine BMCMCs can be generated reliably from canine bone marrow. These cells likely will prove useful for investigating normal mast cell biology as well as disorders of mast cells in dogs.

2. Materials and methods

2.1. Reagents

The monoclonal antibodies directed against canine MHC II, CD1a, CD1c, CD11a, CD11b, CD11c,

CD11d, CD18, and CD45 were kindly provided by Dr. Peter Moore, Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, UC Davis. The antibodies directed against canine CD14, CD16, CD32, CD44, CD49f and mouse anti-dog IgE were purchased from Serotec, (Raleigh, NC) and rat anti-mouse CD117 (ACK45), which cross reacts with dog CD117, was obtained from BD Biosciences (Franklin lakes, NJ). Dog IgE and goat anti-dog IgE were purchased from Bethyl Labs (Montgomery, TX). Recombinant canine stem cell factor (rcSCF) was purchased from R&D Systems (Minneapolis, MN). The following chemicals and enzymes were purchased from Sigma (St. Louis, MO): calcium ionophore A23187, Concanavalin A (Con A), substance P (SP), compound 48/80, Tyrode's basal salt solution, Fast Red Violet LB base solution, Fast Blue BB base solution, 37% formaldehyde, and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide dihydrate.

2.2. Bone marrow collection

Bone marrow was collected from 15 canine patients undergoing routine ovariohysterectomy or castration at the Veterinary Medical Teaching Hospital (VMTH) at UC Davis following appropriate client consent. Approximately 10–15 ml of bone marrow was obtained from the proximal humerus and collected into 3.8% sodium citrate/PBS solution.

2.3. $CD34^+$ cell isolation

While purification of $CD34^+$ cells from canine bone marrow previously had been reported, we adapted a method that increased the purity of the final cell population (McSweeney et al., 1998; Suter et al., 2004). Briefly, the bone marrow was diluted 1:8 with RPMI (Gibco, Grand Island, NJ), layered onto Ficoll-Paque™ PLUS (Amersham Biosciences, Piscataway, NJ) and centrifuged at 300 g for 35 min at room temperature. The interface cells were collected and the remaining red blood cells were lysed using hypotonic shock treatment. Cells were resuspended in RPMI, seeded into 75 mm^2 tissue culture flasks and incubated at 37 °C in a humidified incubator at 5% CO_2 for 30 min to remove adherent cells. The remaining cells were collected and counted. For a starting population of 100×10^6 total cells, 15 μg biotinylated anti-canine CD34 antibody (1H6, BD Biosciences) was added followed by incubation for 30 min at 4 °C. The cells then were washed in 2 mM EDTA/0.5% BSA/PBS twice; 100 μl streptavidin microbeads (Miltenyi, Auburn, CA) were added and the cells were incubated for 30 min at 4 °C. $CD34^+$ cells were enriched using the MidiMACS (Miltenyi, Auburn, CA) separator and analyzed by flow cytometry for $CD34^+$ purity.

2.4. Generation of canine BMCMCs

The purified $CD34^+$ cells were plated in 24-well plates at a final concentration of 5×10^5/ml in the Stemline® II (Sigma) medium supplemented with 100 ng/ml rcSCF, and penicillin/streptomycin/L-glutamine. Cultures were incubated at 37 °C in a humidified incubator at 5% CO_2. Half of the medium was replaced once per week, with the addition of fresh rcSCF at 100 ng/ml. Any remaining adherent cells were eliminated by seeding fresh plates each time during the first 4 weeks of culture. For all experiments, the BMCMCs were harvested after 4–8 weeks of culture in serum-free medium containing rcSCF.

2.5. Assessment of BMCMC purity

BMCMCs were harvested at 4–5 weeks of culture and assessed using both Wright's-Giemsa and toluidine blue staining. Briefly, approximately 1×10^4 cells were collected, cytospins were performed, and the slides were stained by routine Wright's-Giemsa staining at the Clinical Pathology Laboratory of the VMTH at UCD. For toluidine blue staining, 0.1% toluidine blue, pH 4.0, was applied to the cytospin preparations for 10 min followed by a quick incubation in acetic alcohol.

2.6. Enzyme histochemistry

To detect chymase activity, a commercially available detection kit (Sigma) was used with naphthol AS-D chloroacetate as the substrate. The cytospin preparations were fixed in a citrate-acetone-formaldehyde (CAF) solution for 30 s at room temperature followed by incubation with working solution of either the Fast Red Violet LB base solution or the Fast Blue BB base solution (Sigma) at 37 °C for 30 min. The slides then were counterstained with hematoxylin.

2.7. Immunohistochemistry

Approximately 1×10^6 6-week-old BMCMCs were collected, pelleted, and fixed in 10% neutral buffered formalin for 24 h then paraffin embedded. Slides (5 μs) were boiled in citrate buffer for 10–20 min and the LSAB® system-HRP kit (DakoCytomation) was used for staining. Briefly, slides were treated with 5% hydrogen peroxide and washed, followed by the addition of primary antibodies against tryptase (1:300, AA1, DakoCytomation), IL-8 (10 μg/ml, R&D Systems), or MCP-1 (10 μg/ml, R&D Systems). After 1 h incubation at room temperature, the slides were washed and appropriate secondary antibodies were applied at room temperature for another 30 min. Positive staining was detected using a standard avidin-biotin DAB method and counter-stained with hematoxylin. The BR canine malignant mast cell line was used as a positive control for these experiments.

2.8. Phenotypic analysis of BMCMCs

For phenotypic analysis of the BMCMCs, 5×10^4 4–7-week-old cells were collected, washed twice in PBS, and preincubated with 0.5% BSA/PBS containing 10% normal dog serum to block non-specific Fc receptor binding. A panel of primary antibodies was used (see Section 2.1) and appropriate irrelevant isotype control antibodies were used for each. Cells were incubated with primary antibodies for 1 h at 4 °C, washed twice, and incubated with appropriate secondary antibodies (Caltag Lab, Burlingame, CA or BD Biosciences) for 30 min at 4 °C. Cells were analyzed using a FACScalibur (BD Biosciences) and Cell Quest software (BD Biosciences). These experiments were repeated with three sets of BMCMCs from different dogs.

2.9. Determination of IgE receptor expression

To detect FcεRI expression an indirect method was used (Brazis et al., 2002a, 2002b). Approximately 5×10^4 BMCMCs were loaded with 5 μg/ml canine IgE (Bethyl Labs) overnight. After washing, the cells were incubated with monoclonal anti-canine IgE antibody (Serotec) at 4 °C followed by FITC-conjugated goat anti-dog IgE (Bethyl Labs). An irrelevant secondary isotype-matched goat antibody was incubated with IgE-loaded BMCMCs or unloaded BMCMCs as a negative control. These experiments were repeated with three sets of BMCMCs from different dogs.

