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# The Involvement of Protein X in Mast Cell Development and *de novo* Production of Interleukin 6

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## Abstract

Mast cells residing in peripheral tissues are known to be involved in parasite elimination and allergic reactions. Mast cells perform their action through> releasing preformed mediators from their granules and by producing cytokines and chemokines.. The expression of a solute carrier (SLC) protein in mast cells was the main focus in this study since the function is unknown. The use of bone marrow derived mast cells (BMMCs) in a murine model lacking SLC protein (pX) made it possible for us to study the granulation development and the *de novo* production of interleukin 6 (IL-6). However, no difference in granulation development could be seen among BMMCs heterozygous for protein X and BMMCs deficient in protein X. Moreover, the levels of IL-6 production seemed to correspond to another study made with similar conditions, meaning no difference seems to exist. Still, as a pilot study the results are useful as they contribute to the unwinding of this protein's function.

## Abbreviations

| | |
|---|---|
| BMMC | Bone marrow derived mast cell |
| ELISA | Enyzme linked immunosorbent assay |
| FCM | Flow cytometry |
| FCS | Fetal calf serum |
| FMO | Fluorescence minus one |
| Het | Heterozygous |
| Ig | Immunoglobulin |
| IL | Interleukin |
| MCp | Mast cell precursor |
| OVA | Ovalbumin |
| PBS | Phosphate buffered saline |
| SLC | Solute carrier |
| SCF | Stem cell factor |
| TNP | Trinitrophenyl |

# Introduction

## The Mast Cell

Mast cells (MCs) are considered as a part of both the innate immune system and the adaptive immune system where they have a substantial role in allergic reactions and parasitic infections. As sentinels of the immune system, they reside in environmentally exposed tissues, such as skin, lungs and intestines. They originate from hematopoietic stem cells in bone marrow, which differentiate into immature mast cell precursors (MCp) (Metcalfe *et al.* 1997). Unlike the mature MCs, these progenitors contain none or only small amounts of the characteristic granules (secretory vesicles) (Rodewald *et al.* 1996). MCs and their progenitors express c-kit, which is the receptor for stem cell factor, an important survival- and differentiation growth factor and the high affinity receptor for IgE, FcεRI. The MCp migrate from the bone marrow through the blood stream (Dahlin *et al.* 2013), to tissues typically near blood vessels, hair follicles and nerves. In tissues they mature into fully functional cells capable of being activated by a diverse array of stimuli (Metcalfe *et al.* 1997).

## Activation and IgE-crosslinking

The most well studied activation pathway in MCs is the immunoglobulin E (IgE) dependent activation (Metzger 1992, Turner and Kiner 1999). This activation mechanism plays an essential part of an allergic reaction. IgE are antibodies produced by plasma cells derived from antigen-specific B-cells. The main role of IgE is the binding of antigens from parasites, thereby helping the immune system to recognize them as foreign particles and subsequently eliminate them (Abbas and Lichtman 2011). In an allergic reaction, antigen specific IgE bind to FcεRI on the cell surface of MCs causing sensitization of the mast cells. If the FcεRI-bound IgE encounters and binds its specific antigen, an IgE-crosslinking event occurs leading to mast cell activation and degranulation. Cell activation is caused by several FcεRI receptors clustering together to form a complex *i.e* crosslinking, which induces further cascade reactions inside the cell, leading to activation and eventually degranulation (Turner and Kiner 1999). Normally, MCs are coated with a variety of different IgE molecules specific for different antigens. In allergic reactions however, MCs are often coated with large proportions of IgE specific for a certain allergen. This is caused by increased IgE production by antigen-specific B-cells in allergic individuals (Smurthwaite *et al.* 2001).

## Mast Cell Mediators and Their Effects

Mast cells are powerful effector cells causing dramatic changes in their surroundings when activated by IgE crosslinking. Their granules contain a variety of different mediators having various effects on other cell types. The majority of the preformed mediators in the granules consists of vasoactive amines, such as histamine, heparin and different proteases. These vasoactive amines have dilating effects on blood vessels, leading to an increased influx of

blood to the affected tissue. Furthermore, the permeability through the endothelium of blood vessels is increased, facilitating the recruitment of more immune cells. (Abbas and Lichtman 2011).

