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The role of mouse mast cell protease 6 (mMCP-6) in a model of allergic airway inflammation

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Abbreviations

AP	Alkaline phosphatase
APC	Allophycocyanine
BAL	Bronchoalveolar lavage
BMMC	Bone marrow derived mast cell
BMeos	Bone marrow derived eosinophil
BSA	Bovine serum albumin
CD	Cluster of differentiation
Ct	Cycle threshold
ELISA	Enzyme linked immuno-sorbent assay
FcεRI	Fc epsilon receptor I
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
mMCP-6	Mouse mast cell protease- 6
mOD	milli optical density units
OD	Optical density
OVA	Ovalbumin
PAR-2	Protease-activated receptor 2
PBS	Phosphate Buffered Saline
PE	Phosphatidylethanolamine
qRT-PCR	Quantitative real time polymerase chain reaction
RPMI	Roswell park memorial institute (culture medium)
rm	Recombinant mouse
RT	Room temperature
SCF	Stem cell factor
Th1/2	Type 1/2 helper T cell
2-ME	2-mercapto ethanol

Abstract

Allergic asthma is associated with the degranulation of mast cells. One of the granule-associated mast cell mediators is β-tryptase. In mouse, this serine protease is called mouse mast cell protease-6 (mMCP-6). Since mast cell tryptases have been associated with inflammatory responses in many studies, we started to investigate the role of mMCP-6 in a mouse model of allergic airway inflammation. My results suggest that mMCP-6 deficient (mMCP-6-/-) mice have attenuated IgE antibody response and less peritoneal mast cells, but normal FcεRI expression. Our previous study has shown that mMCP-6-/- mice have reduced eosinophilia upon allergic airway inflammation. Using a transwell migration system where mouse eosinophils were allowed to migrate to supernatants from BALB/c and mMCP-6-/- mast cells indicated that an unidentified mediator, present in both supernatants, induced eosinophil migration. This suggests that mMCP-6 is not directly involved in the decreased eosinophil infiltration observed *in vivo*. Quantification of mRNA expression of type 2 helper T cell (Th2) cytokines from lung tissue shows a tendency to be decreased in mMCP-6-/- mice upon inflammation. Altogether, my results show that loss of mMCP-6 results in a weaker immune response that requires further investigation, but meanwhile, suggest an important function of mMCP-6 in this model of allergic airway inflammation.

1 Introduction

1.1 Mast cells and allergic asthma

Allergic asthma is a chronic disorder associated with long term changes in the airways, characterized by bronchoconstriction, airway hyperresponsiveness, airway inflammation and remodeling (Taube *et al*., 2004; Yu *et al*., 2006). The allergic airway inflammation is induced after re-exposure of allergen. Early-phase reaction or the immediate hypersensitivity reaction is induced immediately after allergen challenge and is caused by secretion of proinflammatory mediators from mast cells. Late-phase reaction occurs within several hours after allergen exposure and results in local infiltration of Th2 cells, eosinophils and other leukocytes. Chronic allergic inflammation develops upon persistent allergen challenge and trigger tissue remodeling (Galli *et al*, 2008). Activated Th2 cells produce cytokines to regulate allergic response resulting in IgE production (IL-4, IL-13) and Th2 cells generation (IL-4), eosinophil maturation (IL-5), mast cell progenitor recruitment (IL-9), airway hyperresponsiveness and goblet cell hyperplasia (IL-13) (Holgate *et al*., 2008, Jones *et al*., 2009). In mouse models of acute allergic airway inflammation, many features of clinical asthma have been reproduced, including elevated IgE level, airway inflammatory and hyperresponsiveness and goblet cell hyperplasia (Nials *et al*., 2008).

Mast cells originate from hematopoietic stem cells in the bone marrow then develop into committed mast cell progenitors and home into tissues of peripheral organs including the lung (Hallgren *et al*., 2007). Studies in mice have demonstrated that mast cells promote many inflammatory, structural, and functional changes in lungs that are important features of asthma (Yu *et al*., 2006). Upon allergen provocation, crosslinking of FcεRI-bound IgE with allergen induce mast cells to degranulate and release diverse preformed and synthesized mediators, including histamine, serglycin proteoglycans, proteases, prostaglandins, cysteinyl leukotrienes and various cytokines (Galli *et al*., 2005). Some of the mediators are involved in the recruitment and activation of mast cells, Th2 cells, eosinophils and other leukocytes to orchestrate the inflammatory response.

1.2 Mast cell tryptase and mMCP-6

Mast cell derived proteases have important immunological roles in inflammation, tissue remodeling and bronchial hyperresponsiveness (Bradding *et al*., 2006). The most abundant serine protease is tryptase. Tryptase is a trypsin-like protease with a cleavage preference at the N-terminal of basic amino acids such as arginine or lysine. In human, tryptases are classified into three groups: α-, β-, and γ-tryptase. β-tryptase is the main form of tryptase stored in human mast cells. Sharing similar substrate specificity, mouse mast cell protease-6 (mMCP-6) is the functional counterpart to β-tryptase in human (Hallgren *et al*., 2005). The roles of mast cell tryptases in allergic inflammatory airway response have been implicated from enhanced tryptase level in bronchoalveolar lavage (BAL) fluid in asthmatics patients (Jarjour *et al*.,

1991; Schwartz *et al*., 1994) and through the inhibitory function of tryptase inhibitors to inflammatory responses in allergic sheep (Clark *et al*., 1995). Injection of tryptase also promotes local inflammatory reactions that result in neutrophil and eosinophil infiltration in mouse and guinea pig (Hallgren *et al*., 2000, He *et al*., 1997). Further, tryptases have been demonstrated to stimulate fibroblast (Levi-Schaffer *et al*., 2003) and smooth muscle cell proliferation (Brown *et al*., 1995; Brown *et al*., 2002). These inflammatory effects of tryptase may be regulated by protease-activated receptor 2 (PAR-2).

PAR-2 is one of the known substrates cleaved and thereby activated by tryptase. PAR-2 is expressed on airway epithelial cells, lung fibroblasts, endothelium, bronchial smooth muscle as well as leukocytes, such as eosinophils (Schmidlin *et al*., 2002). By comparing mice lacking and overexpressing PAR-2 to wildtype, Schmidlin *et al* observed a correlation of PAR-2 expression with eosinophil infiltration, airway hyperreactivity and IgE level after ovalbumin (OVA) sensitization and challenge. (Schmidlin *et al*., 2002). These studies demonstrate that PAR-2 play a role in airway inflammation.

