Veterinary Immunology and Immunopathology 135 (2010) 118–127

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Characterization and modulation of canine mast cell derived eicosanoids

Tzu-Yin Lin, Cheryl A. London *

Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 454 VMAB, 1925 Coffey Road, Columbus, OH 43210, USA

ARTICLE INFO

Article history:
Received 9 September 2009
Received in revised form 23 October 2009
Accepted 16 November 2009

Keywords:
Mast cell
Eicosanoid
Leukotrienes
Prostaglandins
NSAID

ABSTRACT

Mast cells play an important role in both innate and acquired immunity as well as several pathological conditions including allergy, arthritis and neoplasia. They influence these processes by producing a variety of mediators including cytokines, chemokines and eicosanoids. Very little is currently known about the spectrum of inflammatory mediators, particularly eicosanoids (prostaglandins and leukotrienes), produced by canine mast cells. This is important since modulating mast cell derived eicosanoids may help in the treatment of autoimmune and inflammatory disorders. The purpose of this study was to investigate the spectrum of eicosanoids produced by normal canine mast cells and to evaluate the effects of cytokines and non-steroidal anti-inflammatory mediators (NSAIDS) on eicosanoid production and release. Canine bone marrow derived cultured mast cells (cBMCMCs) expressed COX-1, COX-2, and 5-LOX and synthesized and released PGD2, PGE2, LTB4, and LTC4 following activation by a variety of stimuli. The selective COX-2 NSAIDs carprofen (Rimadyl®) and deracoxib (Deramaxx®) inhibited PGD2 and PGE2 production but only slightly inhibited LTB4 and LTC4. The mixed COX-1/COX-2 inhibitor piroxicam blocked PGD2 and PGE2 production, but upregulated LTC4 following treatment while tepoxilan (Zubrin®), a pan COX/LOX inhibitor, markedly reduced the production of all eicosanoids. The LOX inhibitor nordihydroguaiaretic acid (NDGA) prevented LTB4/LTC4 release and BMBMC degranulation. Pre-incubation of cBMCMCs with IL-4 and SCF sensitized these cells to degranulation in response to substance P. In conclusion, canine BMCMCs produce an array of eicosanoids similar to those produced by mast cells from other species. Tepoxilan appeared to be the most effective NSAID for blocking eicosanoid production and thus may be useful for modulating mast cell mediated responses in dogs.

1. Introduction

Mast cells actively participate in the induction of innate immune responses and recent evidence suggests they can modulate adaptive immune responses (Kalesnikoff and Galli, 2008; Marshall and Jawdat, 2004). They are also critical players in several pathologic processes including allergy, arthritis, autoimmunity and neoplasia. Mast cells influence both health and disease by producing and releasing a vast array of mediators that help to shape their local microenvironment, promoting tissue remodeling, leukocyte activation and migration, and angiogenesis (Puxeddu et al., 2003). These mediators include several chemokines, cytokines, proteases, histamine, heparin, and lipid based substances termed eicosanoids (Kalesnikoff and Galli, 2008). The eicosanoids, consisting primarily of leukotrienes (LTs) and prostaglandins (PGs), are synthesized from arachidonic acid and collectively modulate effector cell trafficking, antigen presentation, immune cell activation, matrix deposition, fibrosis, and smooth muscle constriction (Boyce, 2005, 2007). Given their wide-ranging effects on numerous biological process, eicosanoids represent a potentially important target for therapeutic intervention in the setting of pathologic processes involving mast cells (Boyce, 2005).

Eicosanoid production is driven by two major pathways that break down arachidonic acid; those involving

* Corresponding author. Tel.: +1 614 292 9554; fax: +1 614 292 7599.
E-mail address: london.20@osu.edu (C.A. London).

0165-2427/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2009.11.010

cyclooxygenase (COX-1 and COX-2) and those involving lipooxygenase (5-LOX) (Boyce, 2005; Diaz et al., 2002). These lead to the generation of prostaglandins (PGD2, PGE2 and others) and leukotrienes (LTB4, LTC4 and others), respectively. COX-1 is constitutively expressed in normal cells, while COX-2 is induced upon stimulation. Mouse and human mast cells release large quantities of PGD2 during allergic and asthmatic anaphylaxis, and this acts as chemoattractant for eosinophils, basophils, and helper T cells expressing a TH2 phenotype (Boyce, 2005; Claria and Romano, 2005; de Mora et al., 2006). PGE2 plays a key role in the development of swelling, pain, and fever associated with inflammation (Claria and Romano, 2005).

In contrast, 5-LOX converts arachidonic acid into LTA4, which is then either converted to LTB4 (the primary event in neutrophils) or to LTC4 (a cysteinyl leukotriene). LTB4 activates neutrophils and promotes neutrophil and T cell recruitment, as well as upregulation of integrin expression on vascular endothelium (Charbeneau and Peters-Golden, 2005; Claria and Romano, 2005). The cysteinyl leukotrienes exhibit powerful pro-inflammatory activities by promoting the production of cytokines, chemokines and other growth factors and inducing vascular leak and subsequent leukocyte migration. These leukotrienes are potent bronchoconstrictors and contribute to airway edema and excessive mucus secretion in asthmatics.

Mast cell eicosanoid production and release has been studied extensively in mouse models and is influenced by a variety of environmental factors (Boyce, 2007). For example, it is known that the neuropeptide substance P can promote release of lipid mediators, primarily leukotrienes from mast cells and thereby contribute to inflammation associated with processes such as atopic dermatitis and osteoarthritis, providing a potential mechanism for neuro-inflammation (Karimi et al., 2000). Interestingly, only connective tissue-type MC (CTMC), but not mucosal MC can be activated by substance P in mice. Additionally, IL-4 has been shown to modulate the response of mast cells to substance P, promoting enhanced release of a variety of mediators in vitro (Karimi et al., 2000).

In previous studies, canine mastocytoma cell lines and purified canine tissue mast cells were shown to release PGD2, PGE2, LTB4 and LTC4 upon antigen and chemical challenges (DeVinney and Gold, 1990; Phillips et al., 1983; Yakabi et al., 2002). However, little is known regarding factors that may influence the production and release of eicosanoids from normal canine mast cells. Furthermore, the potential effects of routinely used non-steroidal anti-inflammatory drugs such as carprofen (Rimadyl®) on canine mast cell derived eicosanoids have not been evaluated. Our laboratory has developed a protocol to generate large numbers of normal canine mast cells from hematopoietic precursors and has demonstrated that these cells exhibit features of mast cells found in vivo including degranulation, production and release of a variety of cytokines, chemokines, and proteases following stimulation, and response to IgE loading and cross-linking (Lin and London, 2006; Lin et al., 2006). The purpose of this study was to use these bone marrow derived cultured mast cells (BMCMCs) to investigate the production and release of eicosanoids from canine mast cells and to determine how this may be altered in the presence of NSAIDs or other mediators such as interleukin-4 and substance P.

