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DISSERTATION

# Chymase-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of mucosal mast cells

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## Abbreviations and important definitions

Abbreviations and explanations of relevant terms are provided below (Table 1).

| | |
|---|---|
| **Bas** | basophils |
| **BM** | bone marrow |
| **BMCMCs** | Bone marrow-derived cultured mast cells |
| **BMCP** | bifunctional basophil-MC lineage progenitor |
| **CD** | Cluster of differentiation |
| **cDNA** | Complementary DNA |
| **c-Kit** | Tyrosine-protein kinase Kit (CD117) |
| **CMC** | cutaneous mast cell |
| **CMP** | common myeloid progenitor |
| **$CO_2$** | Carbon dioxide |
| **Cpa** | Carboxypeptidase |
| **Cre** | Cre recombinase |
| **CTMC** | Connective tissue type mast cells |
| **ddH2O** | Double-distilled water |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **ds** | Double-stranded |
| **DT** | Diphtheria toxin |
| **DTA** | Diphtheria toxin alpha chain |
| **DTR** | Diphtheria toxin receptor |
| **FA** | Food allergy |
| **FACS** | Fluorescence-activated cell sorting |
| **FCS** | Fetal calf serum |
| **Gd** | Gestation day |
| **GMC** | Granulocyte macrophage progenitors |
| **HCl** | Hydrochloric acid |
| **HSC** | Hematopoietic stem cell |
| **IE** | Intronic enhancer |

| | |
|---|---|
| **IgE** | Immunoglobulin E |
| **IL** | Interleukin |
| **MC** | Mast cell |
| **Mcl-1** | Myeloid leukemia cell differentiation protein |
| **$MC_P$** | Mast cell progenitor |
| **MCP** | Mast cell protease |
| **Min** | Minutes |
| **MMC** | Mucosal mast cells |
| **mMCP** | Mouse mast cell protease |
| **NaCl** | Sodium chloride |
| **ns** | Not significant |
| **P/S** | Penicillin/Streptomycin |
| **PBS** | Phosphate-buffered saline |
| **PCR** | Polymerase chain reaction |
| **PE** | R-phycoerythrin |
| **RNA** | Ribonucleic acid |
| **SAs** | Spiral arteries |
| **SCF** | Stem cell factor |
| **SEM** | Standard error of the mean |
| **TGF** | Transforming growth factor |
| **TNF** | Tumor necrosis factor |
| **Treg** | Regulatory T cell |
| **uMC** | Uterine MC |
| **uNK** | Natural killer cell |
| **WT** | Wild type |

**Table 1:. List of abbreviations and relevant terms.**

# Abstract

Mast cells (MCs) are potent inflammatory cells that are found predominantly at the interface between the host and the external environment. Classically, MCs are considered to be key effector cells in the elicitation of allergic symptoms. However, increasing number of studies indicates that MCs are essential players in innate and adaptive immune responses as well.

In mice, two main types of MCs have been described: connective tissue MCs (CTMCs) and mucosal MCs (MMCs). MMCs, under physiological conditions, are found in relatively low numbers in most mucosal tissues, but the expansion of MMC populations can be induced in a T cell-dependent manner, as well as in allergic responses. However, knowledge about the biological functions of MMCs is limited due to the lack of suitable models to investigate MMCs *in vivo*. We, therefore, have generated a new mouse model that exhibits a specific reduction in MMCs, thus allowing for the investigation of MMCs *in vivo*. It has been previously reported that Cre expression driven by a chymase promotor correlates to mature resident MMCs. We mated chymase-Cre transgenic mice with mice bearing a floxed allele of the myeloid cell leukemia sequence 1 (Mcl-1), which encodes for an intracellular antiapoptotic factor in MCs. When comparing Chm-Cre; Mcl-$1^{fl/fl}$ and wild type mice, we found similar proliferation and differentiation rates in bone morrow-derived cultured MCs (BMCMCs) as well as equal numbers of CTMCs such as peritoneal or skin MCs in histological and flow cytometric analyses. In contrast, we found markedly reduced MMCs numbers in the stomach, duodenum as well as in the uterus in Chm-Cre; Mcl-$1^{fl/fl}$ mice.

Taken together, our results show that this new mouse model presents with markedly reduced numbers of MMCs in tissues, i.e. stomach, duodenum and uterus. Therefore, the Chm-Cre; Mcl-$1^{fl/fl}$ model could become a useful tool for the investigation of the pathophysiological functions of MMCs *in vivo*.

# Zusammenfassung

Mastzellen (MZs) sind potente Entzündungszellen, die vorwiegend an der Schnittstelle zwischen dem Wirt und der äußeren Umgebung vorkommen. Klassischerweise werden MZs als wichtige Effektorzellen bei der Auslösung allergischer Symptome angesehen. Eine zunehmende Anzahl von Studien zeigte jedoch, dass MZs auch bei angeborenen und adaptiven Immunantworten eine wichtige Rolle spielen.

Bei Mäusen wurden zwei Haupttypen von MZs beschrieben: Bindegewebs-MZs (CTMZs) und Schleimhaut-MZs (MMZs). MMZs werden unter physiologischen Bedingungen in den meisten mukosalen Geweben in relativ geringen Mengen gefunden, aber die Expansion von MMZ-Populationen kann sowohl T-Zell-abhängig als auch allergisch induziert werden. Das Wissen über die biologischen Funktionen von MMZs ist jedoch begrenzt, da geeignete Modelle zur Untersuchung von MMZs *in vivo* fehlen. Wir haben daher ein neues Mausmodell entwickelt, das eine spezifische Reduktion von MMZs aufweist und somit die Untersuchung von MMZs *in vivo* ermöglicht. Es wurde zuvor berichtet, dass Cre-Expression, die durch einen Chymase-Promotor gesteuert wird, mit reifen residenten MMZs korreliert. Wir kreuzten Chymase-Cre-transgene Mäuse mit Mäuse, die ein floxiertes Allel der myeloischen Zellleukämie-Sequenz 1 (Mcl-1) tragen, welches für einen intrazellulären antiapoptotischen Faktor in MZs kodiert. Beim Vergleich von Chm-Cre;Mcl-$1^{fl/fl}$ und Wildtyp-Mäusen fanden wir ähnliche Proliferations- und Differenzierungsraten in aus Knochenmark kultivierten MZs (BMCMCs) sowie eine gleiche Anzahl von CTMZs wie Peritoneal- oder Haut-MZs in histologischen und durchflusszytometrischen Analysen. Im Gegensatz dazu fanden wir eine deutlich verminderte Anzahl von MMZs im Magen, Zwölffingerdarm sowie im Uterus von Chm-Cre; Mcl-$1^{fl/fl}$ Mäusen.