In recent years, several studies have been published where mMCP-6 deficient mice have been used. Thakurdas *et al* described the generation of mMCP-6-/- mice on the C57/BL6 strain and implicated mMCP-6 to play critical roles in clearance of bacteria (Thakurdas *et al*., 2007). Shin *et al* found that in chronic infection, mMCP-6-/- mice were disabled to recruit normal number of eosinophils to *Trichinella spiralis* larvae in the infected skeletal muscle (Shin *et al*., 2008). Besides infections, mMCP-6 also contributes to autoimmune disease. In a tandem study, McNeil *et al* and Shin *et al* demonstrated that mMCP-6-/- mice have less joint inflammation in an inflammatory arthritis model that was attributed to attenuated neutrophil infiltration (Shin *et al*., 2009; McNeil *et al*., 2008).

1.3 Previous findings

In the present research group, mMCP-6 deficient mice on BALB/c strain is used to investigate the role of mMCP-6 in experimental allergic airway inflammation. Before I joined the group, my supervisor had shown that OVA-sensitized and challenged mMCP-6-/- mice have significantly diminished eosinophil accumulation in BAL, attenuated inflammation and goblet cell hyperplasia. (Data not shown)

1.4 Aims

The aim of this study is to further elucidate the role of mMCP-6 in allergic airway inflammation and to investigate the mechanism behind the attenuated allergic response in mMCP-6-/- mice. Specific goals: 1) to compare OVA-sensitized and challenged BALB/c and mMCP-6 deficient mice in terms of: i. total and OVA-specific antibody responses. ii. mast cell expression of FcεRI. iii. Th2 cytokine production. 2) to test the hypothesis that the observed reduced eosinophil accumulation in BAL is a direct affect of loss of mMCP-6 in mast cells.

2 Materials and Methods

2.1 Mice

mMCP-6-/- mice, backcrossed onto a BALB/c background for 10 generations, were obtained from Dr. David Lee, Brigham and Women's Hospital, Harvard Medical School, Boston, USA. BALB/c wildtype mice were bred in-house. All experimental mice were conducted under approval of the Animal Ethics Committee, Uppsala, Sweden.

2.2 OVA sensitization and challenge

Mice were sensitized on days 0 and 14 by intraperitoneal (i.p.) injection of 20 µg OVA (Albumin from chicken egg white, Grade V, Sigma, 9006-59-1) in 100 µl PBS. On day 28, 29 and 30, mice were challenged with 1% OVA-aerosol in PBS for 30 min per day. Mice were killed by overdose of isoflurane followed by cervical dislocation and studied 24 hours after the last OVA-aerosol treatment.

2.3 ELISA

On day 0, 8, 21, 31, after OVA sensitization or challenge, mice were bled from the tail (day 0, 8, 21) or heart (day 31) and the sera were analyzed for OVA-specific IgG/IgE and total IgG/IgE antibody response by ELISA. After clotting, the aqueous phase of blood was centrifuged at 11×1000 rpm for 5 min to remove the remaining clots and red blood cells. The sera (supernatants) were collected into eppendorf tubes and kept in -20°C. For all ELISAs, 96-well plates (Immunolon 2HB, Thermo) were incubated with antigen or anti-mouse antibody over night at 4°C (details see in table 1). Plates were washed with PBS or 0.05% Tween/PBS after incubation. The washing was typically performed three times between steps. Dry milk or bovine serum albumin (BSA, Sigma) were used for blocking unspecific binding sites on the plates. Sera were diluted in dilution buffer [PBS containing 0.05% Tween, 0.25% dry milk and 0.02 NaN_3] or BSA until appropriate concentration and incubated in the blocked plates over night at 4°C or 2 h at room temperature. For detection, alkaline phosphatase (AP)-conjugated anti-mouse antibody was added. Finally substrate (p-nitro-phenylphosphate, Sigma, 4264-83-9) diluted in diethanolamine buffer [1.0 M diethanolamine, 50 mM $MgCl_2 \times 6H_2O$, PH 9.8] was added and the plate was incubated in the dark for up to 2.5 hours. Absorbance was measured at 405 nm and antibody titer was expressed in OD_{405}±SEM.

Table 1. Materials and Methods for ELISA

ELISA	Coating (4°C over night)	Block	Sample	AP-conjugated Ab
OVA- specific IgG	OVA (50 µg/ml)	dry milk (50 mg/ml) 2 h RT or 4°C over night	1:625 in dilution buffer, 4°C over night	AP- anti-mouse IgG (0.6 µg/ml), 3 h RT*
OVA- specific IgE	rat-anti-mouse IgE (2 µg/ml)	dilution buffer 2 h RT	1:3 in dilution buffer, 4°C over night	Biotin -OVA-TNP (10 µg/ml), 1 h RT ; 1:30,000 AP-streptavidin, 45 min
Total IgG	goat -anti-mouse IgG (2 µg/ml)	BSA (5 mg/ml) 1 h 37°C or 4 °C over night	1:50,000 in BSA 2.5 h RT	AP- anti-mouse IgG 1 h at 37°C
Total IgE	rat-anti-mouse IgE (2 µg/ml)	BSA (5 mg/ml) 1 h 37°C or 4 °C over night	1:10 in BSA 2.5 h RT	AP- anti-mouse IgE 1 h at 37°C

** RT, room temperature*

rat-anti-mouse IgE (BD Biosciences, 553413); goat-anti-mouse IgG (invitrogen, M30100)

Biotinylated OVA-TNP (Biotin-OVA-TNP), prepared as reported (Hjelm et al 2008); BSA (Sigma, 9048-46-8)

AP-conjugated anti-mouse IgG (Jackson ImmunoResearch, 65320); AP- streptavidin (BD Biosciences, 554065);

AP- anti-mouse IgE (Southern Biotech, 1130-04)

2.4 Flow cytometry

At day 31 after last challenge, peritoneal lavage was collected by injecting 10 mL of PBS into the peritoneal cavity of the mouse immediately after sacrifice. The mouse was pinned to a polystyrene foam board and shaken by punching the board ten times to release the cells, and peritoneal lavage was withdrawn from the peritoneal cavities by a 10 ml syringe. The peritoneal lavage cell suspension was centrifuged at 1200 rpm for 5 min, and cell pellets were resuspended in FACS buffer [PBS with 1% FCS] and stored at 4°C. Two million cells from each sample were washed with FACS buffer and probed with the indicated antibody or isotype control in FACS buffer for 40-60 min at 4°C. After staining, the cells were washed with FACS buffer and analyzed by flow cytometry. Data were acquired with a LSRII cytometer (BD biosciences) and analyzed with Flowjo software (Tree Star). Positive cells were identified by comparison to isotype control. Antibodies used included PE-conjugated rat anti-mouse Siglec-F (BD Biosciences, 552126) or PE-conjugated rat IgG2a isotype control (eBioscience, 12-4321-81), FITC-conjugated rat anti-mouse CD45 (BD Biosciences, 553080) or FITC-conjugated rat IgG2a isotype control (eBioscience, 11-4321-81), APC-conjugated hamster anti-mouse CD11c (BD Biosciences, HL3 550261), PE-conjugated anti-mouse FcεRIα (eBioscience, 12-5898-81) or PE-conjugated Armenian hamster IgG isotype control

(eBioscience, 12-4888-81), Horizon V450-conjugated rat anti-mouse c-kit (BD Biosciences, 560558), APC-conjugated rat anti-mouse CD11b (SouthernBiotech, 1561-11).