2. Materials and methods

2.1. Reagents

Recombinant canine stem cell factor (rcSCF) and IL-4 (rcIL-4) were purchased from R&D Systems (Minneapolis, MN). The following reagents and chemicals were obtained from Sigma (St. Louis, MO): calcium ionophore A23187, concanavalin A (ConA), substance P and Tyrode's basal salt solution, Stemline® II and 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dihydrate, piroxicam, nordihydroguaiaretic acid (NGDA), dexomethasone (DEX), carprofen (Rimadyl®) and Stemline®. Tepoxalin (Zubrin®) and deracoxib (Deramaxx®) tablets were obtained from Schering-Plough Animal Health (Boxmeer, Netherlands) and Novartis (Cambridge, MA), respectively. Tablets were dissolved in water and DMSO, respectively to the stock concentration of 10 mM. The drug solids were mixed well before dilution. STA-9090 (HSP90 inhibitor) was kindly provided by Synta Pharmaceuticals Inc. (Lexington, MA).

2.2. Canine bone marrow derived cultured mast cells (BMCMCs) and mastocytoma cell line, C2

The protocol to generate canine BMCMCs has been previously described and used in our laboratory for the past 4 years (Lin et al., 2006). Briefly, 20 ml of fresh bone marrow was collected in fresh 3.8% sodium citrate solution/PBS from healthy dogs that came to the Veterinary Teaching Hospital (VTH) of the Ohio State University for their ovariohysterectomy or castration. The bone marrow collection was approved by the OSU Institutional Care and Use Committee (2006A0222). CD34+ bone marrow cells were purified via MACS® magnetic sorting system (Miltenyi Biotec, Germany) as previously described and cultured in serum-free medium, Stemline® with 100 ng/ml recombinant canine stem cell factor (rcSCF) for 4–8 weeks. The purity of mast cells was evaluated following differentiation and all cultures consisted of greater than 90% mast cells. All the data generated in this study were from 5 different dogs. BMCMCs generated from each animal were never pooled and each experiment was performed with 2–3 sets of BMCMCs; all experiments were performed in triplicate. For the canine C2 mastocytoma cell line, cells were cultured in 10% FBS in RPMI1640 with antibiotics and glutamax® (Invitrogen).

2.3. RT-PCR

To evaluate the expression of COX-1, COX-2 and 5-LOX in canine mast cells, total RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA) from canine BMCMCs cultured in rcSCF and the C2 mast cell line. After quantification, cDNA was generated from 1 μg total RNA using the Superscript III® reverse transcription Kit (Invitrogen) as previously described. PCR amplification was performed with1 μl of the RT product amplification using the primer sets listed in

Table 1
Primer sets used for RT-PCR.

Gene	Primers	Sequence	Genbank
COX-1	COX I-1186F	5′-GCTACTTCTTGCAGCTGAAGTTCG-3′	NM_001003023
	COX I-1464R	5′-GTGCAGGACATGGTGGTCTATG-3′	
COX-2	COX II-1211F	5′-GCCTTACCCAGTTTGTGGAA-3′	NM_001003354
	COX II-1373R	5′-GCCTAAAGCGTTTGCGATA-3′	
5-LOX	5-LOX-873F	5′-AGAGGTCGAGCAAGGAAACA-3′	XM_534950
	5-LOX-1054R	5′-TTTCATCTCCTGGGACTTGG-3′	
GAPDH	GAPDH 55F	5′ACCACAGTTCCATGCCATCAC-3′	NM_001003142
	GAPDH 162R	5′-TCCACCACCCTGTTGCTGTA-3′	

Table 1. All primers were designed to either cross exon–intron boundaries, or to span a large intron to exclude the potential genomic DNA contamination. The PCR cycle was as follows: 95 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min for 35 cycles. The negative control for each PCR reaction consisted of water instead of cDNA.

2.4. Mast cell degranulation assay

Canine BMCMCs were cultured in serum-free medium with 100 ng/ml rcSCF. In some experiments 25 ng/ml recombinant canine IL4 (rcSCF) was added for at least 7 days prior to collection. Cells were then washed and re-suspended in Tyrode's salt solution at a concentration of 1×10^4/ml. Substance P was added to each well at indicated concentrations for 30 min prior to analysis. The substance P concentrations used here were based on previous studies using BR cells and canine skin purified mast cells, and are considered biologically relevant concentrations (Garcia et al., 1998). To assess the effects of NSAIDs and dexamethasone (DEX) on BMCMCs, cells were treated with NSAIDs or DEX at the concentrations listed in Table 2 for 1 h followed by chemical activation (1 μg/ml ConA or 1 μM A23187) for another 30 min. Both supernatants and pellet lysates were collected from treated BMCMCs. The degree of mast cell degranulation was evaluated by the percentage of β-hexosaminidase released following stimulation as previously described (Lin et al., 2006). Briefly, 25 μl of supernatant from the treated BMCMCs was incubated with 100 μl of 1.2 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide in 0.05 M sodium acetate buffer (pH 4.4). Pre-cooled glycine carbonate buffer (175 μl, pH 10.0) was then added to quench the reaction. The fluorescence was measured using an ELISA plate reader (Molecular Devices). The formula was used to calculate the percentage of β-hexosaminidase released: (fluorescence of supernatant − blank)/[(fluorescence of supernatant − blank) + (fluorescence of pellet − blank)] × 100. All experiments were performed in triplicate.

Table 2
Concentration of drugs used in this study.

Selectivity	Drugs	Concentration (μM)	Reference
COX-2	Carprofen	10	McCann et al. (2004)
COX-2	Deracoxib	1	McCann et al. (2004)
COX-1/-2	Piroxicam	10	Wilson et al. (2004)
5-LOX	NDGA	3	Glitsch et al. (2002)
COX/LOX	Tepoxilan	10	Macrory et al. (2009)
COX/LOX	Dexamethasone	1	Robin et al. (1985)

2.5. Mast cell activation with and without NSAID and DEX pre-treatments

Mature canine BMCMCs were washed and re-suspended in Tyrode's salt solution at a concentration of 1×10^6/ml. Cells were treated with increasing concentrations of ConA, calcium ionophore A23187, or substance P (using cells culturing with IL-4 for at least 1 week prior to analysis) for 30 min. This time point has been previously established to be relevant for assessing degranulation of canine BMCMCs (Lin and London, 2006). The cell-free supernatant was collected and stored for analysis. To evaluate the effects of NSAIDs and DEX on canine BMCMCs in response to chemical challenge, BMCMCs were treated with various NSAIDs or DEX in the Tyrode's salt solution at the concentrations indicated in Table 2 for 1 h. The concentrations of carprofen, deracoxib and piroxicam were chosen based on previous canine whole blood assays of these NSAIDs (McCann et al., 2004; Wilson et al., 2004). NGDA at 2 μM has previously been used to inhibit LOX activity in RBL cells (Glitsch et al., 2002). Lastly, 10 μM of tepoxalin was previously shown representing a therapeutic level of drug in dogs (Macrory et al., 2009). Thus, the concentration of NSAIDs used in this study were considered to be biologically relevant based on previous work, most of which was performed in dogs. Following NSAID exposure, cells were washed and treated with chemicals (1 μg/ml ConA, 1 μM A23187 or 100 μM substance P) for 30 min as previously described. The cell-free supernatant was collected and stored for analysis. All drugs were diluted in 0.1% DMSO for use in vitro, and the negative control wells consisted of cells treated with 0.1% DMSO alone. As previously discussed, BMCMCs stimulated with substance P were pre-treated with IL-4 for 7 days prior to use.