Upon IgE-mediated MC activation, some mediators are synthesized *de novo*. These mainly consist of cytokines, chemokines and metabolites of arachidonic acid such as prostaglandins and leukotrienes. These contribute to effects seen in a later phase following MC activation. These further promotes the dilation of blood vessels and the contraction of smooth muscle cells, and aid in the recruitment of other immune cells (Metcalfe *et al.* 1997).

**IL-6**

MCs secrete a cocktail of different cytokines which all have large contributing effects to inflammation processes and recruiting of immune cells. Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced by MCs and a number of other cell types, such as macrophages, endothelial cells and fibroblasts (Van Snick 1990). The protein is secreted in the blood stream where it initiates several reactions. It is known to have the ability to cross the blood brain barrier, which is linked to its capacity to increase the body set temperature as a part of the inflammation process (Herrmann *et al.* 2003). The liver, IL-6 among other cytokines activates the so-called acute phase response. Production of numerous proteins known to have anti-microbial effects is drastically increased, while others are decreased (Van Snick 1990).

**The Solute Carrier Family**

The solute carrier (SLC) gene superfamily consists of a broad spectrum of membrane transport proteins. Membrane transporters are generally large proteins with many different domains that exert their function either intra- or extracellulary or inside the membrane (He *et al.* 2009). Their function is to transport different ligands over the cell membrane. They can transport their ligand(s) passively or actively, with or against the concentration gradient of the ligand and membrane potential (Hediger *et al.* 2003). At present, there are 55 families organized within the SLC family in humans and rodents, with no less than 362 functional genes, coding for proteins. Genetic analyses suggest the SLC family is a very ancient group of proteins since they share ancestry and homology with proteins with similar functions in prokaryotes (He *et al.* 2009). Over the course of evolution, this family has continuously been growing into a present diverse super family with members having widely different functions. Protein family 10 (SLC10) consists of seven members, some of them with known function. All proteins in this group have been shown to have homologs in rat and mouse, two common animal models (HUGO Gene Nomenclature Committee Solute Carrie Family Series: http://www.genenames.org/genefamilies/SLC). This family has been labeled as bile acid transporters (Geyer *et al.* 2006), though only two members have shown signs of transporting such substances (SLC10a1 and SLC10a2) (Dawson 2011). The third known member, SLC10a6 is a putative transporter for sulfo-conjugated steroid hormones (Geyer *et al.* 2007).

The other four members are orphan proteins with so far unknown functions. In this study we investigated the role of one of these orphan SLC10-member, referred to as protein X (pX), in mast cell maturation and activation using a mouse strain lacking the functional gene for pX.

**Aims**

In this study, we focus on the function of one of the orphan proteins (pX) belonging to the SLC10 family. We hypothesized that pX is important for the maturation and activation of mast cells. By using knock-out (KO) mice lacking pX and murine bone marrow derived mast cells (BMMCs) as a source of MCs we attempt to elucidate pXs role and function in mast cells.

## Material and Methods

### Preparation and Culture of BMMCs

Bone marrow was harvested from femur and tibia of 6-10 weeks old mice using a 4 mm syringe to flush the bones with complete medium (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ mL penicillin 100 µg/ mL streptomycin, 1 mM sodium pyruvate, 0.1 mM Non-essential amino acid, 10 mM HEPES , 50 µM β-mercaptoethanol and 10 µg/ mL Gentamicin) (All from Sigma-Aldrich). The cell harvest was spun at 1400g for 5 min and the pellet resuspended in complete medium supplemented with recombinant stem cell factor (SCF (Peprotech); 50 ng/mL) and murine interleukin 3 (IL-3; 50 ng/mL). Murine IL-6 was obtained as supernatant from the X-63 B-cell line. (Karasuyama 1988) The bone marrow cells were counted using trypan blue exclusion with a hemocytometer and the final cell concentration was adjusted to 0.5 million cells per mL media. The bone marrow derived mast cells (BMMCs) were at 37°C with 5% $CO_2$ for three weeks before the cultures were analyzed by flow cytometry to determine the mast cell purity of the culture. The medium was changed every fifth day and the cell concentration adjusted to 0.5 million cells per mL.