Zusammenfassend zeigen unsere Ergebnisse, dass dieses neue Mausmodell eine deutlich verringerte Anzahl von MMZs in Geweben, d. h. Magen, Zwölffingerdarm und Uterus, aufweist. Daher könnte das Chm-Cre; Mcl-$1^{fl/fl}$ Modell ein nützliches Instrument für die Untersuchung der pathophysiologischen Funktionen von MMZs *in vivo* werden.

# 1 Introduction

## 1.1 Biology of mast cells

### 1.1.1 General introduction

Although mast cells (MCs) were identified as granular cells in the mesentery of the frog by Dr von Recklinghausen in 1863 (Recklinghausen, 1863), the first description and denomination of these cells were attributed to Ehrlich in 1878 (Ehrlich, 1878). Since then, MCs have come into our view and have become a source of fascination to innumerable researchers.

For the first decades after von Recklinghausen's report, early studies on MCs were focusing on morphological features, distribution, and abundance in health and disease, which were mostly only the concern of histologists and pathologists. Whereas von Recklinghausen reported his original observations on unstained specimens from frog mesenteries in which he described cells with granules preferentially located along the blood vessels, Ehrlich made the important discovery that mast cells stained well and specifically with certain triphenylmethane and thiazine dyes of the aniline family. The functional implications of Ehrlich's initial view of mast cells as metachromatic, granulated cells implicated in the nutrition of the surrounding tissue evolved gradually.

In 1937, Holmgren and Willander (Holmgren, 1937) first observed tissues that displayed a great number of MCs were enriched in heparin, established the presence of heparin in MCs granules. Subsequently, researchers discovered the presence of histamine in MCs (Riley and West, 1952), and revealed during anaphylactic shock, histamine released along with heparin in MCs (Barger and Dale, 1910; Rocha et al., 1947). These studies established the relationship between MCs and allergic reactions, which has been long term viewed as the most important function of MCs. Then, with the discovery of slow-reacting substance of anaphylaxis (now leukotrienes) (Feldberg and Kellaway, 1938), and IgE (Ishizaka et al., 1966), the role that MCs play in allergic reactions, especially in type I hypersensitivity reactions, became clearer. These reactions, best

known as IgE-mediated allergic reactions, are induced when multivalent antigens crosslink antigen-specific IgE bound to high-affinity IgE receptors (FcεRI) on the mast cell surface. Thereby aggregating FcεRI promotes the immediate release of mast cell mediators and the successive adverse events most commonly associated with allergy, i.e., increased vascular permeability, smooth muscle contraction, and mucus secretion (Kinet, 1999; Metzger, 1992; Siraganian, 2003).

Today, MCs are considered as key effector cells in the elicitation of allergic symptoms and widely known as "allergy cells" (Grimbaldeston et al., 2006; Hofmann and Abraham, 2009; Rivera and Gilfillan, 2006). However, after more than 150 years of intensive research, an increasing number of studies indicate that MCs are more than just the immediate IgE-mediated reaction in the context of an allergy, but also essential players in various processes. These processes including innate and adaptive immune responses (Abraham and St John, 2010; Brown, 2011; Siebenhaar et al., 2007; St John and Abraham, 2013), wound healing (Noli and Miolo, 2001), homoeostasis of hair growth and bone remodeling (Maurer et al., 1995; Maurer et al., 2003; Silberstein et al., 1991), pregnancy (Meyer et al., 2017a; Meyer et al., 2017b; Woidacki et al., 2015; Woidacki et al., 2014a; Woidacki et al., 2014b), inflammatory processes, angiogenesis (Weller et al., 2011), as well as brain edema in ischemic processes (Strbian et al., 2006) and degradation of snake venoms (Metz et al., 2006).

### 1.1.2 Characteristics of mast cells

MCs are characterized by their high content of electron-dense secretory granules, which contain large amounts of preformed and pre-activated immunomodulatory compounds (Fig.1). These include inflammatory mediators (such as histamine), proteoglycans (such as heparin), as well as enzymes, chemokines, various polypeptides, phospholipid mediators and a range of cytokines. Besides granules, MCs also can be identified by the coexistence of membrane-bound Fc epsilon receptors (FcεRI) and c-Kit receptors. (Beaven and Metzger, 1993). FcεRI is a high-affinity immunoglobulin E (IgE) receptor, which is necessary for the IgE-mediated allergic reactions. Cross-linking of FcεRI expressed on MCs causes the release of various inflammatory mediators, which trigger allergic reactions (Dembo et al., 1979). The c-Kit receptor is the receptor for its ligand stem cell factor (SCF), an essential growth factor for MCs.

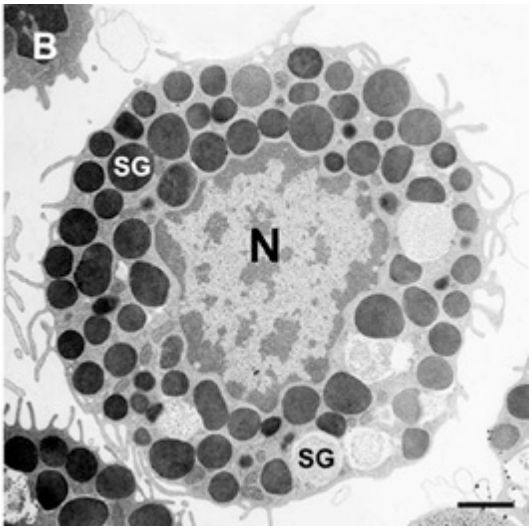


**Figure 1: Mature peritoneal mast cell with electron- dense secretory granules.** N, nucleus; SG, secretory granule. Transmission electron microscopy. (da Silva et al., 2014)

### 1.1.3 Origin and development of mast cells

Although mammalian MCs were first described more than 150 years ago, their origin remained controversial for several decades. The origin of MCs was first considered as undifferentiated mesenchymal cells (Combs 1966), then lymphocytes, multipotent progenitors, myeloid cells and basophils (Bas) have also been suggested as MC precursors (da Silva et al., 2014).