2.5 Cell culture

Bone marrow was obtained from the femurs and tibiae of naive BALB/c and mMCP-6-/- mice by flushing the opened bones with RPMI 1640 complete medium. For bone marrow derived mast cell (BMMC), the bone marrow cells were cultured at 0.5×10^6/ml in RPMI 1640 complete medium [RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, 1 mM sodium pyruvate and 50 μM 2-mercapto ethanol (2-ME) (All from Sigma)] supplemented with 50 ng/ml interleukin 3 (IL-3) (conditional media from X-63 cell line producing IL-3) and 50 ng/ml recombinant mouse stem cell factor (rmSCF, PeproTech, 250-03) for 4 – 5 weeks. Twice a week, medium was replaced with fresh medium containing IL-3 and SCF and the cell concentration was adjusted to 0.5×10^6/ml. For bone marrow derived eosinophil (BMeos), the bone marrow cells were cultured at 10^6/ml in RPMI 1640 complete medium supplemented with 10 ng/ml recombinant mouse (rm) IL-5 (R&D Systems, 405-ML) for 9 days. Every other day, one half of the medium was replaced with fresh medium containing rmIL-5 and the cell concentration was adjusted to 10^6/ml. At harvest day, 50,000 cells were subjected to cytospin (Thermo) and stained using Diff Quick stain set (Labex AB).

2.6 BMMC activation and tryptase activity analysis

Calcium ionophore A23187 (Sigma, 52665-69-7) was dissolved in Dimethyl sulfoxide (DMSO) to 2 mM and stored in freezer. When used, 2 mM A23187 was further diluted to 2 μM in RPMI 1640 medium. Two hundred microliters of BMMC at 5×10^6 cells/ml from BALB/c or mMCP-6-/- mice were activated with 2 μM calcium ionophore A23187 at 37°C for 1 h. After centrifugation at 300g for 5 min, the supernatants were assayed for tryptase activity toward chromogenic peptide substrate S-2288 (H-D-lle-Pro-Arg-pNA·2HCl, Chromogenix, Intrumentation Laboratory). Twenty microliters of supernatants were added to 100 μl PBS, PH 7.4, followed by 10 μl S-2288 at 2.5 mg/ml in H_2O. Absorbance was measured at 405 nm, and tryptase activity was expressed in milli optical density units (mOD) per min.

2.7 Chemotaxis assay

The assay was performed in a transwell plate (Neuro Probe, ChemoTx® 106-5) with a 5 μm pore size polycarbonate membrane. Recombinant mouse eotaxin (PeproTech, 250-01) was dissolved in 0.5% BSA/PBS to 20 μg/ml and then further diluted to 1000 ng/ml in medium containing rmIL-5. Twenty-nine microliters of BALB/c or mMCP-6-/- BMMC supernatants containing rmIL-5 or rmIL5 supplemented medium with or without eotaxin was placed in the

lower well. Fifty microliters of BMeos at 2×10^6 cells/ml in IL-5 containing media was placed in the upper site. Cells were incubated at 37°C for 3 h to permit migration across the membrane in response to the above agents. Cells migrated to the lower well were enumerated in a hemocytometer. Data were presented as percentage of control (medium containing rmIL-5).

2.8 Isolation of RNA from lung homogenates and quantitative RT-PCR

OVA-treated lung of BALB/c (n=3) and mMCP-6-/- (n=4) mice were homogenized in 1 ml TRI REAGENT™ (Sigma, T9424) and extraction preceded according to manufacturer's instructions. Four microliters of RNA prepared as described was subjected to reverse transcription using iScript cDNA synthesis kit (Bio-Rad, 170-8890). Two microliters of the obtained cDNA was subjected to real-time PCR amplifications using SYBR Green kit (Agilent Technologies, 600548) according to the manufacturers' instructions. Real-time PCR was performed using the following cycles: segment 1, 95 °C for 15 min, 1 cycle; segment 2, 95 °C for 15 s, 60 °C for 1 min, 72°C for 30 s, 40 cycles; segment 3, 95°C for 1 min, 55°C for 30 s, 95°C for 30 s, 1 cycle. All primer probe sets (IL-4, IL-5, IL-10, IL-13, GAPDH) were designed as reported (Overbergh *et al*, 1999). The housekeeping gene GAPDH was used as the endogenous control. The relative gene expression among different samples was determined using comparative Ct (cycle threshold) method.

2.9 Statistical analysis

Data were analyzed using Student's t test. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. All data are reported as the mean ± SEM.

3 Results

3.1 mMCP-6-/- mice have impaired antibody responses

We use a mast cell dependent "acute asthma" protocol to study the role of mMCP-6 in allergic airway inflammation. Using this protocol, mast cell deficient mice $Kit^{w/w-v}$ and $Kit^{W-sh/W-sh}$ were observed developing diminished pulmonary inflammation, airway resistance and goblet cell hyperplasia (Reuter *et al*, 2008). We sensitized the mice on days 0 and 14 and challenged on days 28, 29 and 30 with OVA in the absence of adjuvant. On day 0, one week after each immunization (day 8, 21) and 24 h after the final challenge (day 31), sera were isolated and antibody response was analyzed with ELISA. The mMCP-6-/- and BALB/c mice showed similar serum IgG before or after OVA treatment (Fig. 1A). However, the total IgE in mMCP-6-/- serum was significantly reduced (Fig. 1B). Interestingly, the attenuated IgE level was observed even before the immunization (day 0). Quantification of OVA- specific antibodies consistently displayed lower antibody titers in mMCP-6-/- mice than in BALB/c mice, although not all of the time point reached a significant difference (Fig. 1C and D). OVA-specific IgG showed significant reduction on day 31, i.e. 24 h after the final challenge (Fig. 1C) whereas a significant diminished OVA- specific IgE (Fig. 1D) was observed both one week post the second immunization (day21) and 24 after the final challenge (day 31). These data together indicate that loss of mMCP-6 attenuates the antibody response in our model of allergic airway inflammation.