### Preparation of Cytospins and May-Grünwald/Giemsa Staining

Approximately 50 000 cells/sample were cytospun at 500 rpm for 6 minutes onto glass slides using a Shandon Cytospin 2. During the cytospin, cells become attached onto a glass slide by the centrifugal force and excess medium is absorbed by a filter paper. For every sampling, triplicates were produced from each BMMC cell culture. May-Grünwald/Giemsa staining was performed for visualization and examination of the inner components of the BMMCs (*e.g.* granule maturation). Soaking of glass slides in pure May-Grünwald staining solution (Sigma-Aldrich) for five minutes allowed negatively charged cell compartments to be stained. This was followed by rinsing in PBS (pH 7.2), for 1.5 minutes and finally a 20 minutes soaking in 5% Giemsa solution in PBS (Sigma-Aldrich) for nucleus staining. After staining, the slides

were washed in deionized water two times before drying and examination. BMMC samples taken from the same day were stained in the same batch to eliminate variation in staining.

**Microscopy and Digital Analysis**

All the May-Grünwald/Giemsa stained glass slides were photographed using a Nikon Eclipse microscope with 400X magnification. The same position of the slides was photographed to limit variations in the slides. The software NSI-Elements BR 64-bit was used for capturing and editing the pictures, with automatic exposure time and medium contrast.
All stained samples were scored as fully granulated, intermediate or none granulated cells, respectively. A digital grid was applied to every picture taken and the different classes of cells were counted in nine squares, to a total minimum of 30 cells per slide. A percentage of every cell class present on the slides was obtained and used for calculating a total score of the three replicates taken on the same date.

**Flow Cytometry**

Flow cytomtery was performed to check the mast cell purity in the BMMC cultures. This was done using two specific antibodies conjugated to fluorescent dyes recognizing c-kit (c-kit PE-Cy-7; clone 2B8; eBioscience) and FcεR1 (FcεRI-PE; clone MAR-1; eBioscience) found on MC membrane surface. One tube containing 1 million cells was prepared from each culture and two fluorescence minus one (FMO) controls and one tube with unstained cells. Anti-armenian hamster IgG-PE (eBio299Arm, eBioscience) was used for isotype control for c-kit-PE-Cy-7 and anti-rat IgG-PE-Cy-7 (eB149/10H5, eBioscience) as an isotype control for FcεRI-PE. 2 mL fluorescence activated cell sorting (FACS)-buffer (PBS pH 7.4+2% FCS) was added to each tube followed by centrifugation at 1400 g for 10 min. The supernatant in each tube was discarded and the pellet resuspended in 50 µL FACS-buffer containing 1% Fc-block (anti-mouse FcRγII/III (0.5 mg/mL)). Antibodies (FcεRI-PE and c-kit-PE-Cy7) were added to the four test tubes in an amount of 0.2 µg. For compensation controls the BD-compbead system (BD bioscience) was used. Briefly, 100 µL FACS-buffer was added to two tubes (compensation tubes) along with one drop of binding beads (anti-rat and anti-hamster Ig κ) and 1 drop of non-binding beads. 1 µL of c-kit-PE-Cy7 was added to one of the compensation tubes and 1 µL of CD4-PE was added to the other. After 30 min of incubation, the cells and beads were washed twice with 2-3 mL FACS-buffer and centrifuged at 1400g for 10 min. The test tubes were washed twice in the same manner but with 2 mL FACS-buffer instead. After the washing the cells and beads were resuspended with 300µL FACS-buffer. The tubes were analyzed using a BD LSR II multi-laser analytical flow cytometer with lasers emitting 355, 405, 561 and 633 nm light respectively. The software FlowJo was used for analysis.

## Mast Cell Activation

30 million BMMCs were sensitized with 2µg/ml immunoglobulin E (IgE)-anti-trinitrophenyl (TNP) purified in house from a mouse hybridoma (IgELb4) at a concentration of 1 million cells/ml complete medium supplemented with SCF and IL-3 as earlier described for 24 h in 37°C and 5% $CO_2$. Cells were counted and added to 24-well trays in a concentration of 1.4 million cells/ml. IgE-crosslinking was performed by addition of the antigen ovalbumin (OVA)-TNP [100 ng/mL] diluted in 1 mL supplemented complete media per well. As a negative control, cells were incubated with equal volume of vehicle (media without antigen). The IgE-crosslinking step was allowed to continue for 24 h. The samples were centrifuged at 500 g for 5 min at +4°C. The supernatants were collected and stored at -80°C until analysis.