The hematopoietic origin of MCs is derived from the bone marrow (BM). Kitamura et al. found (Kitamura et al., 1977)that tissue MCs can be derived from grafted bone marrow cells in irradiated mice, suggesting that MCs were derived from bone marrow precursor cells. A further study supports this opinion by observing that MC deficient $Kit^{W}/Kit^{W\text{-}v}$ mice could be reconstituted by bone marrow from wild type mice (Kitamura et al., 1978). Furthermore, by using *in vitro* colony forming assays and *in vivo* bone marrow transplantation, it has been shown that cells with MC-generating activity are present in the bone marrow and certain peripheral tissues (Kitamura, 1989; Nabel et al., 1981). These and other evidences indicating that, unlike other myeloid-derived cells which differentiate and mature in the bone marrow before being released to the bloodstream,

MCs leave the bone marrow and circulate as immature progenitor cells, perform transendothelial migration and finally complete their development in peripheral tissues (Gurish and Austen, 2012; Hallgren and Gurish, 2007; Kitamura et al., 1985).

It is suggested that the myeloid origin of MCs is derived from hematopoietic stem cells (HSC), which give rise to common myeloid progenitors (CMP) and then develop into granulocyte macrophage progenitors (GMC). In C57BL/6 mice, it appears that either CMPs or GMPs can directly differentiate into mast cell progenitors (MCps) and basophils (Bas) (Chen et al., 2005; Rodewald et al., 1996). In BALB/c mice, both $Kit^{+}Fc\varepsilon RI^{+}$ MC progenitors (MCps) and Kit-FcεRI+ basophils (Bas) are found in the bone marrow (BM). In both strains, CMPs enter the circulation, before their transendothelial migration into peripheral tissues where they can reside and complete their maturation. Besides, a distinct progenitor cell type called bifunctional basophil-MC lineage progenitor (BMCP) has been identified in the spleen. These cells give rise to either basophils (express high levels of FcεRI and no Kit) or MCs (expressed FcεRI and Kit) (Arinobu et al., 2005; Ohmori et al., 2009) (Fig. 2).

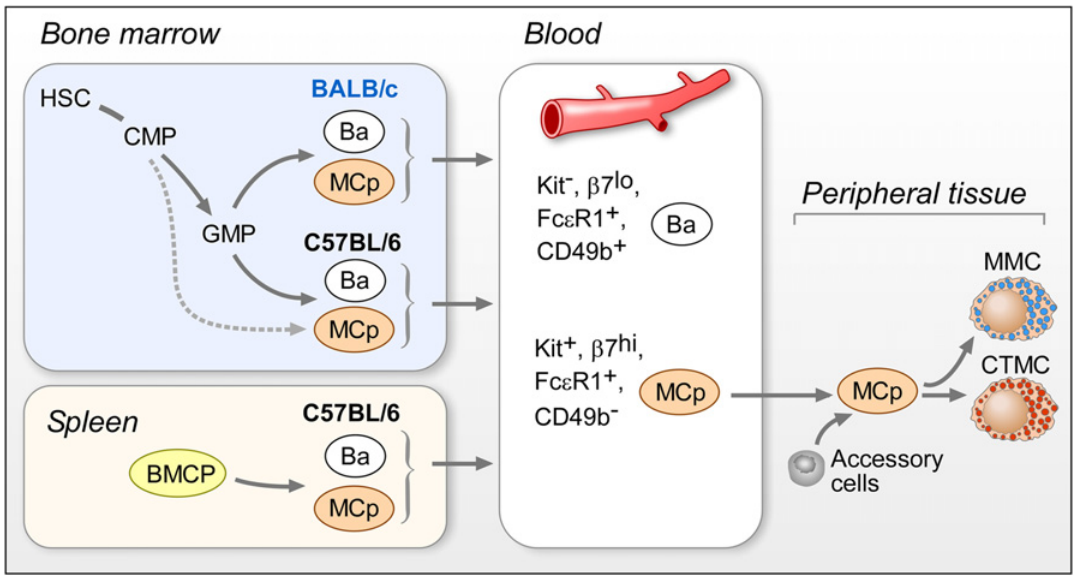


**Figure 2: Mast Cell Lineage Development** (Gurish and Austen, 2012)

MCs have a widespread distribution and are found predominantly at the interface between the host and the external environment (Galli et al., 2005a; Metcalfe et al., 1997). It is increasingly evident that MC maturation, phenotype, and function are a direct consequence of the local microenvironment (Galli et al., 2011). The microenvironment

(such as growth factors and cytokines) can influence the development and maturation of MCs and ultimately contribute to the MC phenotype (Jamur and Oliver, 2011). Different subsets of mature MCs have been described based on their location and functional, structural, and biochemical characteristics.

### 1.1.4 Heterogeneity of mast cells

#### 1.1.4.1 General introduction

In 1878, Paul Ehrlich discovered the classical MC, the connective mast cell (CTMC), in human tissues, by staining with aniline dyes (Ehrlich, 1878). Whereas CTMCs could be fixed by all fixatives tested and stained with either of the cationic dyes, alcian blue or safranin, the mucosal type mast cell (MMC) could be detected only with Carnoy's or a weak formaldehyde and acetic acid fixation and alcian blue staining. Almost a century later, Enerback showed CTMC to be one of two major classes of MC in helminth-infected rats (Enerback, 1966a; Enerback, 1966b).

Unlike most myeloid cells which complete their differentiation within hematopoietic tissues, MCps leave hematopoietic tissues, migrate in peripheral blood, invade connective or mucosal tissue, and then proliferate and differentiate into morphologically identifiable mast cells. It's the microenvironment that contributes to and ultimately leads to their mature phenotype (Jamur and Oliver, 2011).

In mice, MCps differentiate into two major subclasses of mature tissue MCs , which are classified mainly according to their anatomical distributions: a classical constitutive subclass composed of CTMCs, often located around venules and nerve endings, and a subclass composed of MMCs, intraepithelial in gastrointestinal and respiratory mucosa (Enerback, 1966b). There is considerable evidence that these two subclasses are also distinguished from each other according to morphology, protease expression profiles, biochemical and functional properties (Galli et al., 2005a; Galli et al., 2005b; Gurish and Austen, 2012; Moon et al., 2010) (for detailed information see Table 2).