2.6. Measurement of PGD2, PGE2, LTB4, and LTC4

To measure the eicosanoids, commercial competitive ELISA kits were used: LTB4 (R&D Systems, Minneapolis, MN), PGD2, PGE2 (Cayman Chemicals, Ann Arbor, MI) and LTC4 (Neogen, Lexington, KY). Briefly, supernatants were either analyzed neat or serially diluted prior to assessment. Approximately 50–100 μl of samples or PGD2/PGE2/LTB4/

LTC4 standards (diluted in Tyrode's salt solution), and 50 μl of primary antibody were incubated in the pre-coated detection plates with gentle shaking. Controls consisted of wells with Tyrode's salt solution alone and wells containing 50 μl of primary antibody (except for LTC4) as the maximum binding control. Following 1–2 h of incubation, 50 μl of the PGD2/PGE2 tracer or LTB4/LTC4 conjugate solution was added for another 1 h with shaking. Plates were washed 4 times and the color was developed by adding 100 μl of developing buffer for 30 min at room temperature. The reaction was stopped by adding 50 μl of acid solution and the plates were read at wavelengths of 450 and 570 nm using an ELISA plate reader (Molecular Devices). The standard curve was generated using the standards on each plate and the PGD2/PGE2/LTB4/LTC4 concentrations in each sample were then calculated. Each experiment was repeated for 3 times and all samples were analyzed in triplicate.

2.7. Casepase3/7 activity assay

BMCMCs (4×10^4) were treated with NSAIDs or DEX as indicated in Table 2 for 24 h in 96-well plates (Costar, Cambridge, MA). The caspase 3/7 activity was evaluated using the SensoLyte™ Homogeneous AMC Caspase-3/7 assay kit (AnaSpec, San Jose, CA). Working substrate (Ac-Asp-Glu-Val-Asp-AMC, 50 μl) was added to each well. After 1.5 h of incubation, the fluorescence was measured using an ELISA plate reader (Molecular Devices). Wells containing medium and substrate alone were used as controls; the fluorescence from these wells was measured as background and subtracted from the values obtained from mast cell supernatants. Cells treated with 100 nM STA-9090 (HSP90 inhibitor, Lin et al., 2008) served as positive control for caspase3/7 activation.

2.8. Statistics

For analysis of prostaglandin and leukotriene release, all experiments were repeated using different sets of canine BMCMCs and data were presented as mean values ± S.D. Significance was calculated using the Student's *t*-test. Values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Generation and morphological analysis of canine BMCMCs

We previously developed a technique to consistently generate canine BMCMCs that exhibit normal mast cell morphology and functional properties (Lin and London, 2006; Lin et al., 2006). Following 4 weeks of culture of CD34+ canine bone marrow cells in the presence of rcSCF and Stemline®, over 90% of the differentiated cells used for the following experiments were mast cells, as evidenced by the characteristic appearance of large round cells with centrally placed round nuclei and many purple small cytoplasmic granules (Fig. 1a). Upon stimulation with the calcium ionophore A23187, the BMCMCs rapidly lost their cytoplasmic granules, demonstrating morphological evidence of normal degranulation (Fig. 1b).

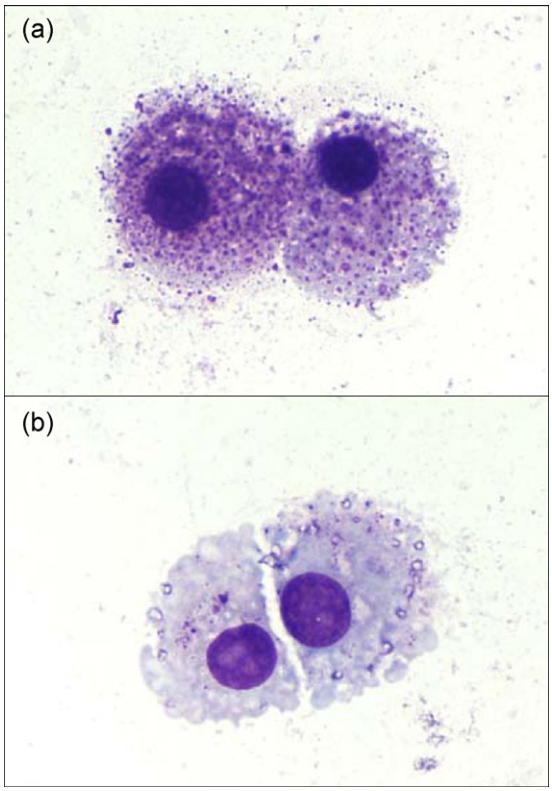

Fig. 1. Morphology and electronic microscopic image of canine BMCMCs. Mature canine BMCMCs contain multiple small purple granules in the cytoplasm (a), and lose these granules 30 min following activation with A23187 (b). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3.2. IL-4 sensitizes canine BMCMCs to stimulation with substance P

The cytokine IL-4 has multiple effects on normal canine mast cells including alteration of their proliferative capacity, Kit expression, histamine content and sensitivity to degranulation (Lin and London, 2006). After incubation of human or mouse mast cells in IL-4 for 3–7 days in combination with standard culture conditions (SCF or IL-3, respectively), an increased sensitivity of these cells to stimulation with substance P was found, suggesting that IL-4 may sensitize mast cells to this compound (Karimi et al., 2000). In our previous work, canine BMCMCs failed to degranulate after stimulation with substance P (Lin et al., 2006). To determine whether canine BMCMCs were capable of being stimulated by substance P after IL-4 sensitization, cells were cultured in serum-free medium with 100 ng/ml rcSCF in the presence or absence of 25 ng/ml rcIL4 for 7 days. Cells were then stimulated by substance P and degranulation was evaluated by the β-hexosaminidase assay. As shown in Fig. 2, only cells cultured with both rcSCF and rcIL-4 responded to substance P in a dose dependent manner. In summary,

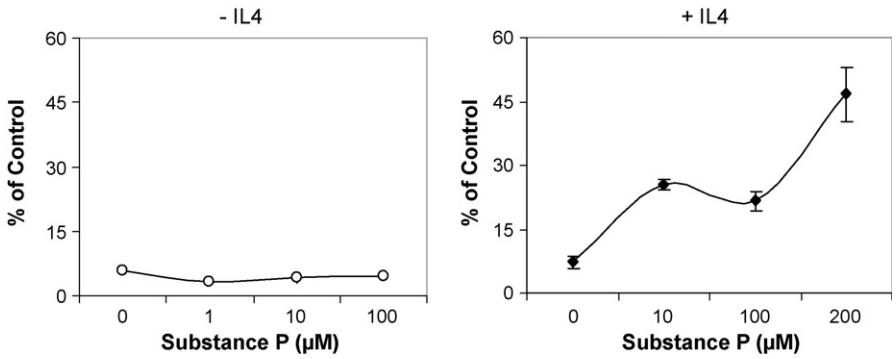

Fig. 2. IL-4 sensitizes canine BMCMCs toward substance P stimulation.
Mature canine BMCMCs were cultured in 100 ng/ml cSCF with (a) or without (b) 25 ng/ml rcIL4 for 1 week. Cells were washed and re-suspended at 1×10^5/ml in the Tyrode's salt solution. Cells were then stimulated with increasing concentrations of substance P for 30 min, the percent degranulation was determined using the β-hexosaminidase assay. This experiment was repeated for 3 times from 2 different dogs. The data is presented as mean ± S.D. Each experiment was performed in triplicate.

like mouse and human mast cells, IL-4 also modulated the releasing ability of canine mast cells. Cells pre-treated with rcSCF and rcIL-4 were used for the following experiments evaluating substance P induced lipid-derived mediator production.