2.10. SCF-dependent proliferation, survival, and migration

To assess the effects of rcSCF on BMCMC proliferation, 7×10^3 5–7-week-old cells were plated in 96-well plates in 150 μl Stemline® II medium in the presence or absence of 5, 50, or 100 ng/ml of rcSCF. Plates were collected at Days 0, 1, 3, or 7 of culture 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. Fluorescence then was 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 fluorescence. Samples were analyzed in quadruplicate and experiments were repeated with three sets of BMCMCs from different dogs.

To evaluate the effects of rcSCF on BMCMC cell cycle and survival, 5×10^4 5–7-week-old cells were cultured in 1 ml Stemline® II in 100 ng/ml rcSCF, no rcSCF, or 100 ng/ml rcSCF plus 0.1 μM PHA-291639E (SU11654, a small molecule kit inhibitor) (Liao et al., 2002; Pryer et al., 2003) in 24-well plates. After 24 and 48 h culture periods, the cells were collected, washed twice in 0.1% glucose-PBS solution and fixed in 1 ml of cold 75% ethanol. To determine cell cycle status, the cells were centrifuged and 0.5 ml of propidium iodide (PI) staining solution (50 μg/ml PI and 10 μg/ml RNAse in 0.1% glucose-PBS solution) was added and cells were analyzed by flow cytometry. To detect apoptosis using flow cytometry, the Annexin-V-FTITC kit was used (BD Biosciences) according to the manufacturer's specifications. Experiments were repeated with three sets of BMCMCs from different dogs.

To assess canine BMCMC migratory responses to rcSCF, transwell migration (Transwell®, Costar, Corning, NY) was performed in 48-well plates using a pore size of 8.0 μm. Briefly, 600 μl Stemline® II medium containing 0, 1, 5, 20, or 50 ng/ml rcSCF was placed into the lower chamber. Approximately

5×10^4 6–8-week-old BMCMCs in 200 μl Stemline® II were added to the upper chambers. Positive controls consisted of 5×10^4 BMCMCs in 200 μl Stemline® II with 5 ng/ml rcSCF and no transwells (i.e., 100% of cells in lower chamber) and negative controls consisted of wells without BMCMCs (i.e., 0% of cells in lower chamber). After 16 h at 37 °C, the transwells were removed and the cells in the lower chambers were quantitated using the CyQuant® cell proliferation assay kit (Molecular Probes, Carlsbad, California). Migration rate was calculated as the percent of cells that had migrated through the filter after subtracting out the blank (fluorescence lower chamber − blank)/(average of fluorescence control chamber − blank) × 100. To provide a direct quantitation of cell migration, the experiments described above were repeated and cells that had migrated into the lower chamber were counted; numbers were expressed as cells per high power field with 10 fields counted and the average reported. Experiments were performed in duplicate and repeated with three sets of BMCMCs from different dogs.

2.11. Stimulation of histamine release from BMCMCs

BMCMC degranulation was induced using both IgE cross-linking as well as direct chemical stimulation. At 5–8 weeks of age, BMCMCs were loaded with 5 μg/ml of dog IgE (Bethyl Labs) overnight at 37 °C. Unbound IgE was removed by washing with PBS and 1×10^4 BMCMCs were then plated in V-bottom 96-well plates in 80 μl Tyrode's basal salt solution. Goat anti-dog IgE antibody at 0.1–30 μg/ml (Bethyl Labs) was added to the wells and after 10 min of incubation at room temperature, 10 μl TrueBlot TM anti-goat Ig IP beads was added to each well followed by incubation for 1 h at 37 °C. The plates were centrifuged and the supernatants were collected and stored at −20 °C for assessment of β-hexosaminidase and histamine release (see below). Next, 75 μl of 0.5% Tween-20/PBS was added and the plates were incubated for 10 min at room temperature to lyse the cell pellets. The plates were centrifuged again and the supernatants (representing unreleased histamine from cells) were collected and stored.

To stimulate chemical degranulation of the BMCMCs, 1×10^4 cells were washed and placed into V-bottom 96-well plates in 90 μl Tyrode's salt solution. The cells were stimulated by adding 10 μl of a 10× solution of each stimulating agent (ConA, Substance P, calcium ionophore A23187 and compound 48/80) in increasing concentrations. After incubation at 37 °C for 30 min, the plates were centrifuged and the supernatants were collected and stored at −20 °C for assessment of β-hexosaminidase and histamine release (see below). The cells then were lysed as described above, the plates were centrifuged, and the supernatants were collected and stored. All experiments were performed in triplicate with BMCMCs generated from three different dogs.

2.12. β-Hexosaminidase assay and histamine measurement

Measurement of β-hexosaminidase in the supernatant was used as a surrogate marker of histamine release and was performed using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as previously described (Naal et al., 2004). Briefly, 100 μl of 1.2 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide in 0.05 M sodium acetate buffer (pH 4.4) was added to 25 μl of supernatants collected after BMCMC stimulation or IgE cross-linking, and incubated for 30 min at 37 °C. The reaction was quenched by adding 175 μl of cold 0.1 M glycine-carbonate buffer (pH 10.0) and adsorbance was measured using an ELISA plate reader (Molecular Devices). The negative control consisted of 25 μl of Tyrode's salt solution instead of supernatants and this background fluorescence was subtracted from all readings. Histamine concentration in supernatants and cell lysates was measured using a commercially available ELISA kit (Neogene, Lexington, KY) according to the manufacturer's instructions. Adsorbance was assessed using an ELISA plate reader (Molecular Devices). The negative control consisted of Tyrode's salt solution instead of supernatants and this background adsorbance was subtracted from all readings. The β-hexosaminidase or histamine released from BMCMCs was calculated using the following formula: (O.D. in cell supernatants − O.D. in blank)/(O.D. in cell supernatants − O.D. in blank + O.D. in cell pellet − O.D. in blank) × 100. All experiments were performed in triplicate with BMCMCs generated from three different dogs.

2.13. Measurement of TNF-α release following IgE cross-linking

TNF-α release following IgE cross-linking was measured using the Quantikine® canine TNF-α/TNFSF1A ELISA kit (R&D Systems) according to the manufacturer's specifications. At 5–7 weeks of age, BMCMCs were loaded with 5 μg/ml of dog IgE (Bethyl Labs) overnight at 37 °C. Unbound IgE was removed by washing the cells in PBS twice. Approximately 2×10^4 BMCMCs were added to wells of V-bottom 96-well plates in Stemline II medium containing rcSCF (50 ng/ml), and IgE was cross-linked as described above. Supernatant (50 μl) was collected at 1, 2, 6, and 8 h after IgE cross-linking and stored at −20 °C prior to use. Controls consisted of Stemline® II medium alone and experiments were performed in triplicate and repeated with three sets of BMCMCs from different dogs.