## Enzyme Linked Immunosorbent Assay (ELISA)

Sandwich ELISA was performed for detection of IL-6. A kit for IL-6 detection from eBioscience was used and the experiment was executed according to the manufacturer's instructions. Briefly, 96 well ELISA plates (Costar 9018) with high affinity protein binding were coated with the capture antibody anti-mouse IL-6 and incubated overnight at +4°C in a moisture chamber. After washing the plates with washing solution (0.05% tween X-100 in PBS) (Sigma Aldrich) all unbound surface was blocked with a blocking solution (PBS supplemented with fetal bovine serum) for 1 hour. Samples and standards were added in the wells in duplicates and incubated for 2 h in room temperature. The plates were washed five times with washing solution to ascertain the removal of unbound samples. The detection antibody, anti-mouse IL-6 biotin was added to each well for binding to IL-6. To remove excess antibody, five more washes with washing solution were done. Horseradish Peroxidase (HRP) enzyme coupled to Avidin was added to all wells and incubated for 30 minutes. After seven washes with washing solution the substrate (1X 3,3',5,5'- tetramethylbenzidine solution) was added. After 15 min incubation the plates were spectophotometrically analyzed using a plate reader (Versamax microplate reader, Molecular Devices) at 450 nm. The amount of IL-6 present was calculated using the standard curve produced (not shown).

## Statistical Analysis

The software GraphPad Prism (ver. 6.04, GraphPad Software, Inc.) was used for making graphical representations and statistical analysis. To test for statistical significance an unpaired Student's t-test was used and $p<0.05$ was considered significant.

# Results

## Mast Cell Development and Maturation

In order to monitor the role of pX on mast cell development and maturation bone marrow cultures were supplemented with SCF and IL-3 to support the growth of BMMCs. Samples of the cultures were taken starting day 6 and then sampled 5 times a week until day 46 in vitro. Cytospins from the samples were stained with May-Grünwald/Giemsa and scored for degree of maturation. The intention was to compare mast cell development and maturation in WT BMMCs to those lacking pX. However, duing the course of this project we found there had been an error in genotyping which caused that the comparison in this diploma work is heterozygous (Het) BMMCs versus BMMCs lacking pX. The results were clustered together to form six different age groups where the percentage of fully matured mast cells were increased from null day 6-9 in vitro until around 90% day 40-46 in both cultures (Figure 1). To visualize the granulation development in BMMCs in vitro representative pictures were taken from each age group, from both Het and knock out (KO) cultures (Figure 2). The stepwise development of granules is visualized well. In day 6-9, no granules are seen, but in later steps of development, accumulation of granules are seen as colourful spots in the cytoplasma. Empty granules which does not contain any mediators are is seen as white spots (day 18-39). In the last age group, almost all mast cells are packed with granules (day 40-46).