CTMCs contain high concentrations of histamine in their granules and mainly contribute to allergic symptoms, such as congestion, itch, urticaria, whereas MMCs contain low

concentrations of histamine (Metcalfe et al., 1997). Increasing evidence reveals the different roles played by CTMCs and MMCs. In food allergy, MMCs are localized in strategic position to enhance a rapid expulsion, the CTMC stabilizers (e.g. tranilast, ketotifen, and cromolyn, which are frequently used for the treatment of various allergic disorders), fail to exert therapeutic effects on food allergy (FA) (Miller et al., 1988). Pronounced hyperplasia, differentiation, and activation of MMCs occur in the mouse gut during nematode Infections (Knight et al., 2000; Miller et al., 1988), and the ablation of an MMC-derived effector molecule compromises the expulsion process (Hofmann and Abraham, 2009).

One of the critical functional distinctions of these two subtypes of MCs is that MMCs expand remarkably during T cell-dependent immune responses (Guy-Grand et al., 1984; Mayrhofer and Fisher, 1979; Ruitenberg and Elgersma, 1976), while CTMCs exhibit little or no T-cell dependence and occur in athymic nude mice or rats in numbers similar to those present in normal animals (Aldenborg and Enerback, 1985). There is evidence that CTMCs represent a constitutive subtype which can persist in a relatively stable population regardless of environmental circumstances (Fukuzumi et al., 1990; Kitamura et al., 1977), while MMCs as a physiologically relatively small population can be induced and accumulate in tissues under certain conditions, such as parasitic infections (Hepworth et al., 2012; McDermott et al., 2003; Ruitenberg and Elgersma, 1976) or food allergy (Brandt et al., 2003). The accumulation of MMCs is dependent on the presence of type 2 cytokines such as interleukin 4 (IL-4), IL-9, IL-10, and IL-13. It is also evident that MMCs can amplify a protective immune response as well as contributing to intestinal pathology.

### 1.1.4.2 Mucosal type mast cells (MMCs)

MMCs, which are located in mucosal tissues such as bronchial or intestinal tissues, contribute to the first line immune response against pathogens. For example, intestinal parasitic infection is usually associated with the expansion and accumulation of MMCs. Although their precise role for the mechanism of parasite expulsion has not been completely defined, there is no doubt that MMCs are key immune mediators, and are associated with a type 2 mucosal immune response. It has been reported that, under Trichinella spiralis infection, MMCs are essential for the resolution of infection. The MC-deficient $Kit^{W}/Kit^{W\text{-}v}$ mice presented destructive intestinal inflammatory response (Alizadeh and Murrell, 1984), however, when reconstituted with MCs, the parasite could be expelled (Oku et al., 1984). Similarly, the administration of IL-3 to Strongyloides ratti-infected mice increases the number of MMCs and accelerates worm expulsion, indicating that IL-3-induced intestinal protection against Strongyloides ratti is mediated by MMCs (Abe et al., 1993). Furthermore, during parasite infection, MC progenitors (MCps) migrate from the submucosa through the lamina propria into the intestinal epithelium where they begin to express mMCP-1, an MMC-specific protease, disrupt epithelial barrier function and allow the influx of solutes and water into the lumen, potentially contributing to parasite expulsion. The type 2 cytokine IL-4 is able to induce this process and induce differentiation, proliferation, and migration of MCs at mucosal sites.

MMCs, under physiological conditions, are found at relatively low numbers in most mucosal tissues. The expansion of MMC is dependent on the presence of type 2 cytokines such as interleukin 4 (IL-4), IL-9, IL-10, and IL-13, and therefore on the CD4+ adaptive T cell response. It has been reported that IL-4-deficient mice infected with parasites, such as *H. polygyrus*, *Strongyloides ratti, N. brasiliensis* and *T. spiralis*, exhibit reduced mucosal accumulation of MCs compared to wild type mice. Similarly, IL-9-deficient mice are unable to establish MC response to *N. brasiliensis* (Townsend et al., 2000). IL-10 is also important for the accumulation of MCs in response to *T. spiralis* (Helmby and Grencis, 2003). Thus, MMCs play a crucial role in T cell-mediated immune responses as well as in allergic reactions. However, knowledge of the biology of MMCs has not yet been fully elucidated and is hampered by the scarcity of these cells in tissues and the lack of proper *in vivo* models.

| | Mucosal mast cell (MMC) | Connective tissue mast cell (CTMC) |
|---|---|---|
| Anatomical distribution (at baseline) | Glandular stomach mucosa (many at baseline), small intestinal and colonic mucosae (very few at baseline), and respiratory mucosa | Skin, serosal cavities, tongue, submucosa, and muscularis of the stomach, trachea & around large airways1 |
| Proteoglycan content | Little or no heparin; Chondroitin sulfate di-B, A, E | Heparin; Chondroitin sulfate E |
| Protease content | Express predominantly the chymases MCPT-1 and 2 | Express predominantly the chymase MCPT-4, the elastase MCPT-5, the tryptases MCPT-6 and 7 and Carboxypeptidase A |
| Bioactive amine content | Low levels of histamine; Serotonin | High levels of histamine; Serotonin |
| Response to cationic compounds | Little or none | Yes |
| Adenosine receptors | A2a; A2b; A3 | A3 |
| Ig receptors | FcεRI; FcγRIIb; FcγRIII | FcεRI; FcγRIIb; FcγRIII |

**Table 2: Characteristics of murine mucosa and connective tissue mast cells** (Reber et al., 2015)

## 1.2 Murine models for investigating mast cells biology

### 1.2.1 General introduction

In order to investigate the biological function of MCs, many approaches have been used have been applied. The use of mast cell lines has greatly facilitated the characterization of various aspects of mast cell function. However, as transformed cells, they present limitations and the results obtained through their use must be interpreted cautiously when extrapolating to mast cell functions *in vivo*. What's more, no agent that can solely and specifically suppress MCs activation has been discovered. There are also findings indicating that even the so-called "MC stabilizer" cromolyn is neither an effective nor selective inhibitor of mouse MCs activation *in vitro* or *in vivo* (Oka et al., 2012). For these reasons, genetic approaches probably represent a more definitive way to identify and characterize MC functions in mice *in vivo*.

### 1.2.2 *Kit*-dependent mouse models

Until now, several MC-deficient mice models have been generated for analyzing the role of MCs *in vivo*. Mice deficient in KIT, the receptor for the main MCs growth and survival factor- stem cell factor (SCF) to analyze the functions of MCs *in vivo* ($Kit^{W/}Kit^{W-v}$ mice and $Kit^{W-sh}/Kit^{W-sh}$ mice) are widely used by researchers (Table 3).