3. Results

3.1. Generation of canine BMCMCs

The generation of mouse BMCMCs is relatively straightforward as bone marrow from C57BL/6 mice tends to have a high percentage (6–7%) of hematopoietic progenitors (Spangrude and Brooks, 1993). In contrast, marrow that we obtained from young adult dogs contained less than 1% $CD34^+$ cells in (Fig. 1(a)) which is slightly lower than previously reported

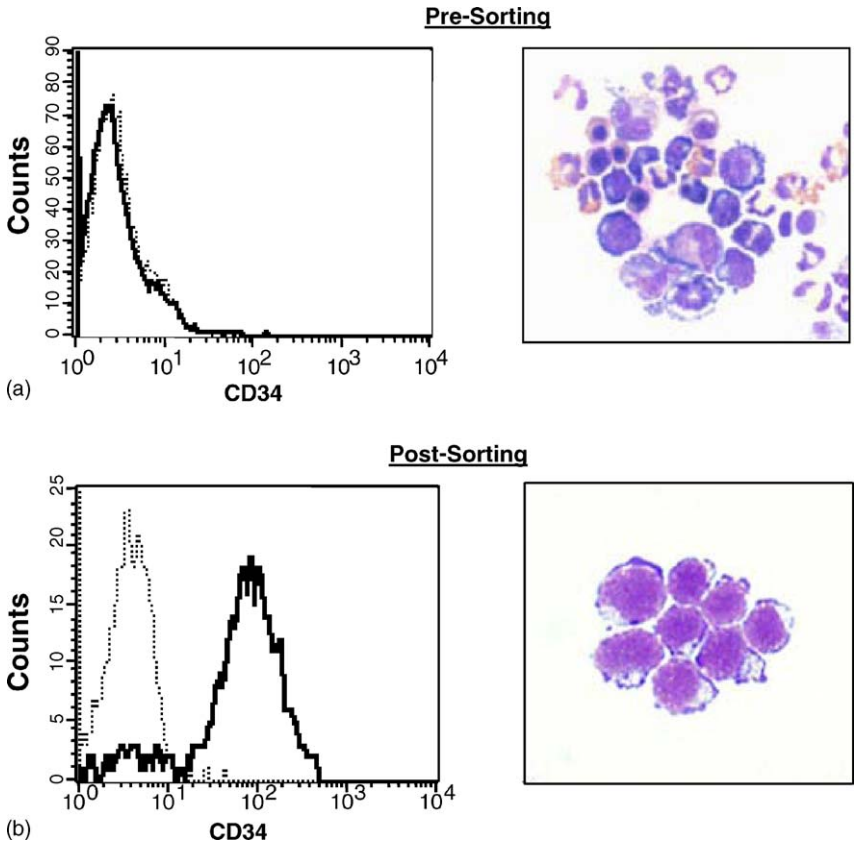

Fig. 1. Purification of $CD34^+$ cells from canine bone marrow. (a) Bone marrow contains less than 1–2% $CD34^+$ cells and multiple lineages are evident. (b) Following red cell lysis, Ficoll-Hypaque density gradient separation, removal of attached cells, and positive selection, the resultant population is 80–90% $CD34^+$ and these cells exhibit morphology typical of hematopoietic stem cells including a round centrally placed nucleus and scant cytoplasm.

concentrations of 1–2% (McSweeney et al., 1998). Our earlier efforts with whole marrow had proved unsuccessful, leading to the growth of primarily neutrophils and macrophages (data not shown). Therefore, to effectively generate canine BMCMCs, it first was necessary to purify the $CD34^+$ cells. As shown in Fig. 1, following a process involving red blood cell lysis, Ficoll-Hypaque density gradient enrichment, removal of attached cells, and positive selection using magnetic bead sorting, approximately