3.3. Canine mast cells express COX-1, COX-2 and 5-LOX mRNA

To evaluate the expression of COX/LOX isomers in normal canine mast cells, canine BMCMCs and the canine mastocytoma cell line C2 cells were collected and expression of COX-1, COX-2 and 5-LOX was assessed by RT-PCR. Fig. 3 shows that both canine BMCMCs and C2 cells express message for COX-1, COX-2 and 5-LOX indicating the ability of these cells to generate a variety of prostaglandins and leukotrienes. Negative controls were also performed.

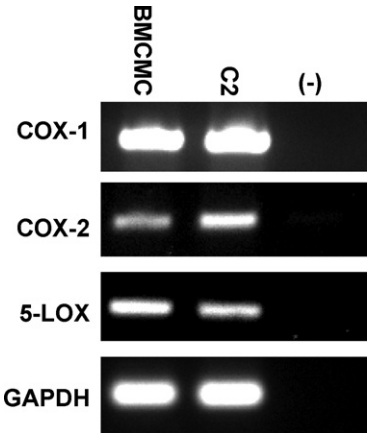

Fig. 3. Canine BMCMCs express COX-1, COX-2 and 5-LOX mRNA.
To detect the expression of COX-1, COX-2 and 5-LOX, canine BMCMCs (in rcSCF) and the canine mastocytoma cell line C2 (control) were harvested, RNA was generated using TRIzol, and RT-PCR was performed. RT-PCR for GAPDH was used as the positive control, and negative control was also included. This experiment was repeated in 3 different sets of BMCMCs. Primer pairs for these reactions are listed in Table 1.

3.4. Canine mast cells release PGD2, PGE2, LTB4, and LTC4 upon activation

Mast cells are a major source for lipid-derived mediators that act to modulate subsequent immune responses (Boyce, 2007). Canine mastocytoma cell lines have previously been found to release several lipid-derived mediators following chemical stimulation and antigen-specific activation (Goetzl et al., 1983; Olivry et al., 2001; Phillips et al., 1983). However, mastocytoma cell lines often exhibit dysregulated functions including loss of FceR expression/response to IgE cross-linking and low histamine levels (Brazis et al., 2002; Garcia et al., 1998). Little is known regarding the lipid-mediator profile of normal canine mast cells as historically, these have been difficult to obtain in large enough quantities for use in multiple assays. To evaluate the ability of normal canine mast cells to produce and release lipid mediators, canine BMCMCs were stimulated with ConA (mimics IgE cross-linking), A23187 (calcium ionophore) or substance P (following culture of cells with IL-4) and supernatants were collected. The BMCMCs released PGD2 (Fig. 4a), PGE2 (Fig. 4b), LTB4 (Fig. 4c), and LTC4 (Fig. 4d) in response to ConA and A23187. The rcIL-4 treated canine BMCMCs released PGD2, PGE2 and LTC4 in response to substance P, but failed to release detectable levels of LTB4 (Fig. 4c). In summary, similar to mast cells from other species, canine mast cells produce and release an array of lipid mediators in response to a variety of stimuli.

3.5. NSAIDs and DEX modulate canine mast cell derived eicosanoids

NSAIDs inhibit COX and LOX enzymes with varying degrees of selectivity. As mast cells are a major source of eicosanoids during the initiation of an immune response and may also participate in several pathologic conditions, we were interested to determine the effects of currently available NSAIDs on canine mast cells. Canine BMCMCs were pre-treated with various NSAIDs or dexamethasone (DEX) for 1 h at the concentrations indicated in Table 2 and left untreated or activated by 1 μM A23187, 1 μg/ml ConA