3.2 Mast cell number but not the expression of FcεRI on mast cells during OVA treatment is regulated by mMCP-6

It has been shown that IgE upregulates mouse mast cell FcεRI expression (Yamaguchi *et al*, 1997). Since we have observed decreased IgE level in mMCP-6-/- mice, we investigated FcεRI expression on peritoneal mast cell to exclude the possibility that the attenuated inflammatory response in the mMCP-6-/- mice was due to less mast cell activation. Mast cells were defined as $FcεRI^{+}$ $c\text{-}kit^{+}$ cells (Fig. 2A). Unexpectedly, FcεRI expression was similar in BALB/c and mMCP-6-/- mice following OVA treatment (Fig. 2B) but the number of peritoneal mast cells was reduced in mMCP-6-/- mice (mMCP-6-/- 301.5±46.5 vs. BALB/c 953.9±156.6 per 10^6 peritoneal cells, Fig. 2C).

3.3 Lungs from mMCP-6-/- mice show a tendency to decreased mRNA expression of Th2 cytokines

The allergic inflammation is characterized by Th2 cell polarization. In a pilot experiment, we compared mRNA expression of Th2 cytokines in lung homogenates from OVA-sensitized and challenged BALB/c and mMCP-6-/- mice (Fig. 3). We found that the key Th2 cytokine IL-4 mRNA levels showed a trend to be decreased in mMCP-6-/- versus BALB/s mice (2.89±1.02

vs. 4.23±0.74, p=0.369), although the observed decrement did not reach significance. Moreover, similar tendency was demonstrated for IL-10 (2.74±0.33 vs. 7.40±2.46, p=0.076) and IL-13 (7.89±1.89 vs. 11.77±2.39, p=0.252). However, IL-5 levels were more or less similar (1.55±0.18 vs. 1.76±0.32, p=0.567).

3.4 Eosinophils migrate equally towards supernatants from BALB/c and mMCP-6-/- mast cells

Our group has previously shown a significant decrement but not abolishment of eosinophil influx to the lung of OVA-sensitized and challenged mMCP-6-/- mice *in vivo* (Hallgren, unpublished data). We hypothesized that mMCP-6 directly attracts eosinophils to the lung. To test this hypothesis, we set up an *in vitro* experiment to examine effects of mast cell supernatant on eosinophil migration.

As a source of mouse eosinophils, we used BMeos. Bone marrow cells were isolated from BALB/c mice and grown in rmIL-5 supplement medium for 9 days. The harvested cells had the characteristic appearance of eosinophil (bilobed blue nucleus with red/orange granules) on microscopic examination after Diff Quick staining (Fig. 4A and B). Further, the harvested cells were >80% positive for Siglec-F and CD45 by flow cytometry (Fig. 4C).

We used BMMC from BALB/c and mMCP-6-/- mice as a source of mature mast cells. To obtain BMMC, bone marrow cells were grown in rmSCF and IL-3 supplement medium. After 4-5 weeks of culture, mature BMMCs were activated with calcium ionophore A23187 to release mast cell mediators. Supernatants were collected and analyzed for tryptase activity using a chromogenic substrate. Supernatants from vehicle treated BALB/c and mMCP-6-/- BMMC were used as negative control. Tryptase activity from BALB/c or mMCP-6-/- BMMC (day27) was 4.06±0.035 and 0.39±0.004 mOD/min respectively while negative control was 0.14±0.012 mOD/min (Fig. 5). This indicated that the lack of mMCP-6 led to diminished tryptase activity from degranulated BMMC.

Figure 6 shows the results of the BMeos migration experiment. In a transwell migration system, mouse eosinophils were allowed to migrate to supernatants from BALB/c and mMCP-6-/- BMMCs. BMeos migration toward culture medium (rmIL-5 supplement medium) was indicated as baseline. BMeos exhibited significant increment of migration (~2 fold) toward eotaxin (positive control) while BMMC supernatant from BALB/c or mMCP-6-/- induced a ~3 fold migration of BMeos. However, BMeos migrated equally well toward supernatant of degranulated BMMC from BALB/c or mMCP-6-/- mice.

4 Discussion

After crosslinking of FcεRI-bound IgE with antigen, mast cells degranulate and release mediators that contribute to the allergic response, characterized by airway inflammation, eosinophil and lymphocyte infiltration and airway remodeling. Antigen-induced allergic airway inflammation is associated to Th2 response with the production of IL-4, IL-5 and IL-13 as well as goblet cell hyperplasia, eosinophil infiltration, airway inflammation and remodeling. Using an acute mast cell dependent protocol of allergic airway inflammation, our preliminary observations have shown that OVA-sensitized and challenged mMCP-6-/- mice have less eosinophil influx, attenued inflammation and goblet cell hyperplasia *in vivo* (data not shown), suggesting the contribution of mast cells to airway inflammation could be at least partly explained by mMCP-6. We thereafter began this study by measuring antibody response in serum for serum total or antigen specific IgG/IgE. mMCP-6-/- mice were demonstrated to have diminished total IgE and OVA specific IgE but normal level of total IgG compared to BALB/c wildtype mice. Interestingly, an attenuated total IgE level was observed even before the immunization (day 0). This suggests that mMCP-6 may regulate antibody production triggered by innate stimulators. OVA specific IgG level was also tested in our study but the results were inconsistent. Figure 1C shows a significant reduction in mMCP-6-/- mice after final challenge (day 31). However, some of our experiments indicate that OVA specific IgG antibody responses were normal in all time points. Therefore, experiments with larger groups are necessary to ascertain whether OVA specific IgG level is impaired in mMCP-6-/- mice.

It has been shown that IgE upregulates the expression of the high affinity receptor for IgE, FcεRI. To explore our finding that mMCP-6-/- has lower level of IgE, we further investigated the expression of FcεRI. Since peritoneal mast cells represent about 2% of the peritoneal cell population (Danon *et al*. 1966) and peritoneal lavage is easily to obtain, our pilot study examined FcεRI expression on peritoneal mast cells. Unexpectedly, FcεRI expression was unchanged in OVA-sensitized and challenged mMCP-6-/- mice (n=5) compared to BALB/c (n=4) mice. One possible explanation could be that mMCP-6 -/- mice have significantly lower IgE antibody response, but the level is still competent to drive normal FcεRI expression. Studies by Smurthwaite *et al*. demonstrated that local IgE synthesis rather those present in circulation is important for FcεRI upregulation. Therefore beside serum antibody level, it would be logical to examine local antibody response, if possible, in the airways. Surprisingly, the number of peritoneal mast cells was reduced in mMCP-6-/- mice, an observation that we currently have no explanation for.