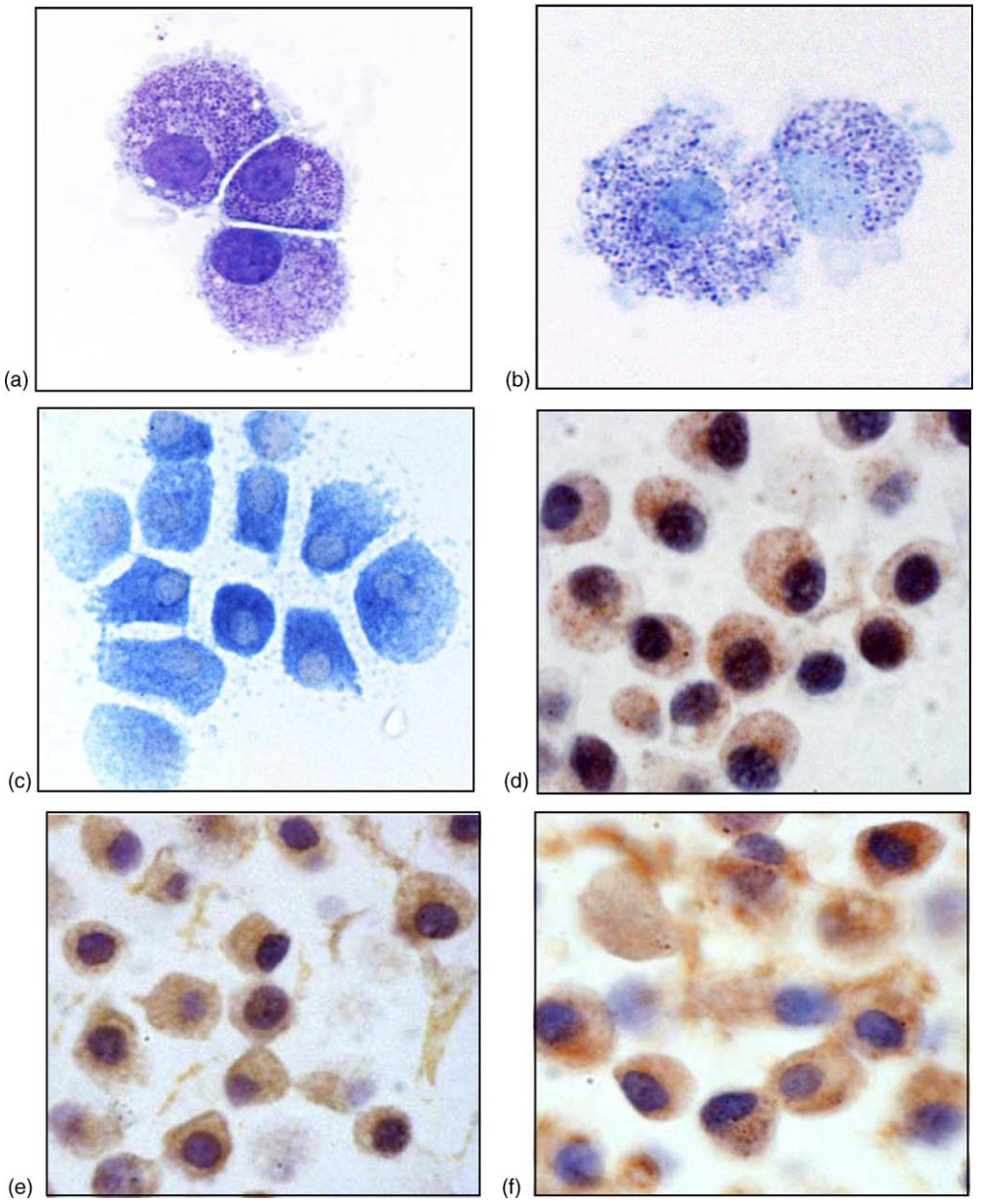

Fig. 2. Canine BMCMC morphology and granule content. Canine BMCMCs at 5–7 weeks of age were prepared for analysis by cytospin or fixation in formalin and subsequent embedding in paraffin. The BMCMCs were stained with Wright's-Giemsa (a); toluidine blue (b); or for chymase (c); tryptase (d); IL-8 (e); or MCP-1 (f) according to the procedures outlined in the Section 2.

1–5 × 10^6 cells can be obtained from 10 to 15 ml of canine bone marrow. The cell population obtained was 80–90% $CD34^+$, and had the typical appearance of mononuclear hematopoietic progenitors (Fig. 1(b)).

We found that even with the use of an enriched population of $CD34^+$ cells, initial attempts at generating canine BMCMCs from this starting population using standard culture conditions, including animal serum complex or fetal bovine serum supplemented with rcSCF, were largely unsuccessful (data not shown) resulting in a mix of differentiated cell types. However, when the purified $CD34^+$ cells were placed in serum-free medium (Stemline® II) supplemented with rcSCF, a population of mast cells was evident by 3–4 weeks of culture. Abundant proliferation and expansion of this population was observed from Weeks 4–7 of culture. Evaluation of these cells at 5–7 weeks of culture revealed that greater than 90% contained metachromatic granules following Wright's-Giemsa staining and greater than 90% also were toluidine blue positive (Fig. 2(a and b)). On average, approximately 5–10 × 10^6 BMCMCs routinely could be generated from a starting population of 5 × 10^5 $CD34^+$ cells using the purification and culture conditions described above.

3.2. Phenotype of BMCMCs

To determine whether the canine BMCMCs expressed phenotypic markers characteristic of mast cells found *in vivo*, we performed a variety of analyses. First, we evaluated the BMCMCs for expression of several products known to be packaged in granules. As shown in Fig. 2, the canine BMCMCs contained

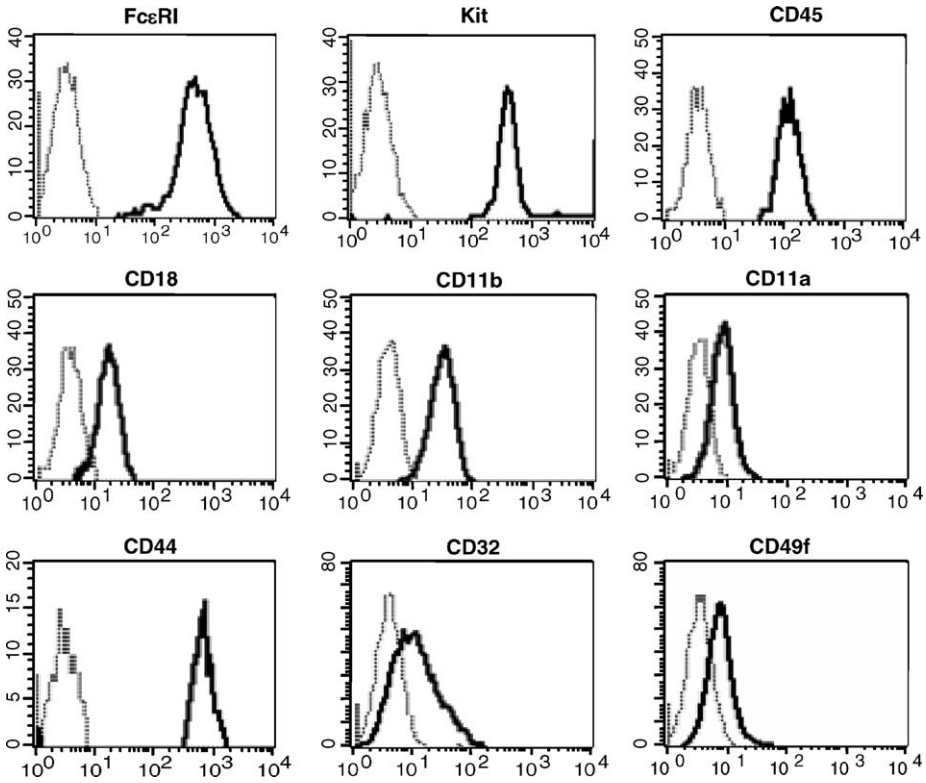

Fig. 3. Cell surface phenotype of canine BMCMCs. Canine BMCMCs at 5–7 weeks of age were collected, washed, and incubated with FITC or PE-labeled antibodies directed against a variety of cell surface antigens. For FcεRI detection, cells first were loaded with canine IgE, then a labeled goat anti-dog IgE was used for detection. Controls consisted of cells stained with labeled isotype-matched control antibodies. Samples were analyzed by flow cytometry using a BD FACScan. The phenotypic analysis was repeated three times using BMCMCs generated from three different dogs.

abundant granules that were positive for chymase (Fig. 2(c)), tryptase (Fig. 2(d)), IL-8 (Fig. 2(e)) and MCP-1 (Fig. 2(f)) using either immunohistochemical or cytochemical methods, consistent with the phenotype of human and mouse BMCMCs, as well as mast cells found *in vivo* from a variety of species, including dogs (Nadel, 1991; Baghestanian et al., 1997; Grutzkau et al., 1997; Kinoshita et al., 1999). We next quantified the amount of histamine in BMCMCs after 6 weeks of culture and found them to contain an average of 1.1–1.2 pg histamine/cell. To characterize these cells further, the canine BMCMCs were incubated with a panel of antibodies specific for cell surface markers such as the IgE receptor, integrins and MHC molecules. As shown in Fig. 3, canine BMCMCs consistently expressed high levels of Kit, FcεRI, CD44 (the homing receptor Pgp-1), CD45 (common leukocyte marker), and lower levels of CD11a (LFA-1), CD11b (CR3), CD18 (Integrin alpha M, Mac-1 alpha), CD32 and CD49f. In addition, the cells were negative for CD1a, CD1c, CD11c, CD11d, CD14, CD34 and MHC-II (data not shown), which was anticipated because mast cells from other species do not express the majority of these markers (Escribano et al., 1998; Welker et al., 2000; Dahl et al., 2004; Krauth et al., 2005). Therefore, the canine BMCMCs exhibit phenotypic characteristics consistent with mast cells found *in vivo*.