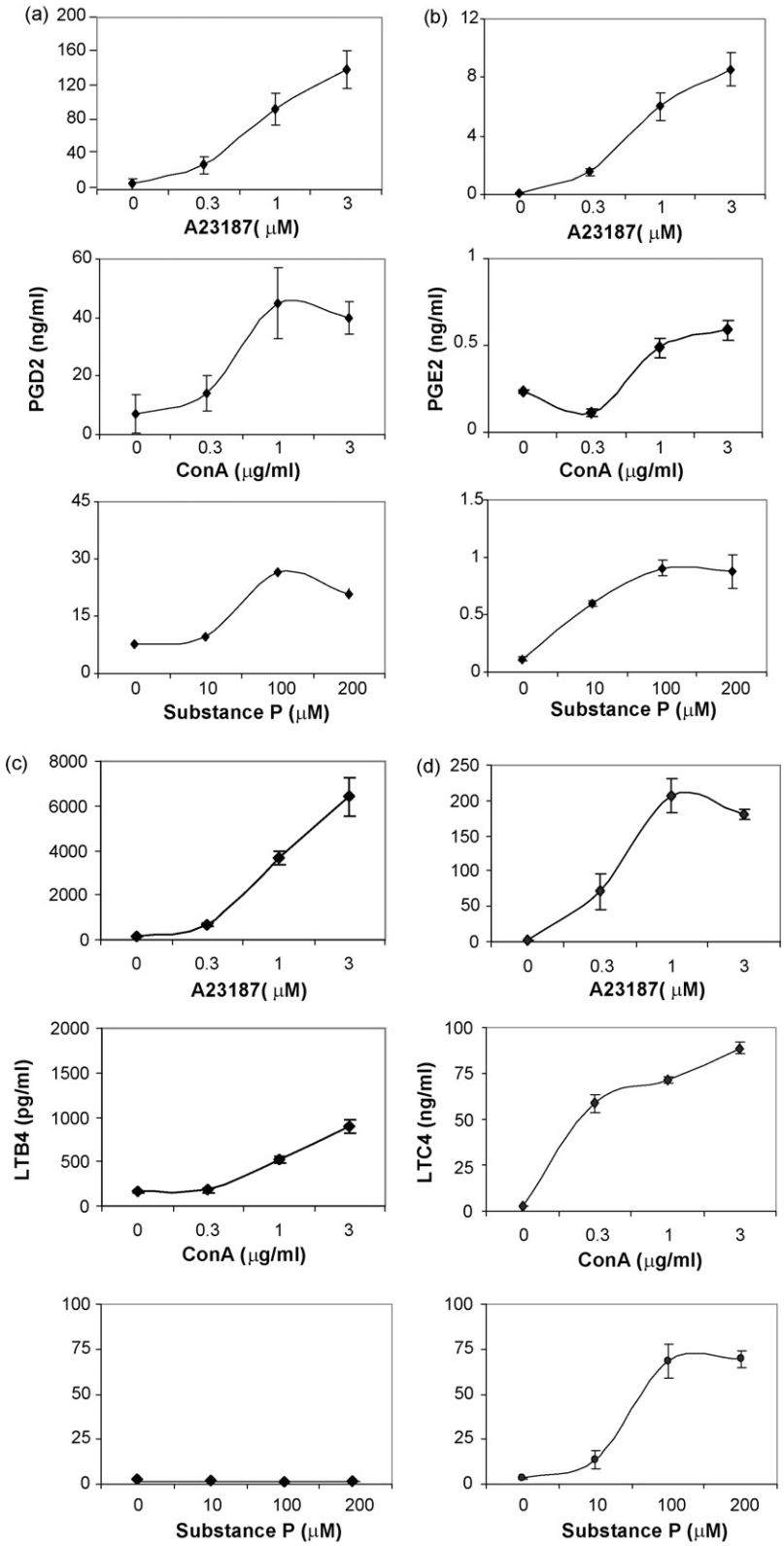

Fig. 4. Canine BMCMCs release PGD2, PGE2, LTB4 and LTC4 upon activation.
Canine BMCMCs were washed and re-suspended at a concentration of 1×10^6/ml in Tyrode's salt solution then treated with increasing concentrations of A23187, ConA, or substance P for 30 min. The cell-free supernatants were then collected and the PGD2 (a), PGE2 (b), LTB4 (c), and LTC4 (d) concentrations in each sample were determined by commercial competitive ELISA kits. Substance P treated BMCMCs were cultured in the presence of IL-4 for 1 week prior to use in this assay. Each experiment was repeated 2–3 times from 4 different dogs. The data is presented as mean ± S.D. Each experiment was performed in triplicate.

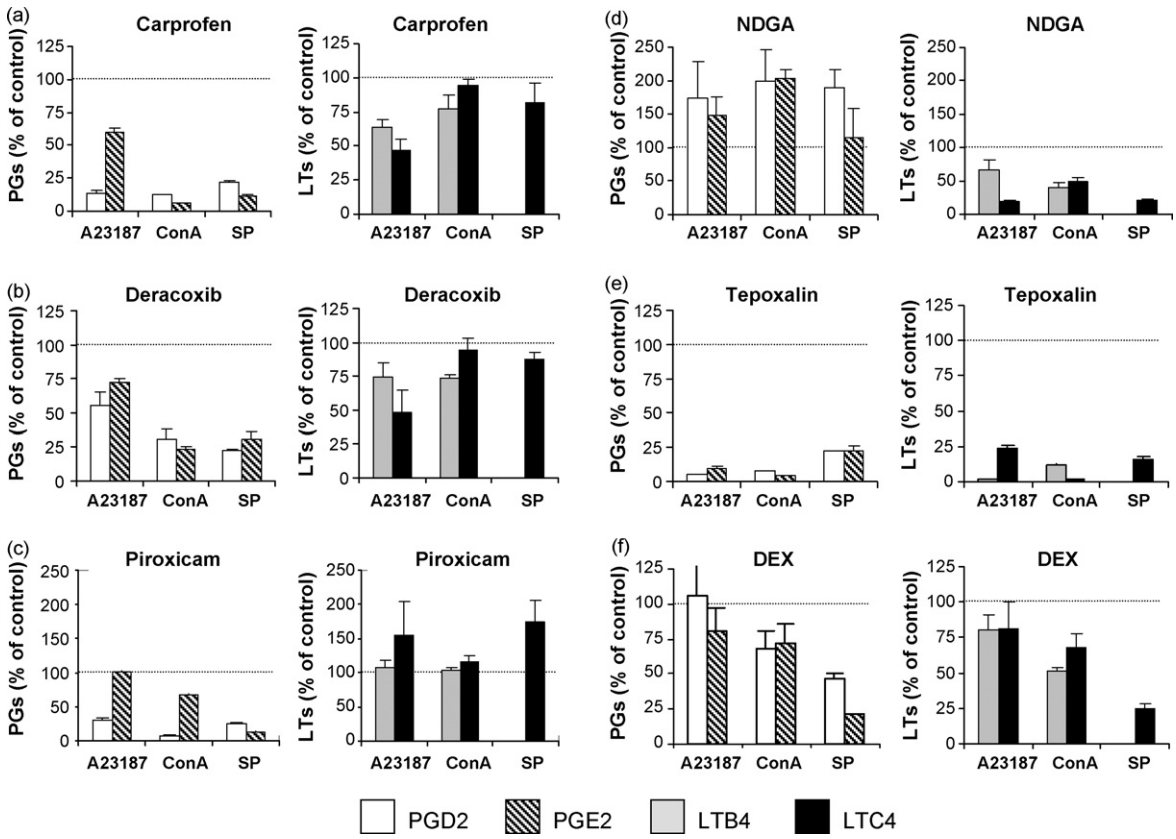

Fig. 5. NSAIDs and dexamethasone modulate the release of prostaglandins and leukotrienes from canine BMCMCs following activation. Canine BMCMCs were washed and re-suspended in Tyrode's salt solution at a concentration of 1×10^6/mL. Cells were left untreated or treated with NSAIDs or DEX at the concentrations listed in Table 2 for 1 h, then activated by 1 μg/ml ConA, 1 μM A23187 or 100 μM substance P for 30 min. Cell-free supernatants were then collected and prostaglandins and leukotrienes were measured using commercial competitive ELISA kits. Substance P treated BMCMCs were cultured in the presence of IL-4 for 1 week prior to use in this assay. This experiment was repeated 3 times from 2 different dogs. The data is presented as mean ± S.D. Each experiment was performed in triplicate.

or 100 μM substance P for 30 min and PGD2, PGE2, LTB4 and LTC4 were measured in the cell-free supernatants. As shown in Fig. 5a and b, the selective COX-2 inhibitors carprofen (Fig. 5a) and deracoxib (Fig. 5b) reduced PGD2 and PGE2 production but only slightly inhibited LTB4 and LTC4. The COX-1/COX-2 inhibitor piroxicam strongly reduced PGD2 production and had variable effects on PGE2 dependent on the method of stimulation (Fig. 5c). Although LTB4 was unaffected, LTC4 production was upregulated after piroxicam treatment (Fig. 5c). The selective 5-LOX inhibitor NGDA substantially reduced LTB4 and LTC4 release but promoted increased release of PGD2 and PGE2 from canine BMCMCs (Fig. 5d). The dual COX/LOX inhibitor tepoxilan strongly blocked PGD2, PGE2, LTB4, and LTC4 release from canine BMCMCs following their activation (Fig. 5e). Lastly, DEX exhibited variable effect on the release on eicosanoids from mast cells depending on the method of stimulation (Fig. 5f). To demonstrate that the inhibitory effects we observed were not secondary to mast cell death, we measured the activation of caspases 3 and 7 in mast cells following incubation with NSAIDs or DEX. As shown in Fig. 6, no caspase3/7 activity was detected after 24 h of incubation of mast cells with the various drugs, while STA-9090 induced significant caspase3/7 activity.