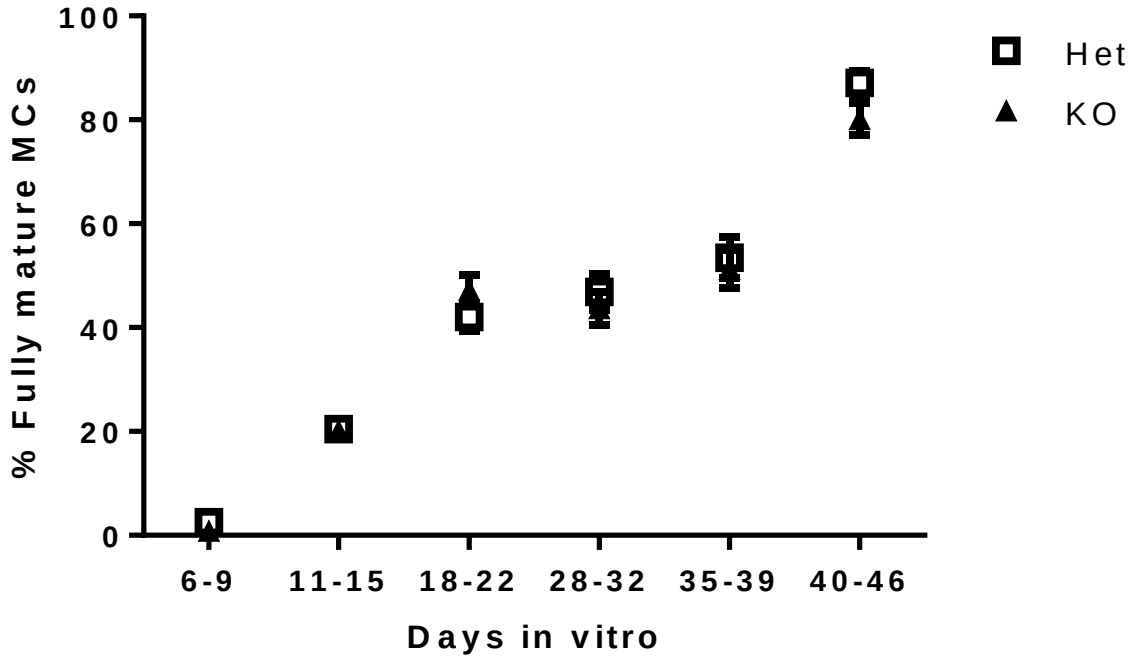


**Figure 1.** Scoring for the percentage of fully maturated BMMCs cytospin stained with May-Grünwald/Giemsa stain. Samples from Het BMMCs and BMMCs obtained from knockout mice of pX (KO) were divided into six different groups; 6-9, 11-15, 18-22, 28-32, 35-39 and 40-46 days in vitro respectively. 8-16 samples are included into every point illustrated.