3.3. Role of rcSCF in BMCMC survival, proliferation, and migration

SCF is a critical growth factor for mast cell development and acts to prevent apoptosis of mature mast cells (Galli et al., 1995; Puxeddu et al., 2003; Femenia et al., 2005). To assess the role of rcSCF in canine BMCMC survival, cells were cultured in the presence or absence of rcSCF and assessed for evidence of apoptosis. Following 24 and 48 h of rcSCF withdrawal, 53% (Fig. 4(a)) and 75% (Fig. 4(b)) of the cells had undergone apoptosis, respectively, compared to less than 5% of the cells maintained in rcSCF at either time point. Furthermore, when the BMCMCs were incubated with rcSCF in the presence of a small molecule kit inhibitor (PHA-291639E), they rapidly underwent a significant degree of apoptosis (30.2% and 61% at 24 and 48 h, respectively), demonstrating that SCF induced kit signaling was directly responsible for promoting BMCMC survival. Therefore, similar to BMCMCs from other species, canine BMCMCs are dependent on SCF for survival.

In addition to supporting mast cell survival, SCF is known to stimulate mast cell proliferation both *in vitro* and *in vivo* in other species. We evaluated the effects of increasing concentrations of rcSCF on canine BMCMCs at 1, 3, and 7 days of culture. Our results demonstrated that a low concentration (5 ng/ml) of rcSCF was able to promote survival but not proliferation of canine BMCMC, while cultures supplemented with 50 or 100 ng/ml rcSCF underwent significant proliferation (Fig. 5(a)). This effect was dose-dependent since 100 ng/ml rcSCF resulted in larger numbers of BMCMCs than 50 ng/ml at Day 7 of culture (Fig. 5(a)). Therefore, as with mouse and human BMCMCs, SCF induces proliferation of canine BMCMCs and likely supports their proliferation *in vivo*.

Accumulation of mast cells in various tissues is not only due to local proliferation, but is also a consequence of migration from other sites. To assess the ability of canine BMCMCs to migrate along an SCF gradient, a standard transwell assay was employed. As shown in Fig. 5(b), the canine BMCMCs readily migrated through transwells with a 8.0 μm pore in the presence of low concentrations of rcSCF (5–20 ng/ml). Interestingly, migration was inhibited in the presence of high concentrations of rcSCF (50 ng/ml). In summary, canine BMCMCs exhibit a similar spectrum of responses to SCF as do BMCMCs from other species.

3.4. Functional response of canine BMCMCs

One fundamental property that defines the mast cell is the ability to release histamine, either through IgE cross-linking or chemical stimulation. Previous work using mast cells isolated from canine skin demonstrated that these cells could be loaded with IgE and cross-linked, or stimulated with chemicals, resulting in degranulation and the release of histamine and cytokines such as TNF-α (Garcia et al., 1998; Brazis et al., 2002a, 2002b). We performed a similar analysis of the canine BMCMCs, loading them with canine IgE then cross-linking the IgE and measuring histamine and TNFα release into the tissue culture medium. For