WBB6F1-$Kit^{W}/Kit^{W-v}$ mouse line is a spontaneous mutation of both c-Kit gene alleles. This MC-deficient mouse model is a milestone in mast cell research. $Kit^{W}$ is a point mutation that produces a truncated KIT that is not expressed on the cell surface (Hayashi et al., 1991); $Kit^{W-v}$ is a mutation in the c-Kit tyrosine kinase domain that substantially reduces the kinase activity of the receptor (Nocka et al., 1990). Due to the mutation of Kit, MC progenitor cells are affected by proliferation and differentiation, thus resulting in MC deficiency (Nakano et al., 1985). C57BL/6-$Kit^{W-sh}/Kit^{W-sh}$ is a mouse line with an inversion mutation that affects the transcriptional regulatory elements upstream of the c-Kit transcription start site on mouse chromosome 5 (Nagle et al., 1995; Nigrovic et al., 2008).

These two mouse lines are commonly used as MC-deficient mouse models for analyzing MC functions *in vivo*. Nevertheless, because the MC-deficiency is based on the mutations affecting c-Kit structure or expression, these mice present with a variety of other phenotypic abnormalities, including alterations of hematopoietic cells unlike MCs that contribute to innate or adaptive immune responses (Galli et al., 2005b; Grimbaldeston et al., 2005; Nakano et al., 1985). For example, WBB6F1-$Kit^{W}/Kit^{W-v}$ mice are anemic, present reduced numbers of neutrophils, basophils, and are sterile. By contrast, C57BL/6-$Kit^{W-sh}/Kit^{W-sh}$ mice are neither anemic nor sterile but have increased numbers of neutrophils (Galli et al., 2015).

| Strain | Systemic abnormalities | Celluar abnormalities | Limitations | Level of deficiency |
|---|---|---|---|---|
| $Kit^{W}/Kit^{W-v}$ | White coat, macrocytic anaemia, sterility | Lack of melanocytes, age dependent changes in intestinal intra-epithelial lymphocyte populations, variable deficiency of interstitial cells of Cajal, impaired T-cell development in the thymus, shift in intra-epithelial T cells in the gut in favor of TCR alpha beta+ and against TCR gama sigma+ cells, neutropenia and 75%-90% reduced basophil numbers, idiopathic dermatitis, stomach papilloma and ulcers | Bystander effects, reduced muscular fitness, weight loss and survival | Depletion > 99% |

| | | | | |
|---|---|---|---|---|
| $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ | White coat, splenomegaly, histological aberrations of the spleen | Neutrophilia megakaryocytosis and thrombocytosis. | Bystander effects, reduced muscular fitness, weight loss and survival | Depletion > 99% |

**Table 3: *Kit*-dependent MC-deficient mouse models** (Siebenhaar et al., 2015)

### 1.2.3 Cre/loxP system

In order to have a certain tissue-specific gene-targeted mouse model, the Cre/loxP recombination system has been widely used. (Kos, 2004; Rajewsky et al., 1996). In this system, two different transgenic mouse lines are required to achieve a tissue-specific gene inactivation. One mouse strain contains a targeted gene flanked by two loxP sites (floxed gene), another transgenic mouse strain expressing the Cre recombinase under the control of a promoter specific targeted at a particular cell or tissue (detailed terms information in table 4). When the mouse strain harboring the floxed targeted gene is crossed with the strain of mice expressing Cre recombinase, the targeted gene is inactivated only in tissues expressing Cre and remains active in all other tissue of the body.

| | |
|---|---|
| Transgenic mouse | A mouse into which cloned genetic material has been transferred |
| Cre recombinase | A 38-kDa enzyme that recognizes specific nucleotide sequences called loxP sites. Cre recombinase catalyzes DNA recombination between two loxP sites, resulting in either deletion of the intervening DNA segment and one loxP site or inversion (flipping) of the intervening DNA segment and two loxP sites. |

| | |
|---|---|
| Promoter | A DNA sequence that is recognized (directly or indirectly) and bound by a DNA dependent RNA polymerase during the initiation of RNA transcription. |
| LoxP site | A 34-base pair nucleotide sequence that contains an 8-base pair core sequence (that confers directionality) in the center of two 13-base pair palindromic repeats. Two loxP sites are brought together by the Cre recombinase. |
| Floxed gene | A gene flanked by loxP sites. |
| Recombination | The exchange of two portions of DNA segments. |

**Table 4: Definition of terms of Cre/loxP system** (Kos, 2004)

### 1.2.4 *Kit*-independent mast cell- deficient mouse models

#### 1.2.4.1 *Kit*-independent constitutive mast cell-deficient mouse models

Because of the potential complexities in interpreting findings based on work employing *Kit* mutant MC-deficient mice, several groups have developed mice that are MC-deficient but which lack abnormalities related to *Kit* structure or expression. By the development of Cre/loxP system, the approach to generate mouse model to have a certain tissue-specific gene targeted, transgenic mice have been generated. In these mouse models, Cre-recombinase were expressed under control of a MC associated promoter, and presented with MC-deficiency. Until now, several *Kit*-independent constitutive MC-deficient mouse models have been reported (Table 5).

For example, Cpa3-Cre; Mcl-$1^{fl/fl}$ mice represent one of the *Kit*-independent MC-deficient models (Lilla et al., 2011), which contain the absence of mutations affecting *Kit* and, thereby, lack *Kit*-related phenotypic alterations. This transgenic mouse stain expresses Cre recombinase under the control of an MC-associated protease carboxypeptidase A3 (Cpa3) promoter. Cpa3-Cre; Mcl-$1^{fl/fl}$ mice exhibited a marked *Kit*-independent constitutive reduction in numbers of MCs (92%-100% depletion).

Another MC-deficient mouse line which does not *Kit* was generated by Dudeck et al. named Mcpt5-Cre; R-DTA mice (Dudeck et al., 2011). This mouse strain is generated by crossing Mcpt5-Cre transgenic mouse with R-DTA$^{fl/fl}$ mouse, so the diphtheria toxin alpha chain (DTA) is produced only in Cre-expressing cells, thereby driving Cre-specific ablation of such cells. Mcpt5-Cre; R-DTA mice displayed a constitutive lack of peritoneal and ear skin MCs as well as >90% reduction of abdominal and back skin MCs in comparison to the Cre$^{-}$ counterparts, indicating that this mouse strain presented with a constitutive deficiency of MCs.