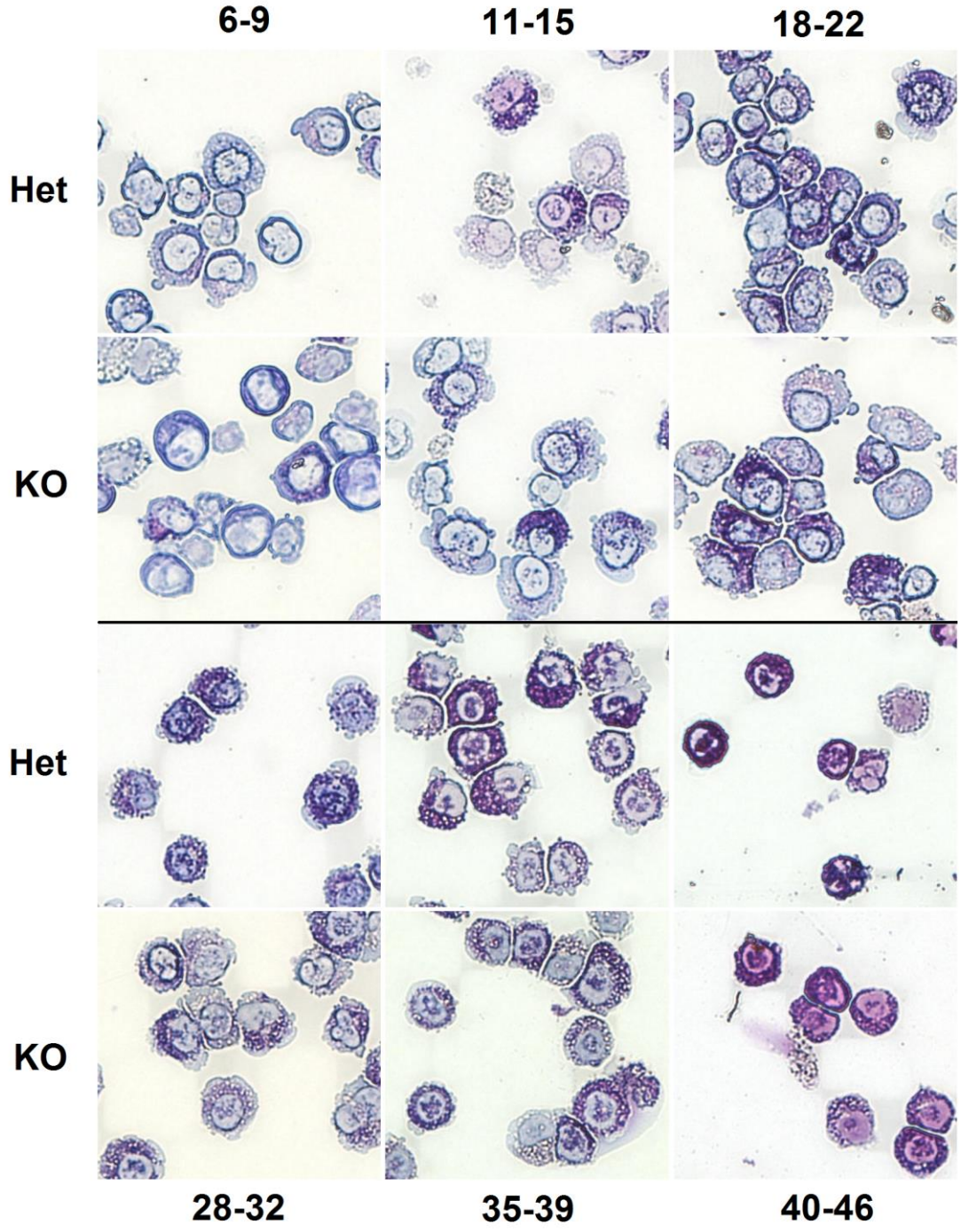


**Figure 2.** Het BMMCs and BMMCs lacking pX (KO) were grown in complete medium supplemented with IL-3 and stem cell factor and stained with May-Grünwald/Giemsa staining. Six different maturation groups were constructed; 6-9, 11-15, 18-22, 28-32, 35-39 and 40-46 days *in vitro* respectively. Representative pictures for the phenotypic appearance of developing mast cells**.**

## Flow Cytometry

The maturation of BMMCs was confirmed by flow cytometry for c-kit and FcεRI after three weeks *in vitro*. C-kit$^{+}$ FcεRI$^{+}$ cells were considered mast cells. All BMMC cultures demonstrated a high degree (> 90%) of mature mast cells. Representative graphs are shown in (Figure 3).

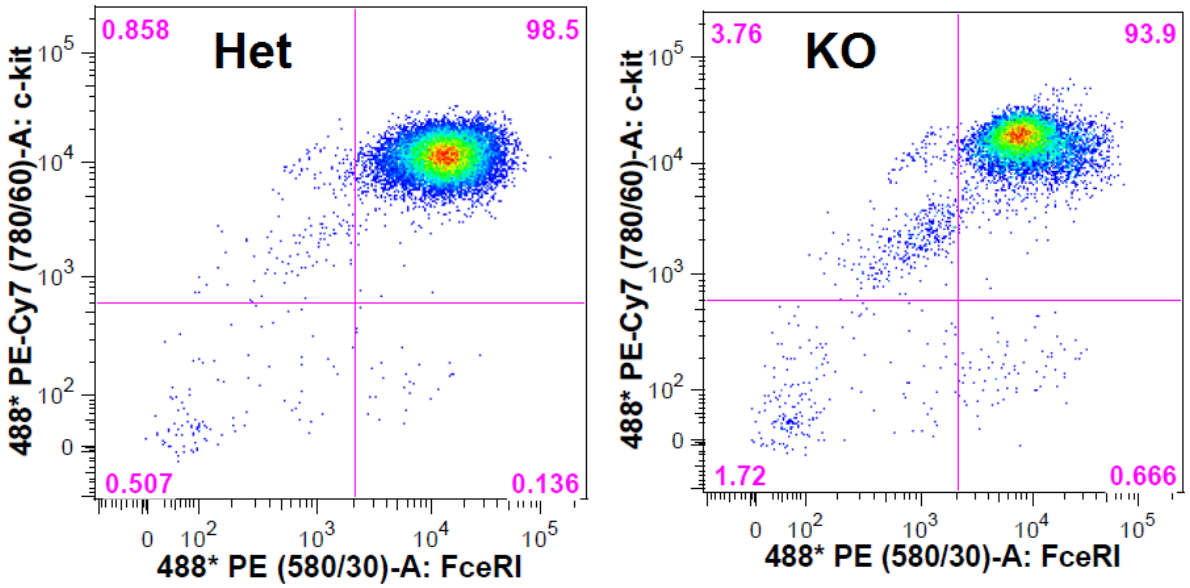


**Figure 3. A high degree of mast cells are present after 27 days old cell culture.** Het and KO of pX BMMCs were grown 27 days in vitro with IL-3 and SCF stained with PE-anti-FcεR1 (x-axis) and PE-Cy7-anti-c-kit (y-axis) and analyzed by flow cytometry.The pink numbers show the percentage of cells present in each quadrant.