In Th2 prone individuals or in mouse models with OVA as an allergen, dendritic cells present processed allergen to naïve T cells, thereby driving them to Th2 cell phenotype. Activated Th2 cells produce cytokines to orchestrate allergic response, including B cell class switching to

produce allergen specific IgE which can be measured after sensitization. Upon re-exposure of allergen in the airway, the Th2 responses drive eosinophil and lymphocyte infiltration, airway hyperresponsiveness and goblet cell hyperplasia (Holgate *et al*., 2008). In our study, both cytokine protein levels in BAL (data not shown) and mRNA expression in the lung were determined. Sensitized and challenged mMCP-6-/- mice displayed a tendency of less mRNA expression of all examined Th2 cytokines compared to similarly treated BALB/c mice. This observation may explain the attenuated airway inflammation observed *in vivo*. Again, larger groups are necessary to ascertain the observed results. Meanwhile, we assayed cytokine proteins using a Multiplex mouse Th1/Th2 10plex kit from Bender MedSystems and found that most cytokine proteins from BAL were under detection limit. IL-10 was the only detectable cytokine (data not shown) and showed a trend to be decreased in mMCP-6-/- mice, consistent to the mRNA expression. IL-10 is a suppressive cytokine, produced by Th2 cells, that inhibits type 1 helper T cell (Th1) responses by inhibiting macrophage and dendritic cell production of IL-12. Studies have shown that T regulatory cells and Th1 cells also secrete IL-10. IL-10 has also been identified to have a role in the induction of respiratory tolerance (Hawrylowicz *et al*., 2005, Holgate *et al*., 2008). To be able to study cytokine levels further, we need to find a way to concentrate specimen or apply instrument/methods with higher sensitivity.

As mentioned, our previous results observed a decrement of eosinophil influx to the lung BAL in OVA-sensitized and challenged mMCP-6-/- mice. To investigate the possible direct effect of mMCP-6 on the migration of eosinophils, an *in vitro* transwell cell migration assay was used. We used BMeos as a source of mouse eosinophil. The BMeos were defined as Siglec-F^+ cells by flow cytometry (Dyer *et al*., 2008; Ohmori *et al*., 2009) and exhibited eosinophil morphology and migrated toward eotaxin. Interestingly, our data demonstrated that BMeos migrated equally well toward supernatant from degranulated BALB/c and mMCP-6-/- BMMC suggesting that mMCP-6 does not itself induce migration of eosinophil. However, the finding that eosinophils migrate to mast cell supernatant suggests that an unidentified mast cell mediator, but not mMCP-6, induce eosinophil migration. Early studies found so called eosinophil chemoattractant protein in mast cells. But latter, this protein was proved to be the production of TNF-α and IL-8 from mast cells acting on stromal cells to release eotaxin (Leonardi *et al*., 2003). But in our system, we do not have cells other than mast cells and eosinophils so that cannot account as an explanation to our data. Recently, chymase, another main protease in mast cells, has been found to promote the expression of the eosinophil adhesion molecule CD18 and exerting chemokinetic rather than chemotactic migration on eosinophils (Wong *et al*., 2009). However, since we did not perform a check board analysis to differentiate between chemokinesis (enhanced random movement in the presence of chemical agents) and chemotaxis (directed migration toward a chemotactic factor), we cannot rule out that chymase rather than tryptase could be the mediator inducing eosinophil migration. There

are many alternative explanations for the observed reduced eosinophil accumulation in BAL of OVA-sensitized and challenged mMCP-6-/- mice. For example, Jaruga *et al* demonstrated that IL-4 from mast cells upregulate eotaxin expression from endothelium and epithelium, thereby developing eosinophil chemotaxis (Jaruga *et al*., 2007). Therefore, the attenuated eosinophil infiltration in mMCP-6-/- could be partially explained by diminished IL-4 production, which is consistent to our IL-4 mRNA expression data. Meanwhile, Pang *et al*. revealed a role of mast cell β-tryptase to cleave eotaxin and abrogate chemotactic function of eosinophil. In mice, mMCP-6 is the functional counterpart to human β-tryptase. Therefore, it would be interesting to examine eosinophil migration toward eotaxin-treated BMMC supernatant.

Serine protease, such as mast cell tryptase, has been shown to activate PAR-2 (Molino *et al*., 1997). Recent studies demonstrate that PAR-2 signaling triggers dendritic cell development *in vitro* (Fields *et al*., 2003) and *in vivo* (Ramelli *et al*., 2010). Upon allergen challenge, dendritic cells migrate to draining lymph node and activate T cells. Our current hypothesis is that the impaired inflammatory responses in OVA-sensitized and challenged mMCP-6-/- mice could be due to impaired PAR-2 activation on dendritic cells. As dendritic cells are professional antigen presenting cells, which are important for sensitization, less dendritic cells maturation in mMCP-6-/- mice may also explain the lower antibody response in mMCP-6-/- mice. Moreover, Reuter *et al* demonstrated that the sensitized mast cell deficient mice have diminished migration of dendritic cells to draining lymph nodes after allergen challenge (Reuter *et al*., 2009), although this effect attributes to loss of mast cell derived TNFα. Thus our future plan is to further investigate the allergic airway inflammation responses in mMCP-6 deficient mice and to study the number and activation status of dendritic cells in lung draining lymph nodes to explore a possible PAR-2 related mechanism.