What's more, a mouse model with depletion of MCs mediated by Cre-induced genotoxicity, Cpa3$^{Cre/+}$—"Cre-Master" mice was generated. (Feyerabend et al., 2011). This mouse strain has Cre expression under the control of the Cpa3 promoter. The heterozygous Cpa3$^{Cre/+}$ mouse exhibited a virtually complete lack of MCs under both steady-state and inflammation conditions.

These mouse models provide new possibilities of investigating MCs *in vivo*; however, there are also limitations about affecting other cell types and populations.

| Strain | Systemic abnormalities | Cellular abnormalities | limitations | Level of deficiency |
|---|---|---|---|---|
| Cpa3-Cre; Mcl-1$^{fl/fl}$ | Mild macrocytic anemia | Reductions in basophil numbers in spleen (58%), blood (74%) and bone marrow (75%), splenic neutrophilia | Other Cpa3-expressing cells and depending whether Mcl-1 to survive may be affected, for example basophils | Depletion of 92%-100% |
| Mcpt5-Cre R-DTA | Not reported | Not reported | Mcpt5 expression in | Around 96% in peritoneum |

| | | | | |
|---|---|---|---|---|
| | | | other population in the thymus. Efficiency of DTA to deplete MC in inflammation conditions unclear | and ear skin. 89% in the back and abdominal skin |
| $Cpa3^{cre/+}$ | Not reported | Basophils reduction around 40% | Other Cpa3-expressing cells may be affected, that is, basophils and T cells | Total depletion under steady-state or inflammation conditions |

**Table 5: *Kit*-independent constitutive MC-deficient mouse models** (Siebenhaar et al., 2015)

### 1.2.4.2 *Kit*-independent inducible mast cell-deficient mouse models

In order to improve the models for *in vivo* investigation of MCs biology and study on a particular cell population, inducible ablation has been developed. One common approach for generating this inducible mouse model with specific deficiency of a certain cell population, is to inject the diphtheria toxin (DT) into transgenic mice bearing the DT receptor (DTR) only in that particular cell type (Buch et al., 2005).

With the help of this approach, Dudeck et al. mated Mcpt5-Cre mice with $iDTR^{fl/fl}$ mice, generated Mcpt5-Cre; iDTR mice, which contains Cre-dependent expression of DTR in MCs (Dudeck et al., 2011). After DT inducing, Mcpt5-Cre; iDTR mice showed around 97% MCs depletion in ear skin, and depletion of subepithelial CTMCs in the intestine and stomach.

Another strain named “Mas-TRECK”, is a mouse model in which expression of the human DTR gene is under the control of an intronic enhancer (IE) element, which was essential for IL-4 expression in MCs. After DT treatment, Mas-TRECK showed completely depleted MCs in the skin, peritoneal cavity, stomach and mesenteric windows.

| Strain | Systemic abnormalities | Cellular abnormalities | Limitations | Level of deficiency |
|---|---|---|---|---|
| Mcpt5-Cre iDTR | Small size, Cre instability | No report about basophil numbers in PBMC | Side-effects of DT injection | Around 97% in ear skin |
| Mas-TRECK | Not reported | Basophils are also transiently depleted | Side-effects of DT injection | Total depletion |

**Table 6: *Kit*-independent inducible MC-deficient mouse models** (Siebenhaar et al., 2015)

## 1.3 Aim of the study

Although mammalian MCs were first described more than a century ago, their crucial functions are still being elucidated in detail; especially the biological function of MMCs remains widely unclear. One of the reasons for this situation is the lack of suitable *in vivo* models for allowing further understanding of the biology of MMCs. To date, mice with a specific focus on the MMC population have not yet been developed. We, therefore, aimed to generate a new mouse model that exhibits a specific reduction in MMCs, thus allowing for the investigation of MMCs *in vivo*.

# 2 Materials and methods

The appliances, chemicals, antibodies, media, buffers, kits and disposables used in this study are listed below. The concentrations and specific applications of the listed materials are specified in the corresponding method section.

## 2.1 Appliances

| Appliance name | Distributor |
|---|---|
| Autoclave | MELAG, Berlin, Germany |
| Centrifuge,<br>Megafuge 1.0 ST R | Heraeus, Hanau, Germany |
| $CO_2$-incubator<br>HeraCell 150 | Heraeus, Hanau, Germany |
| Drying cabinet<br>Hera Cell | Heraeus, Hanau, Germany |
| Electronic pipette boy<br>Handy Step® | Brandt GmbH & Co, Wertheim, Germany |
| Electronic weighing machine<br>LA 4200 and PT120 | Satorius, Göttingen, Germany |
| Flow Cytometer,<br>MACSquant Analyzer | Miltenyi Biotec, Bergisch Gladbach, Germany |
| Inverted microscope<br>CKX 41 | Olympus, Hamburg, Germany |
| Micro reaction vessel | Frötek, Osterode, Germany |
| Microtome Finesse 325 | Shandon, Waltham, USA |
| Multi-channel pipette<br>5µl-50µl, 50µl-300µl | Eppendorf, Hamburg, Germany |
| Neubauer chamber<br>Deep 0.1mm, Big square 1mm$^2$ | Marienfeld, Lauda Königshofen, Germany |
| pH-Meter<br>PB-11 | Sartorius, Göttingen, Germany |

| | |
|---|---|
| Pipettes<br>2µl, 10µl, 20µl, 100µl, 200µl, 1000µl | Brandt GmbH & Co, Wertheim, Germany;<br>Eppendorf, Hamburg, Germany;<br>Gilson, Middleton, USA |
| Plate reader<br>VICTOR X | Heidolph, Schwabach, Germany |
| Power Supply<br>Power Pac 300 | BioRad, München, Germany |
| Shaver | |
| Surgical instruments,<br>Scissors, forceps, scalpel | Aesculab B. Braun, Tuttlingen, Germany |
| Thermomixer<br>Thermomixer R | Eppendorf, Hamburg, Germany |
| Tissue embedding-machine<br>Shandon CitadelTM 1000 | Thermo Fisher Scientific, Walldorf, Germany |
| Vortexer<br>Model 72020 | NeoLab, Heidelberg, Germany |
| Water bath<br>WBT 222 | Medingen, Dresden, Germany |