3.6. NGDA inhibits canine mast cell degranulation

In previous studies, NSAIDs were shown to inhibit eicosanoid production through blocking of COX and/or LOX activities. However, the effects of NSAIDs on mast cell degranulation directly or following stimulation have not been well-investigated (Grosman, 2007). To evaluate the effects of NSAIDs and DEX on histamine release from canine mast cells we treated cBMCMCs with drugs for 1 h at the concentrations indicated in Table 2. Both NSAIDs and DEX did not induce spontaneous degranulation compared to the controls (data not shown). After stimulation of mast cells with ConA or A23187 for 30 min, only NGDA significantly inhibited β-hexosaminidase release (Fig. 7). Piroxicam exhibited no effect on the mast cells. The effects of carprofen, deracoxib, tepoxilan and DEX exhibited substantial variability over 5 sets of independent experiments (i.e., in some experiments they had no effect, in others they did induce a slight increase or decrease in β-hexosaminidase release, data not shown).

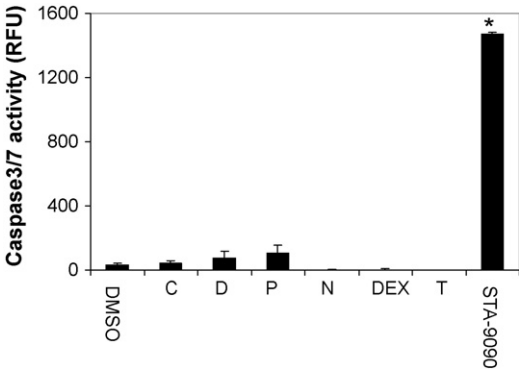

Fig. 6. Caspase3/7 activity is not induced following treatment of BMCMCs with NSAIDs or dexamethasone.
Canine BMCMCs (4×10^4) were treated with carprofen (C), deracoxib (D), piroxicam (P), NGDA (N), tepoxalin (T) or dexamethasone (DEX) at the concentrations listed in Table 2 with 5 ng/ml rcSCF in Stemline, and incubated for 24 h. Cells treated with 100 nM STA-9090 (HSP90 inhibitor) were used as positive control, while cells without drug treatment were used as negative control. The caspase3/7 activity was evaluated using the SensoLyte™ Homogeneous AMC Caspase-3/7 assay kit; 50 μl of substrate (Ac-Asp-Glu-Val-Asp-AMC) was added to each well and plates were incubated for 1.5 h before detecting fluorescence. The experiments were repeated 3 times in triplicate from 2 different dogs. Medium only plus substrate served as the blank control (*$p < 0.05$).

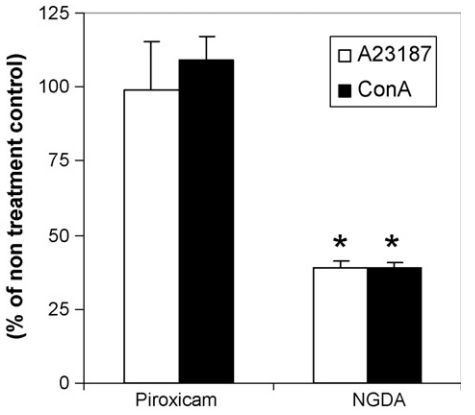

Fig. 7. NDGA, but not piroxicam inhibits degranulation of BMCMCs.
Canine BMCMCs (1×10^4) were left untreated or treated with 0.1% DMSO, piroxicam or NDGA as indicated concentrations in Table 2 for 1 h, then activated with 1 μg/ml ConA or 1 μM A23187 for 30 min. The percent degranulation was determined using the β-hexosaminidase assay, and the cells treated with 0.1% DMSO served as 100% control. This experiment was performed in triplicate 4 times from 2 different dogs (*$p < 0.05$).

4. Discussion

Mast cells play an important role in normal immune responses to bacterial and parasite infections, and likely contribute to the induction of adaptive immunity (Galli et al., 2005; Marshall and Jawdat, 2004). They also participate in a variety of pathological processes such as asthma and allergy (Beaven, 2009; Edwards, 2008). Given that mast cells have the ability to produce and release several inflammatory products including cytokines, chemokines and eicosanoids, mast cells and their derived mediators have been recognized as a potential target for therapeutic intervention. In particular, eicosanoids, including the prostaglandins and leukotrienes are attractive targets as they are critical players in early and late inflammation. While much is known regarding the biology of mast cell derived eicosanoids in mice and humans, the biology of eicosanoids has not been investigated in normal canine mast cells. The purpose of this study was to investigate the spectrum of eicosanoids produced by normal canine mast cells and to evaluate the effects of cytokines and non-steroidal anti-inflammatory mediators (NSAIDS) on eicosanoid production and release. Our data demonstrate that similar to mast cells derived from mice and humans, canine bone marrow derived mast cells (cBMCMCs) express COX-1, COX-2, and 5-LOX and synthesize and release PGD2, PGE2, LTB4, and LTC4 following activation by a variety of stimuli (Fig. 4).

Evidence suggests that the neuropeptide substance P is an activator of mast cells inducing granule exocytosis both in vitro and in vivo in vivo (Hua et al., 1996; Okabe et al., 2006). In this study we demonstrated that canine BMCMCs responded to substance P activation only after incubation with IL-4. This is consistent data generated from human, rat and mouse mast cells in which IL-4 promoted degranulation in response to substance P (Karimi et al., 2000; Moon et al., 2003). IL-4 is known to have various effects on mast cell biology including altering their proliferation, granule content and degranulation potential (Banks and Coleman, 1996; Lin and London, 2006). In our previous work, canine IL-4 upregulated Kit expression and promoted canine BMCMC proliferation (Lin and London, 2006). The increased sensitivity of mast cells toward substance P may be partially due to IL-4 upregulated neurokinin 1 receptors on mast cells (Kraneveld et al., 2002). These data indicate that as has been demonstrated in other species, canine mast cells may alter neurologic responses under appropriate conditions of stimulation.

A variety of NSAIDs have been developed to either broadly or selectively target prostaglandin and leukotriene production through inhibition of COX-1, COX-2, and/or LOX. In our study, the selective COX-2 inhibitors carprofen and deracoxib strongly inhibited PGD2 and PGE2 production but only slightly inhibited LTC4 production. In contrast, the nonselective COX-1/COX-2 inhibitor piroxicam decreased prostaglandin production from canine mast cells, but the production of leukotrienes appeared to increase following treatment. This is consistent with previous data suggesting that inhibition of COX pathways may induce an upregulation of 5-LOX based leukotrienes (Julemont et al., 2004; Mao et al., 2004). In contrast, NDGA, a selective 5-LOX inhibitor induced the opposite effect on canine mast cells, decreasing leukotriene production while increasing prostaglandin production. Previous studies evaluating inhibition of 5-LOX by Zileuton® demonstrated enhanced thromboxane A2 production and increased platelet aggregation (Wu et al., 2003), implying a similar "shunting" of lipid mediators to the COX pathway after 5-LOX inhibition. Given the potential paradoxical effects of COX-only or LOX-only inhibition, dual inhibitors of COX and LOX have been suggested to be potentially more useful for the clinical management of inflammatory conditions without gastrointestinal toxicity (Julemont et al., 2004).