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7 Figures

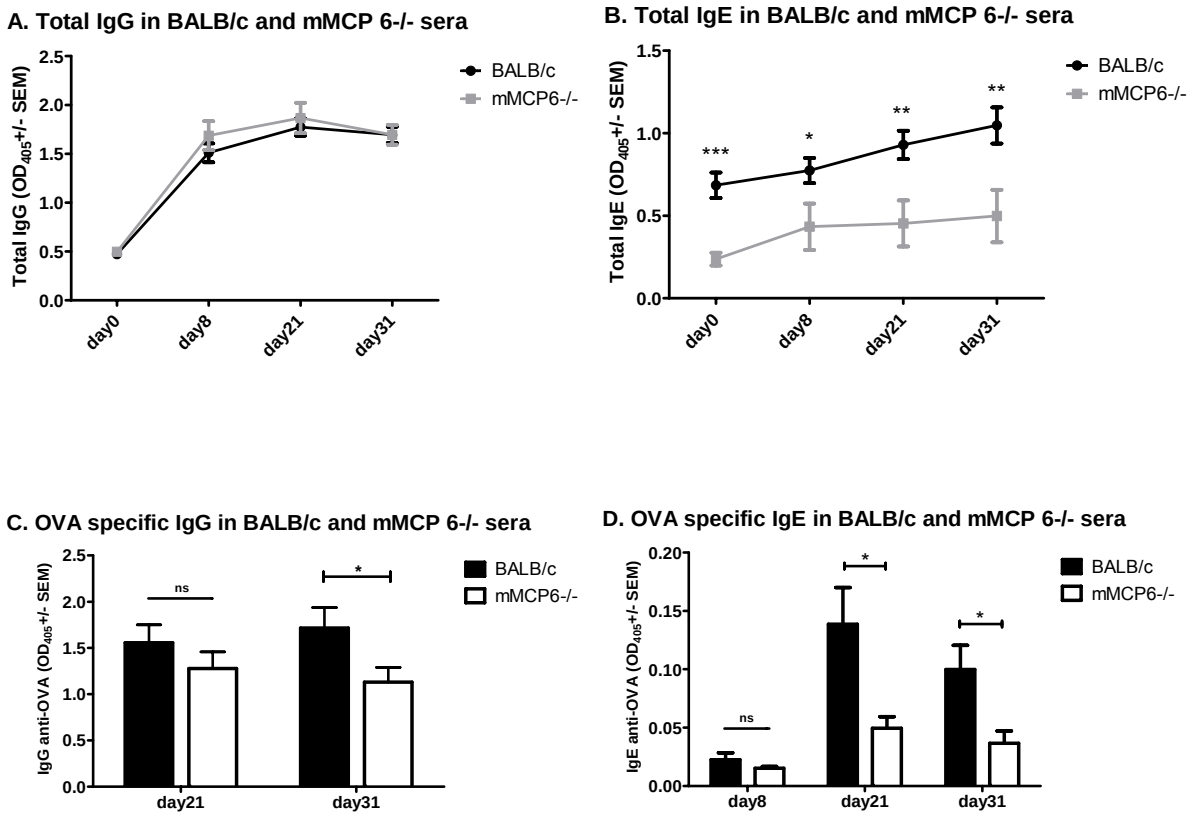

Figure 1. mMCP-6-/- mice have impaired total and OVA specific IgE but normal total IgG antibody responses. Sera were isolated from OVA treated BALB/c (n=15) and mMCP-6-/- (n=14) mice on indicated time points and antibody titers were determined by ELISA. (A) Total IgG. (B) Total IgE. (C) IgG anti-OVA. (D) IgE anti-OVA. Statistics were analyzed using Student's t test. ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

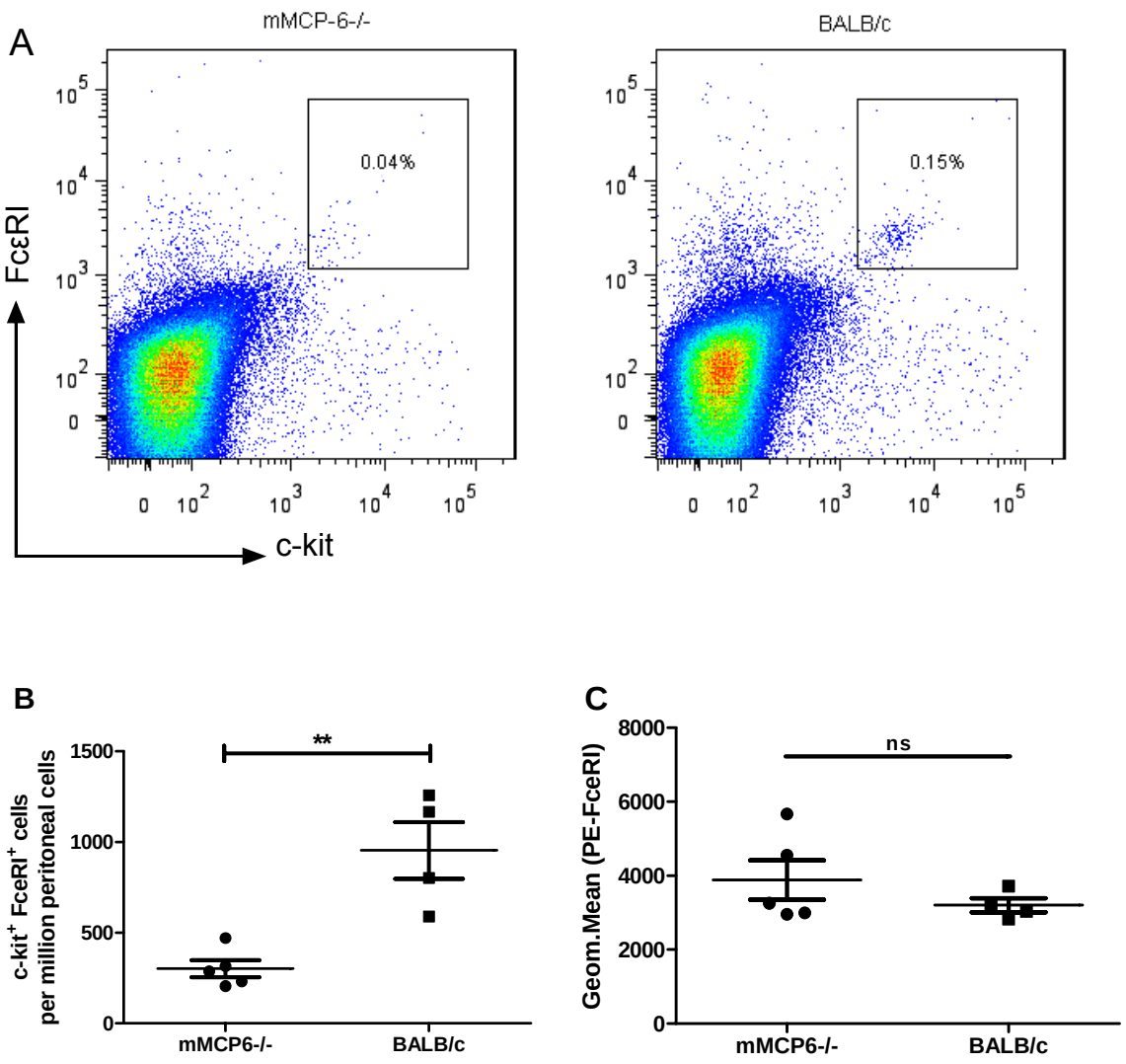

Figure 2. mMCP-6-/- mice have less peritoneal mast cells with normal FcεRI expression. Peritoneal cells were isolated from OVA-sensitized and challenged BALB/c (n=4) and mMCP-6-/- (n=5) mice and stained with PE-conjugated FcεRIα and Horizon V450-conjugated c-kit antibodies for mast cell detection. Mast cell number and FcεRI expression were analyzed by flow cytometry. (A) FACS plot showed identified mast cells as $FcεRI^+$ $c\text{-}kit^+$ cells. One mouse from each group is shown as representative. (B) Average number of peritoneal mast cells from mMCP-6-/- and BALB/c mice. ns, $p>0.05$; **, $p<0.01$. (C) Median fluorescence intensity of FcεRI expression on $FcεRI^+$ $c\text{-}kit^+$ peritoneal cells from mMCP-6-/- and BALB/c mice.