**Table 7: List of appliances used for this study**

## 2.2 Chemicals and reagents

| Chemicals/Reagents name | Distributor |
|---|---|
| 4% buffered formaldehyde | Herbeta Arzneimittel, Berlin, Germany |
| 4’,6-diamidino-2-phenylindole,<br>DAPI | Roche, Mannheim, Germany |
| Alcian blue 8GX | Sigma-Aldrich, Steinheim, Germany |
| Aqua ad iniectabilia | Braun, Melsungen, Germany |
| Bovine serum albumin<br>BSA | Serva, Heidelberg, Germany |
| Dimethyl sulfoxide | Sigma-Aldrich, Steinheim, Germany |

| | |
|---|---|
| DMSO | |
| Ethylenediaminetetraacetic acid<br>EDTA | Merck, Darmstadt, Germany |
| Giemsa stain solution | Sigma-Aldrich, Steinheim, Germany |
| $H_2O$, steril | Braun, Melsungen, Germany |
| MACSFlow | Miltenyi Biotec Bergisch Gladbach<br>Germany |
| Methanol | Merck, Darmstadt, Germany |
| Natriumhydroxid<br>NaOH | Merck, Darmstadt, Germany |
| Paraformaldehyde<br>PFA | Sigma-Aldrich, Steinheim, Germany |
| PBS without $Ca^{2+}$ and $Mg^{2+}$ | PAA, Pasching, Osterreich |
| Proteinase K | DAKO, Hamburg, Germany |
| Streptavidin-FITC | DAKO, Hamburg, Germany |
| Streptavidin-TexasRed | Roche, Mannheim, Germany |
| Tris-buffer saline | Sigma-Aldrich, Steinheim, Germany |
| α-Monothioglycerol | Sigma-Aldrich, Steinheim, Germany |

**Table 8: List of chemicals and reagents for the study**

## 2.3 Antibodies

| Antibodies name | Distributor |
|---|---|
| Armenian Hamster IgG-FITC/ PerCP-Cy5.5 | eBioscience, Frankfurt, Germany |
| CD117-APC-Cy7 | eBioscience, Frankfurt, Germany |
| Fc-Block-FITC | Biolegend, London, United Kingdom |
| FcεRI | Biolegend, London, United Kingdom |
| Rat IgG1-Biotin | BD Pharmingen, Heidelberg, Germany |
| Rat IgG2a,k-PE | BD Pharmingen, Heidelberg, Germany |
| Rat IgG2b-PE/APC/APC-Cy7/PE-Cy7 | BD Pharmingen, Heidelberg, Germany |

**Table 9: List of antibodies for the study**

## 2.4 Medium and buffers

| Medium name | Distributor |
|---|---|
| FCS | Biochrom AG, Berlin, Germany |
| Iscove's Modified Dulbecco's Medium<br>IMDM | Invitrogen, Carlsbad, USA |
| Mouse recombinant interleukin<br>IL3 | BD Pharmingen, Heidelberg, Germany |
| Penicillin/ Streptomycin | Biochrom AG, Berlin, Germany |

**Table 10: List of medium for the study**

## 2.5 Mouse strain

### 2.5.1 Chm-Cre; Mcl-$1^{fl/fl}$ mice

Genetically, Chm-Cre; Mcl-$1^{fl/fl}$ mice and congenic normal Chm-Cre; Mcl-$1^{+/+}$ mice (8 to 12 weeks old) were obtained from breeding colonies of the animal facilities of Charité-Universitätsmedizin Berlin. The animals were kept in group cages under constant and externally shielded climatic conditions. All mice received standard laboratory feed, as well as water *ad libitum*, during a constant 12-hour day-night rhythm. Transgenic mice were maintained in the heterozygote status and bred on the C57BL/6 background. All animal care and experimentation was conducted in accordance with current Institutional Animal Care and Use Committee guidelines at the Charité-Universitätsmedizin Berlin under official permissions of the State of Berlin, Germany.

### 2.5.2 Development of mouse model

It has been reported that transgenic mice with Cre expression driven by baboon α-chymase promotor (Chm: Cre), which exhibit Cre expression restricted to lung and colon, indicating that this α-chymase promotor correlates to mature resident MCs in these mucosal tissues (Müsch et al., 2008). Thus, in the present study, we mated the Chm:Cre transgenic mice with mice bearing a floxed allele of the myeloid cell leukemia

sequence 1 (Mcl-1), which encodes for an intracellular antiapoptotic factor in MCs. Using this approach, we generated a new mouse model: Chm-Cre; Mcl-1$^{fl/fl}$ mice (Fig 3).

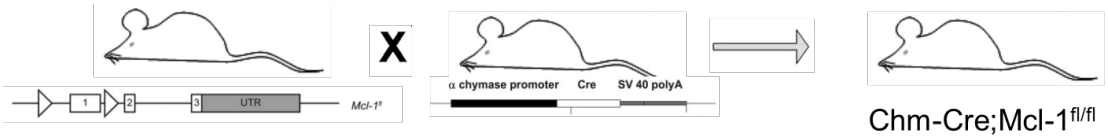


**Figure 3: Map of the Chm-Cre; Mcl-1$^{fl/fl}$ mouse.** In this example, a mouse with a floxed Mcl-1gene is crossed with transgenic mice harboring Cre recombinase under the control of baboon α-chymase promoter, to direct Cre expression only in MMCs. Figure has been modified from (Musch et al., 2008) and (Lilla et al., 2011).

In detail, Chm-Cre mice (Müsch et al., 2008) were crossed with Mcl-1$^{+/fl}$ mice. The offspring were identified by PCR genotyping (see "genotyping"). The heterozygote Chm-Cre; Mcl-1$^{+/fl}$ mice were bred as breeder to obtain Chm-Cre; Mcl-1$^{+/+}$, Chm-Cre; Mcl-1$^{+/fl}$ and Chm-Cre; Mcl-1$^{fl/fl}$ mice.