Our data demonstrate that NSAIDs have the potential to modulate canine mast cell derived eicosanoid production and release, suggesting that dogs with mast cell mediated inflammatory disorders and malignancies may benefit from NSAID treatment. In support of this, human patients with systemic mastocytosis or massive mast cell activation were found to have high levels of PGD2 and its metabolites in both blood and urine (Bochenek et al., 2004) and the administration of aspirin normalized PGD2 levels, helping to resolve clinical symptoms (Butterfield and Weiler, 2008). Furthermore, our results show that tepoxilan (Zubrin®), a dual COX/LOX inhibitor, strongly inhibits the production of both leukotrienes and prostaglandins from canine BMCMCs indicating that this compound may be more likely to prevent the previously noted increase in production of prostaglandins or leukotrienes noted with more selective inhibitors.

Glucocorticoids are known to alter the biology of mast cells including their activation and mediator profile (Nakajima et al., 2002; Sewell et al., 1998; Zhou et al., 2008). We found that dexamethasone inhibited the production of both prostaglandins and leukotrienes by canine BMCMCs following activation with either ConA or substance P, but had less of an effect after A23187 challenge. Prior studies have generated conflicting data regarding the effects of glucocorticoids on mast cell derived eicosanoids. Dexamethasone was found to inhibit the release of LTB4 and LTC4 from mouse BMCMCs but promote PGD2 production (Robin et al., 1985). Interestingly, COX-2 expression was inhibited in RBL-2H3 mast cells upon dexamethasone treatment (Hundley et al., 2001). In contrast, the expression of 5-LOX was substantially upregulated in the malignant human mast cell line (HMC-1) after incubation with dexamethasone for 24 h (Colamorea et al., 1999), but the release of PDG2 and leukotrienes from purified human mast cells that was not affected by incubation with dexamethasone for 24 h (Schleimer et al., 1983). It is therefore evident that treatment with dexamethasone induces varying effects on mast cells under different experimental conditions. It is possible that at least some of these differences may be secondary to species-specific effects, alterations in culture conditions, and/or variation in duration of exposure.

Consistent with previous work (Kuno et al., 1993), NDGA blocked mast cell degranulation. Other studies have shown that piroxicam, meloxicam, NS-398 (COX-2 inhibitor) and acetylsalicylic acid (aspirin, a COX-1/COX-2 inhibitor) treatment can prevent rat mast cells from releasing histamine following antigen-dependent (IgE) mediated stimulation (Grosman, 2007). We did not observe an effect of piroxicam on the ability of canine BMCMCs to degranulate while other NSAIDs (carprofin, deracoxib, and tepoxalin) and dexamethasone, had variable and inconsistent effects on mast cell degranulation. This difference may be due to the fact that the drug concentrations used in our study are considered biologically relevant while concentrations used in the Grossman study were much higher (1–10 μM vs. 100–1000 μM).

In summary, canine mast cells are capable of producing large quantities of both prostaglandins and leukotrienes upon activation with a variety of stimuli. While several currently available NSAIDs were found to modulate mast cell eicosanoid production, only tepoxilan (Zubrin®) appeared to block the production of both prostaglandins and leukotrienes. These data suggest that mast cell derived eicosanoids may be a relevant target for therapeutic intervention in the treatment of inflammatory disorders.

Acknowledgement

This work was supported by a grant from the AKC Canine Health Foundation.

References

Banks, E.M., Coleman, J.W., 1996. A comparative study of peritoneal mast cells from mutant IL-4 deficient and normal mice: evidence that IL-4 is not essential for mast cell development but enhances secretion via control of IgE binding and passive sensitization. Cytokine 8, 190–196.

Beaven, M.A., 2009. Our perception of the mast cell from Paul Ehrlich to now. Eur. J. Immunol. 39, 11–25.

Bochenek, G., Nizankowska, E., Gielicz, A., Swierczynska, M., Szczeklik, A., 2004. Plasma 9alpha, 11beta-PGF2, a PGD2 metabolite, as a sensitive marker of mast cell activation by allergen in bronchial asthma. Thorax 59, 459–464.

Boyce, J.A., 2005. Eicosanoid mediators of mast cells: receptors, regulation of synthesis, and pathobiologic implications. Chem. Immunol. Allergy 87, 59–79.

Boyce, J.A., 2007. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. Immunol. Rev. 217, 168–185.

Brazis, P., Torres, R., Queralt, M., de Mora, F., Ferrer, L., Puigdemont, A., 2002. Evaluation of cell-surface IgE receptors on the canine mastocytoma cell line C2 maintained in continuous culture. Am. J. Vet. Res. 63, 763–766.

Butterfield, J.H., Weiler, C.R., 2008. Prevention of mast cell activation disorder-associated clinical sequelae of excessive prostaglandin D(2) production. Int. Arch. Allergy Immunol. 147, 338–343.

Charbeneau, R.P., Peters-Golden, M., 2005. Eicosanoids: mediators and therapeutic targets in fibrotic lung disease. Clin. Sci. (Lond.) 108, 479–491.

Claria, J., Romano, M., 2005. Pharmacological intervention of cyclooxygenase-2 and 5-lipoxygenase pathways. Impact on inflammation and cancer. Curr. Pharm. Des. 11, 3431–3447.

Colamorea, T., Di Paola, R., Macchia, F., Guerrese, M.C., Tursi, A., Butterfield, J.H., Caiaffa, M.F., Haeggstrom, J.Z., Macchia, L., 1999. 5-Lipoxygenase upregulation by dexamethasone in human mast cells. Biochem. Biophys. Res. Commun. 265, 617–624.

de Mora, F., Puigdemont, A., Torres, R., 2006. The role of mast cells in atopy: what can we learn from canine models? A thorough review of the biology of mast cells in canine and human systems. Br. J. Dermatol. 155, 1109–1123.

DeVinney, R., Gold, W.M., 1990. Establishment of two dog mastocytoma cell lines in continuous culture. Am. J. Respir. Cell Mol. Biol. 3, 413–420.

Diaz, B.L., Fujishima, H., Kanaoka, Y., Urade, Y., Arm, J.P., 2002. Regulation of prostaglandin endoperoxide synthase-2 and IL-6 expression in mouse bone marrow derived mast cells by exogenous but not endogenous prostanoids. J. Immunol. 168, 1397–1404.

Edwards, A., 2008. The mast cell and allergic diseases: role in pathogenesis and implications for therapy. Clin. Exp. Allergy 38, 1063–1064 (author reply 1064–1065).

Galli, S.J., Nakae, S., Tsai, M., 2005. Mast cells in the development of adaptive immune responses. Nat. Immunol. 6, 135–142.

Garcia, G., Brazis, P., Majo, N., Ferrer, L., de Mora, F., Puigdemont, A., 1998. Comparative morphofunctional study of dispersed mature canine cutaneous mast cells and BR cells, a poorly differentiated mast cell line from a dog subcutaneous mastocytoma. Vet. Immunol. Immunopathol. 62, 323–337.

Glitsch, M.D., Bakowski, D., Parekh, A.B., 2002. Effects of inhibitors of the lipo-oxygenase family of enzymes on the store-operated calcium current I(CRAC) in rat basophilic leukaemia cells. J. Physiol. 539, 93–106.

Goetzl, E.J., Phillips, M.J., Gold, W.M., 1983. Stimulus specificity of the generation of leukotrienes by dog mastocytoma cells. J. Exp. Med. 158, 731–737.