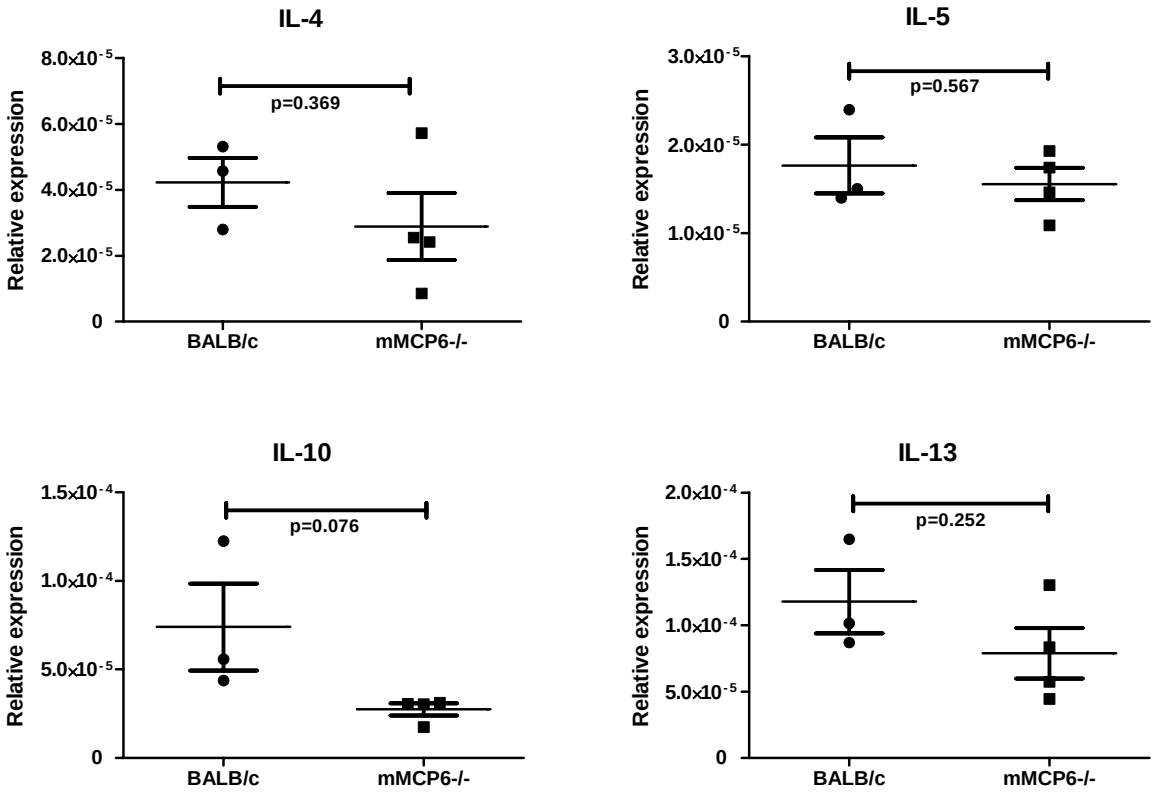

Figure 3. The mRNA expression of Th2 cytokines show a trend to be decreased in lungs from mMCP-6-/- mice. OVA-sensitized and challenged lung of BALB/c (n=3) and mMCP6-/- (n=4) mice were subjected to RNA isolation. IL-4, IL-5, IL-10, IL-13 mRNA were measured using quantitative real-time PCR. The housekeeping gene GAPDH was used as a normalization reference. The relative mRNA expression among different samples was determined using the comparative Ct method.