## IL-6 release after IgE crosslinking

IL-6 is a crucial protein and cytokine that has an important role in inflammation processes both locally and systemically. Mast cells have been shown to be important producers of IL-6 (Bradding *et al.* 1993). To test whether BMMCs deficient in pX would have an excretion of IL-6, MC activation by IgE-crosslinking was performed.. BMMCs were sensitized for 24 h using IgE-anti-TNP [2 µg/million cells]. Previous studies suggest activation for 20 h or more is needed for optimal results, therefore the activation time was set to 24 h. For IgE-crosslinking, OVA-TNP was used with a concentration of 100 nM, shown to have the most effective result (Boudreau *et al.* 2004). Genotyping of the mice used, revealed presumed WT individuals to be Het, leading to the experimental setup of BMMCs either lacking pX completely or being Het for it. After confirmation of maturity by FCM (Figure 3),BMMCs were activated for 24 h and supernatant was collected for analysis by ELISA.. IgE-crosslinking of Het and KO BMMCs stimulated with ~200 pg/ mL IL-6 (Figure 4). The experiments was performed twice with similar results. There was no trend towards a difference in IL-6 production upon IgE-crosslinking. No significant difference could be proven between the two tested groups (p=0.87).

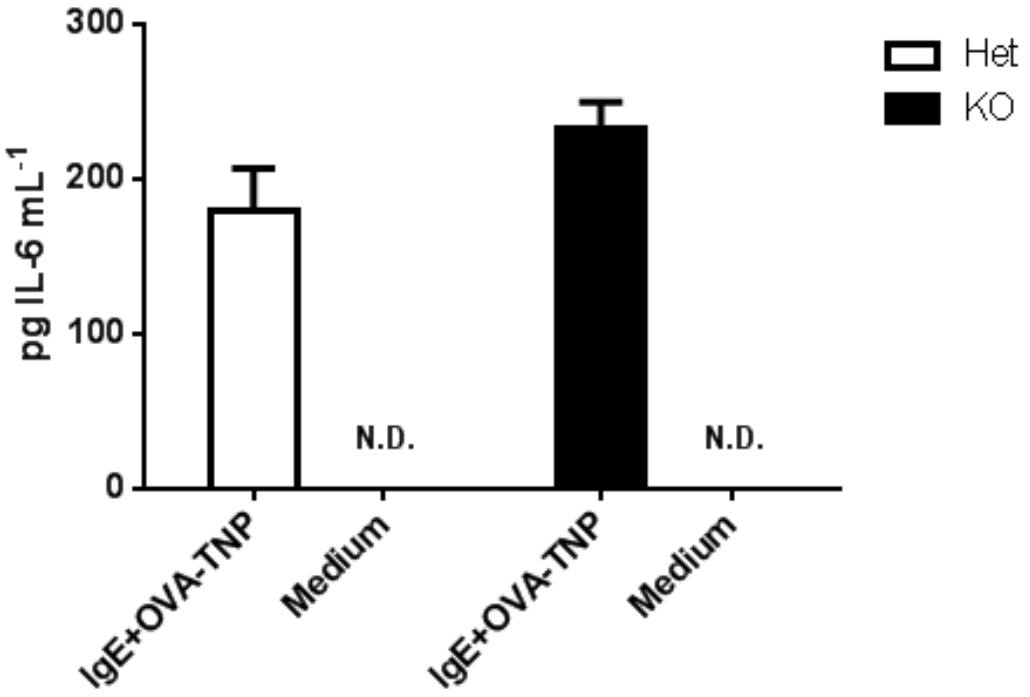


**Figure 4.** Results from ELISA for IL-6 performed after 24 h sensitization with α-TNP IgE, followed by IgE-crosslinking with OVA-TNP for another 24 h. The amount of cells were estimated to 1 million/mL. Medium was used as a negative control where no detection of IL-6 could be seen (N.D.). No significant difference could be seen between the two groups (p=0.87).