Grosman, N., 2007. Comparison of the influence of NSAIDs with different COX-selectivity on histamine release from mast cells isolated from naive and sensitized rats. Int. Immunopharmacol. 7, 532–540.
Hua, X.Y., Back, S.M., Tam, E.K., 1996. Substance P enhances electrical field stimulation-induced mast cell degranulation in rat trachea. Am. J. Physiol. 270, L985–991.
Hundley, T.R., Prasad, A.R., Beaven, M.A., 2001. Elevated levels of cyclooxygenase-2 in antigen-stimulated mast cells is associated with minimal activation of p38 mitogen-activated protein kinase. J. Immunol. 167, 1629–1636.
Julemont, F., Dogne, J.M., Pirotte, B., de Leval, X., 2004. Recent development in the field of dual COX /5-LOX inhibitors. Mini Rev. Med. Chem. 4, 633–638.
Kalesnikoff, J., Galli, S.J., 2008. New developments in mast cell biology. Nat. Immunol. 9, 1215–1223.
Karimi, K., Redegeld, F.A., Blom, R., Nijkamp, F.P., 2000. Stem cell factor and interleukin-4 increase responsiveness of mast cells to substance P. Exp. Hematol. 28, 626–634.
Kraneveld, A.D., van der Kleij, H.P., Kool, M., van Houwelingen, A.H., Weitenberg, A.C., Redegeld, F.A., Nijkamp, F.P., 2002. Key role for mast cells in nonatopic asthma. J. Immunol. 169, 2044–2053.
Kuno, M., Kawawaki, J., Shibata, T., Gotani, H., 1993. Inhibitors of the arachidonic acid cascade dissociate 48/80-induced Ca^{2+} influx and Ca^{2+} release in mast cells. Am. J. Physiol. 264, C912–917.
Lin, T.Y., Bear, M., Du, Z., Foley, K.P., Ying, W., Barsoum, J., London, C., 2008. The novel HSP90 inhibitor STA-9090 exhibits activity against Kit-dependent and -independent malignant mast cell tumors. Exp. Hematol. 36, 1266–1277.
Lin, T.Y., London, C.A., 2006. A functional comparison of canine and murine bone marrow derived cultured mast cells. Vet. Immunol. Immunopathol. 114, 320–334.
Lin, T.Y., Rush, L.J., London, C.A., 2006. Generation and characterization of bone marrow derived cultured canine mast cells. Vet. Immunol. Immunopathol. 113, 37–52.
Macrory, L., Vaughan-Thomas, A., Clegg, P.D., Innes, J.F., 2009. An exploration of the ability of tepoxalin to ameliorate the degradation of articular cartilage in a canine in vitro model. BMC Vet. Res. 5, 25.
Mao, J.T., Tsu, I.H., Dubinett, S.M., Adams, B., Sarafian, T., Baratelli, F., Roth, M.D., Serio, K.J., 2004. Modulation of pulmonary leukotriene B4 production by cyclooxygenase-2 inhibitors and lipopolysaccharide. Clin. Cancer Res. 10, 6872–6878.
Marshall, J.S., Jawdat, D.M., 2004. Mast cells in innate immunity. J. Allergy Clin. Immunol. 114, 21–27.
McCann, M.E., Andersen, D.R., Zhang, D., Brideau, C., Black, W.C., Hanson, P.D., Hickey, G.J., 2004. In vitro effects and in vivo efficacy of a novel cyclooxygenase-2 inhibitor in dogs with experimentally induced synovitis. Am. J. Vet. Res. 65, 503–512.
Moon, T.C., Lee, E., Baek, S.H., Murakami, M., Kudo, I., Kim, N.S., Lee, J.M., Min, H.K., Kambe, N., Chang, H.W., 2003. Degranulation and cytokine expression in human cord blood-derived mast cells cultured in serum-free medium with recombinant human stem cell factor. Mol. Cells 16, 154–160.
Nakajima, T., Inagaki, N., Tanaka, H., Tanaka, A., Yoshikawa, M., Tamari, M., Hasegawa, K., Matsumoto, K., Tachimoto, H., Ebisawa, M., Tsujimoto, G., Matsuda, H., Nagai, H., Saito, H., 2002. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. Blood 100, 3861–3868.
Okabe, T., Hide, M., Hiragun, T., Morita, E., Koro, O., Yamamoto, S., 2006. Bone marrow derived mast cell acquire responsiveness to substance P with Ca(2+) signals and release of leukotriene B(4) via mitogen-activated protein kinase. J. Neuroimmunol. 181, 1–12.
Olivry, T., Marsella, R., Hillier, A., 2001. The ACVD task force on canine atopic dermatitis (XXIII): are essential fatty acids effective? Vet. Immunol. Immunopathol. 81, 347–362.
Phillips, M.J., Gold, W.M., Goetzl, E.J., 1983. IgE-dependent and ionophore-induced generation of leukotrienes by dog mastocytoma cells. J. Immunol. 131, 906–910.
Puxeddu, I., Piliponsky, A.M., Bachelet, I., Levi-Schaffer, F., 2003. Mast cells in allergy and beyond. Int. J. Biochem. Cell Biol. 35, 1601–1607.
Robin, J.L., Seldin, D.C., Austen, K.F., Lewis, R.A., 1985. Regulation of mediator release from mouse bone marrow derived mast cells by glucocorticoids. J. Immunol. 135, 2719–2726.
Schleimer, R.P., Schulman, E.S., MacGlashan Jr., D.W., Peters, S.P., Hayes, E.C., Adams 3rd, G.K., Lichtenstein, L.M., Adkinson Jr., N.F., 1983. Effects of dexamethasone on mediator release from human lung fragments and purified human lung mast cells. J. Clin. Invest. 71, 1830–1835.
Sewell, W.A., Scurr, L.L., Orphanides, H., Kinder, S., Ludowyke, R.I., 1998. Induction of interleukin-4 and interleukin-5 expression in mast cells is inhibited by glucocorticoids. Clin. Diagn. Lab. Immunol. 5, 18–23.
Wilson, J.E., Chandrasekharan, N.V., Westover, K.D., Eager, K.B., Simmons, D.L., 2004. Determination of expression of cyclooxygenase-1 and -2 isozymes in canine tissues and their differential sensitivity to non-steroidal anti-inflammatory drugs. Am. J. Vet. Res. 65, 810–818.
Wu, X., Dev, A., Leong, A.B., 2003. Zileuton, a 5-lipoxygenase inhibitor, increases production of thromboxane A2 and platelet aggregation in patients with asthma. Am. J. Hematol. 74, 23–25.
Yakabi, K., Arimura, T., Koyanagi, M., Uehigashi, Y., Ro, S., Minagawa, Y., Nakamura, T., 2002. Effects of interleukin-8 and Helicobacter pylori on histamine release from isolated canine gastric mucosal mast cells. J. Gastroenterol. 37, 10–16.
Zhou, J., Liu, D.F., Liu, C., Kang, Z.M., Shen, X.H., Chen, Y.Z., Xu, T., Jiang, C.L., 2008. Glucocorticoids inhibit degranulation of mast cells in allergic asthma via nongenomic mechanism. Allergy 63, 1177–1185.