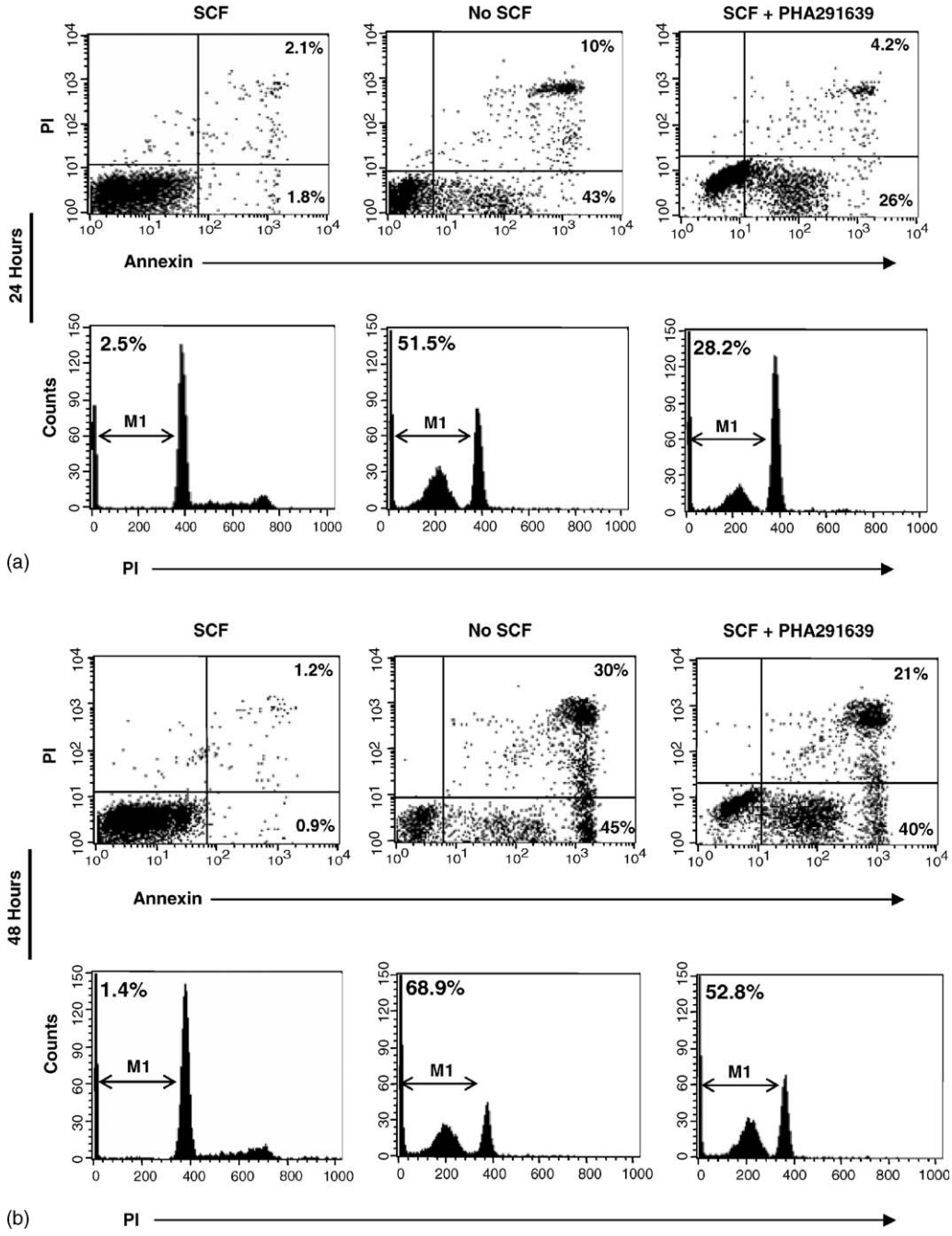

Fig. 4. Canine BMCMCs require rcSCF for cell survival. Canine BMCMCs at 5–7 weeks of age were collected, washed then placed back in complete medium with 100 ng/ml rcSCF, no SCF, or 100 ng/ml rcSCF plus 0.1 μM PHA-291639E (Kit inhibitor). Cells were collected at 24 h (a) and 48 h (b) of treatment. The cells were split into two aliquots, and the first (upper panels) was stained with Annexin V FITC and propidium iodide (PI) to assess the number of cells undergoing early and late apoptosis. The second aliquot (lower panels) was washed in 0.1% glucose in PBS, permeabilized and fixed in 75% ethanol, and PI was added immediately prior to analysis to assess the percentage of cells undergoing cell cycle arrest and late apoptosis. Samples were analyzed by flow cytometry using a BD FACScan. The apoptosis and cell cycle analysis was repeated three times using BMCMCs generated from three different dogs.

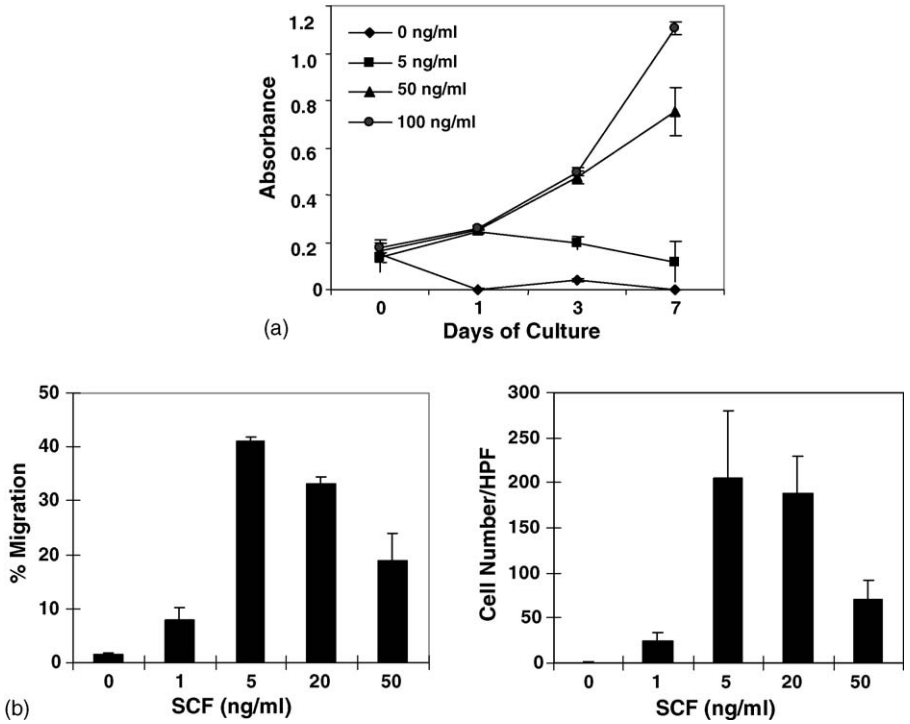

Fig. 5. rcSCF induces proliferation and migration of canine BMCMCs. (a) Canine BMCMCs at 5–7 weeks of age were collected, washed, and then cultured without rcSCF or with increasing concentrations consisting of 5, 50, or 100 ng/ml. Plates were collected on Days 0, 1, 3, and 7 to assess cell numbers using the WST-1 assay. (b) To assess the ability of rcSCF to induce migration of canine BMCMCs, rcSCF at 0, 1, 5, 20, or 50 ng/ml was placed in the lower chamber and the BMCMCs at 5–7 weeks of age were placed in the upper chamber above transwells possessing 8.0 μm pores. The percent of cells that had migrated into the lower chamber after 12 h was then calculated (left panel) as was the number of cells in the lower chamber per high power field (right panel). Shown is a representative of three separate experiments using BMCMCs generated from three different dogs.

most studies, β-hexosaminidase was measured initially because it is released along with histamine and the assay is more rapid and accurate than commercially available histamine ELISA detection kits. The canine BMCMCs released β-hexosaminidase and histamine following stimulation with ConA (Fig. 6(a)) and compound 48/80 (Fig. 6(b)), as has been previously demonstrated with skin-derived canine mast cells. Fig. 6(c) demonstrates that canine BMCMCs also readily released β-hexosaminidase and histamine following cross-linking of cell surface IgE. Furthermore, a time-dependent release of TNFα from BMCMCs was detected following IgE cross-linking (Fig. 7). Interestingly, the canine BMCMCs failed to respond to substance P and exhibited lower histamine release in response to treatment with calcium ionophore A23187 (data not shown). Nevertheless, the canine BMCMCs do exhibit standard functional responses to IgE cross-linking and thus are a relevant cell population in which to study FcεRI-regulated processes.