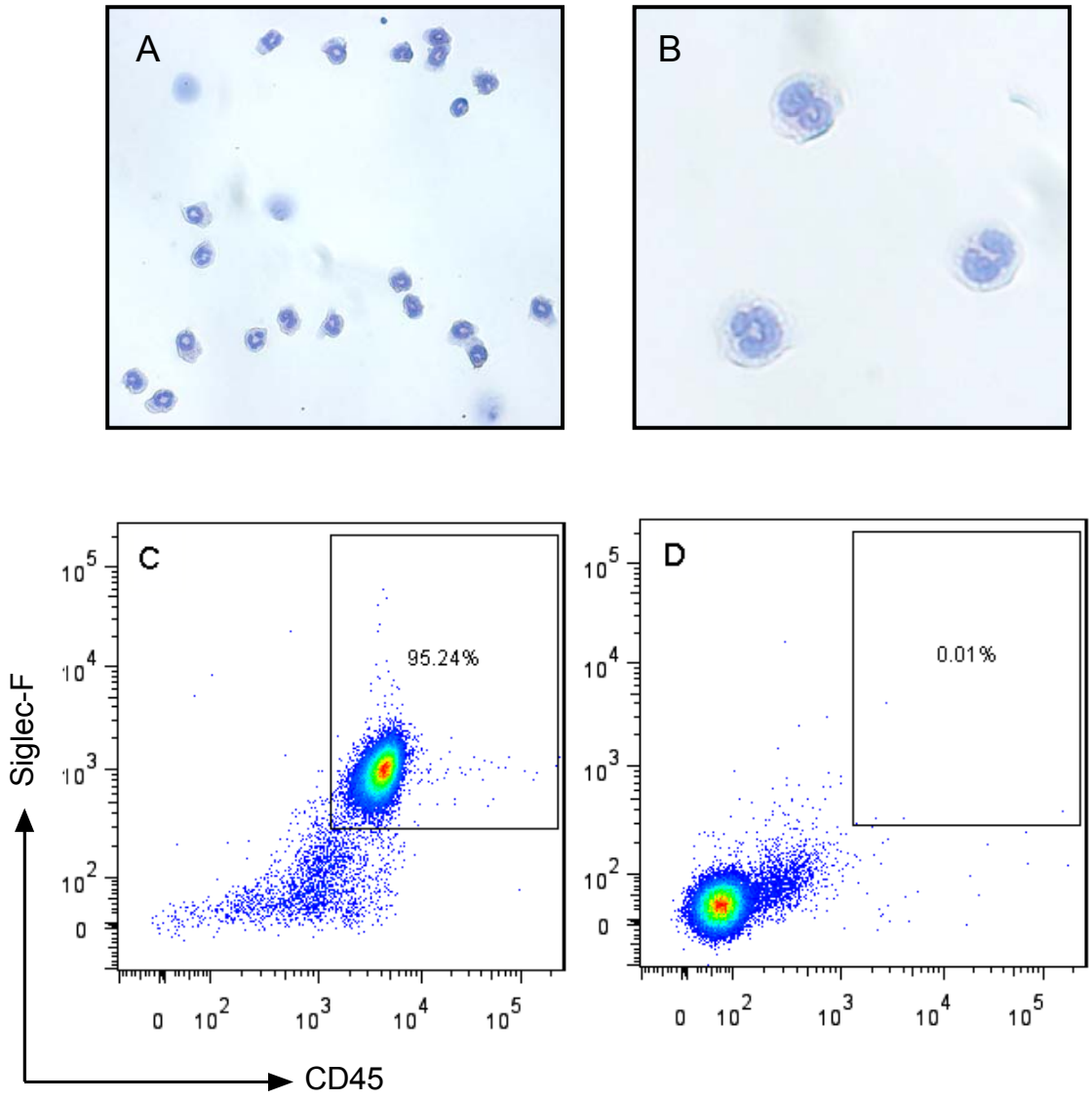

Figure 4. Culture of mouse bone marrow derived eosinophils. Bone marrow cells were cultured in rmIL-5 supplemented medium for 9 days. Light microscopic image of *in vitro* cultured BMeos, culture day 8, stained with Diff Quick. (A) Cell morphology was shown at original magnification ×20. (B) Characteristic appearance of eosinophil (bilobed blue nucleus with red/orange granules) was shown at original magnification ×43. (C, D) At day 9, cultured cells were detected by flow cytometry upon the staining of APC-labeled CD11c, FITC-conjugated CD45 and PE-conjugated Siglec-F. (C) Eosinophils were marked as $CD45^+$ $Siglec\text{-}F^+$ cells. (D) Isotype control for CD45 and Siglec-F staining.

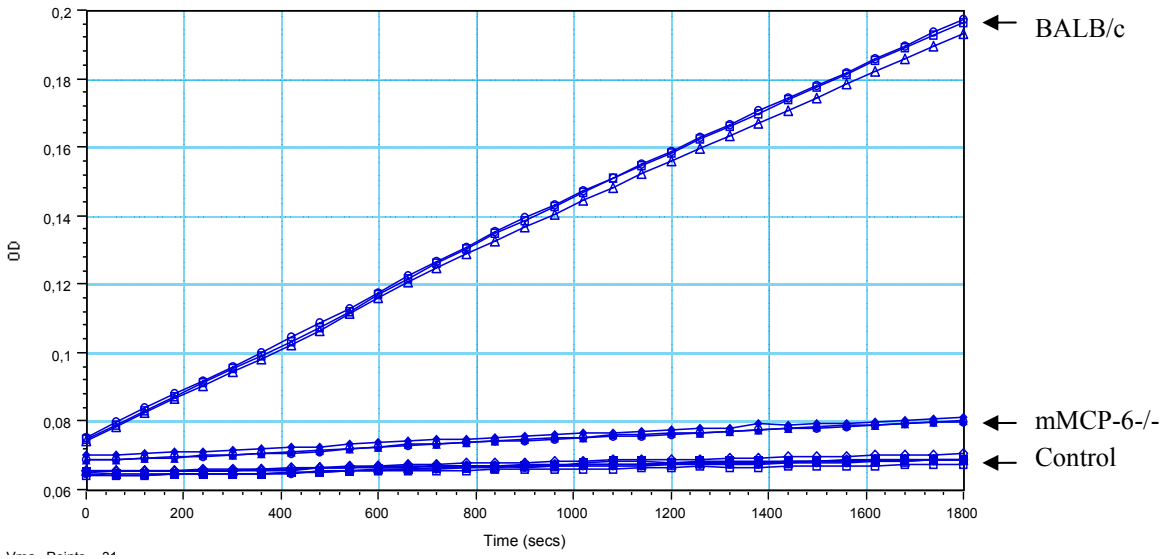

Figure 5. The supernatants from degranulated mMCP-6-/- mast cells have impaired tryptase activity. Cultured BMMC from BALB/c and mMCP-6-/- were degranulated by calcium ionophore A23187. Supernatants were collected to examine tryptase activity towards S-2288 as substrate. Supernatants from vehicle treated BALB/c and mMCP-6-/- BMMC were used as control. Tryptase activity was expressed in milli optical density units (mOD) per min. Triplicates of each treatment were performed. Data are representative of four independent experiments.

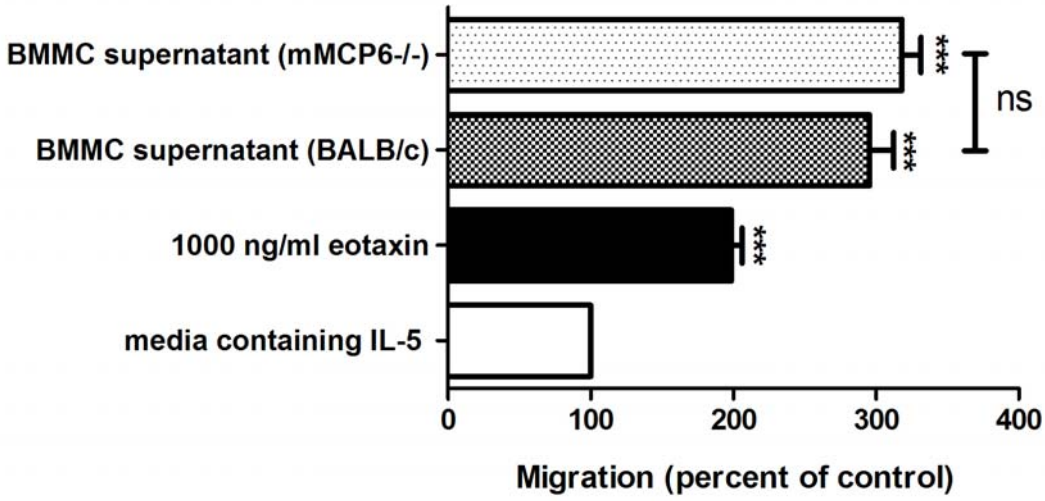

Figure 6. Eosinophils migrate equally toward supernatants from degranulated BALB/c and mMCP-6-/- mast cells. BMeos (BALB/c) from day9 were used to measure transwell migration in presence of supernatant from degranulated BMMC (day27) in BALB/c and mMCP-6-/- respectively, determined as percentage of migrated cells over control (media containing IL-5). Eotaxin was used as positive control. Triplicates of each treatment were performed. Data are representative of four independent experiments. **, $p<0.01$; ***, $p<0.001$.