## Discussion

In this study we aimed to elucidate if maturation and development of BMMCs from MCp was affected by the absence of pX. We also aimed to nivestigate whether *de novo* production and secretion of IL-6 were affected by pX. Unfortunately, the genotyping of the mice was found to be incorrect after 42 days of culturing the BMMCs. Thus, we had been comparing the development of Het BMMCs to BMMCs lacking pX instead. With this knowledge we reformulated the hypothesis to the Het genotype.

In monitoring of the development and maturation processes of BMMCs a May-Grünwald/Giemsa staining approach was used. This was very useful for scoring the cells for granules, since the Giemsa stain has specificity for negative charged cell compartments, granules included. However, one problem with this approach was the unevenness in the different batches stained. Differences arose despite the fact that similar conditions were used for all samples taken. This problem made it difficult to quantify the data to reasonable results. Still, the result from this study can be used as a pilot, though this type of studies generally use

more specific methods to measure development, than May Grünwald/Giemsa staining. In other studies, saturation of IgE bound to FcεRI measurement or development of other specific receptors such as c-kit is used instead as markers for maturation, and May-Grünwald/Giemsa is more used as a general monitoring tool for visualization (Galli *et al.* 1982, Rodewald *et al.* 1996). With the method used in this study, we could conclude that no differences in granule development could be seen between the two groups indicating that pX does not affect the maturation process of BMMCs *i.e.* the granulation formation, at least not in comparison to Het BMMCs However, the genotyping error gave an opportunity to study the BMMCs Het for pX, which never would have happened otherwise since most studies concerning KO models, only include WT and KO individuals. This pilot study suggests that the maturation phenotype of the Het BMMCs is similar to KO BMMCs. On the other hand, the development of both Het and KO BMMCs proceeded as expected with around 90% being fully granulated after 46 days, which could indicate that the development of mast cells is normal in Het and KO BMMCs. If the development of BMMCs lacking pX actually is different from WT, it could only be clarified by additional studies including WT BMMCs.

Since pX belongs to the SLC10 family (bile acid transporters), this implies pX are involved in transporting a ligand over a membrane. This could theoretically suggest that pX are involved in an important mast cell function. The most well-characterized mast cell function is the IgE-mediated activation of mast cells leading to mast cell degranulation and new synthesis and release of cytokines including IL-6. However, there are several mechanisms left to look into to see if pX deficiency affects the function. Currently, studies are being done on these pX-deficient BMMCs to elucidate if the protein has a function in release of granules. Here, IL-6 release after IgE-mediated crosslinking was investigated in Het BMMC and KO BMMC activated in parallel. IL-6 release assayed 24h after IgE-crosslinking showed no difference between the different groups. However, the levels were quite variable and found in an interval of 20-250 pg/mL. The reason for this high variation is unknown, although one explanation could be that the BMMCs used were from mice from a mixed genetic background. Previous studies done on IL-6 production and secretion of BMMCs show results of relatively high secretion of IL-6 in WT BMMCs in magnitudes of thousands of pg/mL compared to hundreds acquired in this study (Boudreau *et al.* 2004, Kalesnikoff *et al.* 2001). One possible explanation for this difference could be the use of C57BL/6 mice with unmixed genetic background, used in one of the studies (Kalesnikoff *et al.*2001). However, another study done by Selander *et al.* 2009 showed similar levels of IL-6 production in WT BMMCs activated by IgE-crosslinking. They also used the same clone for IgE, which makes their results even more applicable. To conclude, the results from this experiment seem to be comparable with previous results produced by Selander *et al.* 2009 which indicates that IL-6 production in pX deficient BMMCs are normal.

In conclusion, it cannot be stressed enough that comparison of data from Het and KO groups with data from WT BMMCs is necessary in order to achieve demonstrate a possible phenotypic consequences on mast cell development arising from lacking this protein. Also the measurement of *de novo* production of IL-6 after IgE-mediated mast cell activation needs to be repeated with WT controls to confirm the results obtained in this study.

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