4. Discussion

Mast cells generated from bone marrow stem cells, peripheral blood stem cells, cord blood stem cells or fetal liver-derived hematopoietic stem cells have been used extensively in mice, humans and more recently pigs, to explore the biology of normal mast cells as well as to investigate their roles in pathological processes such as asthma and arthritis (Shimizu et al.,

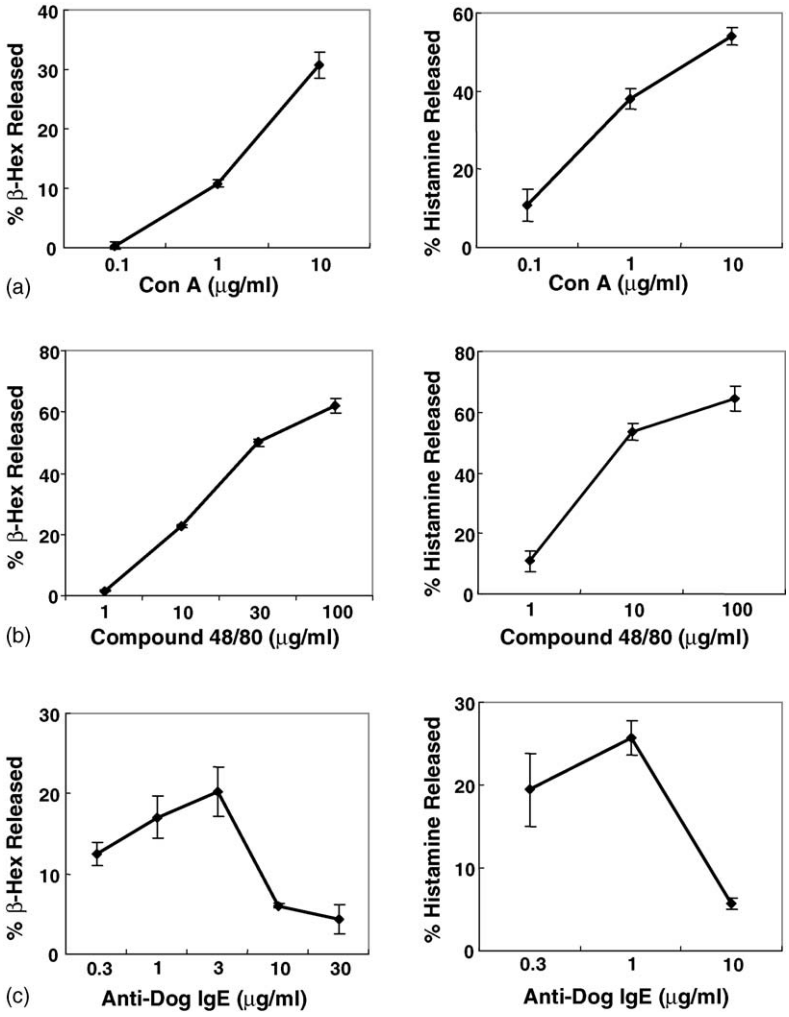

Fig. 6. Release of β-hexosaminidase and histamine from canine BMCMCs following chemical stimulation or IgE cross-linking. (a and b) Canine BMCMCs at 5–7 weeks of age were left untreated or incubated with ConA or compound 48/80 at increasing concentrations for 30 min. The supernatants were collected, then the remaining cells were lysed, the plates were spun, and the lysis supernatant was collected. The amount of β-hexosaminidase and histamine released was assessed using ELISA by measuring the amount of β-hexosaminidase/histamine present in the supernatants and dividing this by the total β-hexosaminidase/histamine present in the BMCMCs (quantity in supernatant plus quantity in lysis supernatant). (c) Canine BMCMCs at 5–7 weeks of age were washed, loaded with canine IgE and the IgE was cross-linked as described in Section 2. After 60 min, supernatants were collected, then the remaining cells were lysed, the plates were spun, and the lysis supernatant was collected. The amount of β-hexosaminidase and histamine released was assessed by ELISA using the method described above. Shown is a representative of three separate experiments using BMCMCs generated from three different dogs.

2002; Corr and Crain, 2002; Kraneveld et al., 2002). The process of generating mast cells *in vitro* is fairly species-specific. For example, murine mast cells can be grown from whole mouse bone marrow in the presence of IL-3, while human mast cells often require serum-free medium, IL-6, SCF and/or other cytokines such as IL-9 (Kinoshita et al., 1999; Matsuzawa et al., 2003). While canine mast cells can be enriched from the skin and other sites, it has been challenging to obtain a pure population and cell number often is limiting, thereby making particular investigations, such as transcriptional profiling, extremely difficult

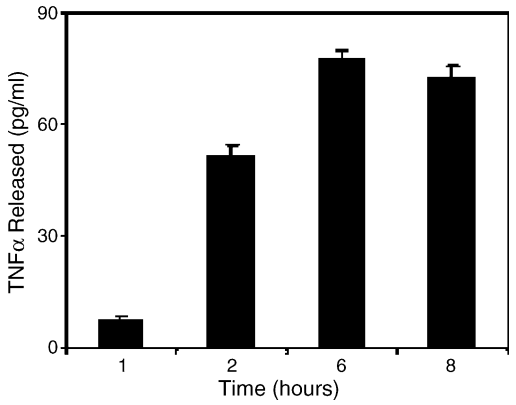

Fig. 7. Canine BMCMCs release TNF-α following IgE cross-linking. Canine BMCMCs at 5–7 weeks of age were washed, loaded with canine IgE and the IgE was cross-linked as described in Section 2. At 1, 2, 6, and 8 h following cross-linking, supernatants were collected. TNFα was measured using a commercially available ELISA kit. Shown is a representative of three separate experiments using BMCMCs generated from three different dogs.

(Soll et al., 1979; de Mora et al., 1993). Furthermore, much of what is known regarding canine mast cell biology has been derived from malignant mast cell lines and thus may not reflect the true functional properties of normal mast cells (Garcia et al., 1998; Brazis et al., 2002a, 2002b). Given the widespread utility of mast cells derived *in vitro* from other species, the purpose of this work was to develop a reliable protocol for the generation of canine bone marrow-derived mast cells to facilitate studies of canine mast cell biology.

Our initial attempts at *in vitro* generation were performed with whole canine bone marrow in the presence of rcSCF, resulting in the differentiation of many cell lineages including neutrophils and macrophages. We reasoned that because the frequency of $CD34^+$ cells was quite low in our marrow samples (typically less than 1%, Fig. 1) the more numerous committed lineages such as granulocyte/monocyte precursors or red cell precursors were interfering with differentiation through overcrowding or the production of other cytokines. Furthermore, it was also possible that growth factors/cytokines present in fetal bovine serum might prevent mast cell development, as is known to be the case for the generation of human BMCMCs from hematopoietic precursors (Kinoshita et al., 2000; Ishida et al., 2003). Therefore, we developed a protocol to purify $CD34^+$ cells from the marrow (Fig. 1) and cultured these cells using a variety of serum-free media supplemented with rcSCF. One of the serum-free media, Stemline II, led to the reliable development of a population of mast cells from the $CD34^+$ hematopoietic precursors (Fig. 2).

The canine BMCMCs we generated *in vitro* exhibited the typical round cell morphology, and possessed metachromatic granules, as assessed by Wright's Giemsa staining (Fig. 2). Furthermore, the canine BMCMC granules possessed tryptase and chymase activity, and expressed IL-8 and MCP-1, characteristic of granules found in mast cells from other species (Fig. 2). It is known that mast cells from different species vary greatly in their expression of proteases. For example, mouse mast cells are known to express at least eleven proteases and this expression varies among inbred strains (Saito, 2005). In contrast, human mast cell granules contain primarily tryptase (T) and chymase (C), and they often are classified based on this expression pattern (MC_T type versus MC_{TC} type) (Galli et al., 2005). Previous work on canine mast cells found *in vivo* identified three populations: MC_T, MC_C, and MC_{TC} (found primarily in canine skin) (Noviana et al., 2004). Since the canine BMCMC we generated *in vitro* expressed both chymase and tryptase, they exhibit characteristics more closely related to mast cells found in canine skin.

With respect to cell surface markers the canine BMCMCs expressed an array of antigens typical of mast cells from other species including the high affinity IgE receptor (FcεRI), Kit, CD11/CD18, CD45, CD44, as well as others (Fig. 3). While we did not detect either CD14 or MHC Class II on the canine BMCMCs, the expression of these antigens has been variably reported on human and mouse mast cells (Welker et al., 2000), and in many instances, requires stimulation with cytokines such as IFNγ before expression can be detected (Banovac et al., 1989). In addition, the cell surface phenotype of human mast cells often varies with the source of hematopoietic stem cells; human cord blood derived mast cells were found to express CD44 and Kit, but only low levels of FcεRI (Kinoshita et al., 1999), while human BMCMC express high levels of FcεRI (Shimizu et al., 2002). The canine BMCMCs we generated did not express CD34, which is consistent with both canine and human mast cells found *in vivo*, but differs from mice in which CD34 can be found on mature cells (Drew et

al., 2005). Lastly, it is interesting to note that while murine BMCMCs can be differentiated from whole marrow using only rmIL-3, the absence of SCF or kit signaling *in vivo* leads to a near complete lack of mast cells (Grimbaldeston et al., 2005). Like human BMCMCs, the canine BMCMCs did not require IL-3 for *in vitro* differentiation. Therefore, based on differentiation requirements and cell phenotype, canine BMCMCs appear to be more similar to human than mouse BMCMCs.

Our data demonstrate that canine BMCMCs are dependent on rcSCF for survival and proliferation (Figs. 4 and 5). This result is in direct contrast to murine BMCMCs that do not require SCF stimulation *in vitro*, but consistent with the biology of human BMCMCs or cord blood derived mast cells (Kinoshita et al., 1999; Mekori et al., 2001). Interestingly, the canine BMCMCs did not survive well beyond 8 weeks of culture (data not shown), and similar results have been obtained with human BMCMCs (Shimizu et al., 2002). However, human mast cells derived from cord blood were found to survive for at least 50 weeks *in vitro* (Kinoshita et al., 1999) suggesting that the hematopoietic stem cells derived from cord blood have a greater proliferative capacity or more immature phenotype than those obtained from adult marrow. Furthermore, we found that low concentrations (5–20 ng/ml) of rcSCF induced migration through a transwell, while high concentrations (50 ng/ml) were inhibitory (Fig. 5). This result was unexpected as canine mast cells derived from skin exhibited their greatest migration at 50 ng/ml SCF. It is possible that this effect is the result of fundamental differences between the BMCMCs and mast cells generated *in vivo*, or may be secondary to the fact that at high concentrations, rcSCF readily diffuses to the well above, thereby eliminating the gradient effect. Furthermore, studies with human mast cells suggested that high concentrations of SCF may inhibit mast cell migration that is induced by antigen-specific IgE cross-linking (Sawada et al., 2005).

The hallmark of mast cells is their ability to release granule contents, particularly histamine, and produce cytokines and lipid mediators upon stimulation (Marshall and Jawdat, 2004). We first measured histamine in the BMCMCs and found that the average histamine content in our canine BMCMCs was lower than that of canine mast cells found *in vivo* (1 pg/cell versus 2.5 pg/cell) (Soll et al., 1979), but was substantially higher than that found in several canine mast cell lines (0.04 pg/cell) (Garcia et al., 1998).

The lower quantity of histamine likely reflects both the level of BMCMC maturity as well as unique culture conditions that do not completely replicate *in vivo* conditions. In support of this notion, human BMCMCs often have a lower concentration of histamine than those found *in vivo*, but this level can be up-regulated with the addition of serum to the tissue culture medium (Dahl et al., 2002).

Classic mast cell activation occurs following antigen-induced IgE cross-linking, but can also be stimulated through a variety of chemical mediators. As antigen-specific canine IgE was not available, it was necessary to load the canine BMCMCs with pooled canine IgE and cross-link this using a tertiary system including a goat anti-canine IgE followed by anti-goat IgG linked to agarose beads. We observed a dose-dependent release of both histamine and β-hexosaminidase that was induced by cross-linking of canine IgE and was inhibited at high doses of secondary anti-IgE (Fig. 6). The high dose inhibition observed is likely due to the fact that excess secondary antibodies do not bind more than one site on IgE, and thus fail to induce cross-linking. The canine BMCMCs also produced TNFα following cross-linking of IgE (Fig. 7). Therefore, the cells exhibited functional responses that matched those of mast cells found *in vivo*.

In addition to classical activation through IgE cross-linking, mast cells derived from several species can be induced to degranulate following stimulation with a variety of mediators. Mast cells purified from canine skin release histamine following stimulation with compound 48/80, substance P, calcium ionophore and ConA (Garcia et al., 1998). The BMCMCs we generated *in vitro* responded vigorously to both ConA and compound 48/80, releasing histamine and β-hexosaminidase in a dose-dependent fashion (Fig. 6). In contrast, only a weak response to calcium ionophore could be elicited and no histamine release was noted following exposure to substance P. The basis for this difference between canine BMCMCs and mast cells isolated from canine skin is not known, but may reflect up-regulation of different sets of particular receptors under the influence of factors found in the skin microenvironment that are not replicated *in vitro*.

Moreover, murine BMCMCs are known to exhibit significant strain-related variations in histamine release secondary to IgE cross-linking or stimulation with mediators (Noguchi et al., 2005), suggesting that BMCMCs generated from different breeds of dog may display differences in functional responses.

In summary, we have developed a reliable protocol for the *in vitro* generation of large numbers of mast cells from purified canine $CD34^+$ cells. These BMCMCs exhibit phenotypic and functional characteristics consistent with mast cells found *in vivo* and are comparable to BMCMCs generated from both human and mouse hematopoietic precursors. Canine BMCMCs are a novel resource that will be a useful tool to investigate basic canine mast cell biology, breed-specific difference in mast cell function, as well as factors that lead to malignant transformation of mast cells.

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