### 2.5.3 Genotyping

Genotyping was performed by PCR. The genotype of transgenic offspring from Chm-Cre; Mcl-1$^{+/fl}$ mice was detected by tail DNA as described earlier (Brandt et al., 2003). Three reactions are performed during the genotyping, in brief: one to test for Chm-Cre; one to test for the wild type Mcl-1 allele (PCR product: 360bp); and one to test for the Mcl-1 flox allele (PCR product: 400bp).

| Primer name | Primer sequence<br>Forward-Primer | Reverse-Primer |
|---|---|---|
| Chm-Cre | 5' CGG CGC TAA GGA TGA CTC TGG TCA G 3´ | 5' GTC CAA CGT TCC GTT CGC GCG G 3´ |
| Mcl-1 | 5' CGA TGC AAC GAG TGA TGA GG 3' | 5' GCA TTG CTG TCA CTT GGT CGT 3' |

**Table 11: Primers sequences**

### 2.5.4 Histology

For histological analysis, each mouse after sacrificing with Isoflurane anesthesia and samples of back skin, ear pinna, were fixed in 4% formaldehyde, stomach, duodenum, ileum, colon was fixed in 4% paraformaldehyde. The paraffin sections were embedded by means of the Shandon Citadel 1000 (Waltham, USA) according to the protocol (Table 12).

| Medium name | Time (mins) |
|---|---|
| Aqua distilled water | 20 |
| 70% ethanol | 50 |
| 70% ethanol | 60 |
| 96% ethanol | 50 |
| 96% ethanol | 60 |
| Absolute ethanol | 50 |
| Absolute ethanol | 60 |
| Absolute ethanol | 60 |
| xylol | 50 |
| xylol | 60 |
| paraffin | 120 |
| paraffin | unlimited |

**Table 12: Dehydration and embedding protocol**

After dehydration and embedding in paraffin and ensuring a cross-sectional orientation of all tissues, and 5-μm sections were stained with Alkaline-Giemsa solution (Table 13) for histologic examination and enumeration of mast cells. To examing stomach mucosal mast cells, stomach samples were stained with alcian blue solution (Table 14). To exam duodenum, jejunum, ileum and colon mast cell, the 5-μm sections of 4% paraformaldehyde intestinal tissue were stained for chloroacetate esterase–positive mucosal mast cells, as previously described (Yamada et al., 2003). MCs were recognized by their specific granules. At least 3 random sections per mouse were analyzed.

| Medium name | Time (mins) |
|---|---|
| 2x xylol | 10 |
| 2x absolute ethanol | 5 |
| 96% ethanol | 5 |
| 70% ethanol | 5 |
| aqua distilled water | 5 |
| Alkaline-Giemsa solution | 15 |
| 0.1% acetic acid | 10s |
| aqua distilled water | 1 |
| 70% ethanol | 5 |
| 96% ethanol | 5 |
| 2x absolute ethanol | 5 |
| 2x xylol | 5 |

**Table 13: Alkaline-Giemsa staining protocol**

| Medium name | Time (mins) |
|---|---|
| 2x xylol | 10 |
| 2x absolute ethanol | 5 |
| 96% ethanol | 5 |
| 70% ethanol | 5 |
| aqua distilled water | 5 |
| Alcian blue solution<br>(1% alcian blue in 3% acetic acid solution) | 15 |
| aqua distilled water | 1 |
| 70% ethanol | 5 |
| 96% ethanol | 5 |
| 2x absolute ethanol | 5 |
| 2x xylol | 5 |

**Table 14: Alcian blue staining protocol**

### 2.5.5 Cells

#### 2.5.5.1 Bone marrow-derived cultured mast cells (BMCMCs)

Chm-Cre; Mcl-$1^{+/+}$ and Chm-Cre; Mcl-$1^{fl/fl}$ mice were anesthetized and sacrificed, the posterior limbs were pulled off, all soft tissue was removed from the bone, both the femur and tibia were wiped with 70% ethanol. The bone marrow of the bones was rinsed out using a 20G syringe needle, centrifuged at 1200 rpm for 10 minutes and placed in sterile filtered cell culture medium supplemented with 10% FCS, 0.002% monothioglycerate, 1% penicillin/streptomycin, and 10ng/ml interleukin 3 (IL-3). By changing the medium every 2 days and adding 10 ng / ml of IL-3, a mast cell suspension was taken from a cell suspension of myeloid progenitor cells. After culture of 28 days at a constant 37°C and a carbon dioxide content of 5%, an over 95% pure mast cell monoculture could be obtained. Morphological analysis of MCs was assessed by Alkaline-Giemsa staining of cytospins. The differentiation status of BMCMCs was assessed by flow cytometry for surface expression of c-Kit and FcεRI.

#### 2.5.5.2 Peritoneal mast cells (PMCs)

Peritoneal mast cells (PMCs) were obtained by peritoneal lavage from at least 12-week old Chm-Cre; Mcl-$1^{+/+}$ and Chm-Cre; Mcl-$1^{fl/fl}$ mice. Mice were anesthetized and sacrificed, the abdominal area was disinfected with 70% ethanol and skin was cut open without injuring the peritoneum. 5ml sodium chloride (NaCl) and 5ml air were injected into the peritoneal cavity. After gently shaking for 2 minutes, the peritoneal cells were carefully aspirated, transferred to a 50ml Falcon tube and placed on ice.

#### 2.5.5.3 Cytospins

To centrifuge cells onto slides, a special centrifugation device with 1.5 ml micro reaction vessels is required. A special matched filter paper was added between the micro reaction vessels and slides. 50µl of the cell suspension was added to each centrifuge tube and centrifuged for 7 minutes at 45g. Then the sheath was removed and the slide was air-dried. The slides were fixed in -20°C acetone freshly before the staining process.

#### 2.5.5.4 Determination of mast cell numbers

In all histological assessments, cell numbers were enumerated by a single observer not aware of the identity (mouse group) of the individual sections. Cell numbers were based on counting 10 medium power fields (200x) or high power fields (400x), and mean values were calculated. Skin mast cells were quantified according to horizontal field length of the dermis, uterus mast cells were quantified according to the area, and gastrointestinal mast cells were quantified according to an anatomical structure (per field of mucosa and submucosa). Images were captured with a Zeiss Axioplan 2 Imaging microscope using a Zeiss AxioCam camera run by AxioVision Rel. 4.8 software.

### 2.5.6 Uterus mast cells in mouse

To investigate the possible role of uterus MCs *in vivo* in mouse, experiments were conducted in collaboration with Otto-von-Guericke University (Magdeburg, Germany) as previous described (Woidacki et al., 2014a). Briefly, animals were maintained in the barrier animal facility of the Medical Faculty, Otto-von-Guericke University (Magdeburg, Germany) with a 12-h light/dark cycle. All females were checked daily for vaginal plugs. The day at which the vaginal plug was detected was considered as day 0 of pregnancy and the females were separated from the males. Female mice were sacrificed on day 5, or 10 of pregnancy, and the uteri were removed and implantation sites were documented.

The determination of the estrous cycle in virgin female mice was performed by vaginal lavage with 0.9% sodium chloride. The stage of the cycle was defined based on the typical cell content of the lavage. Mice were sacrificed when they showed a clear stage.
The determination of the embryo, the decidua basalis, the placental area, thickness, and diameter was conducted by ultrasound (Fig. 4).

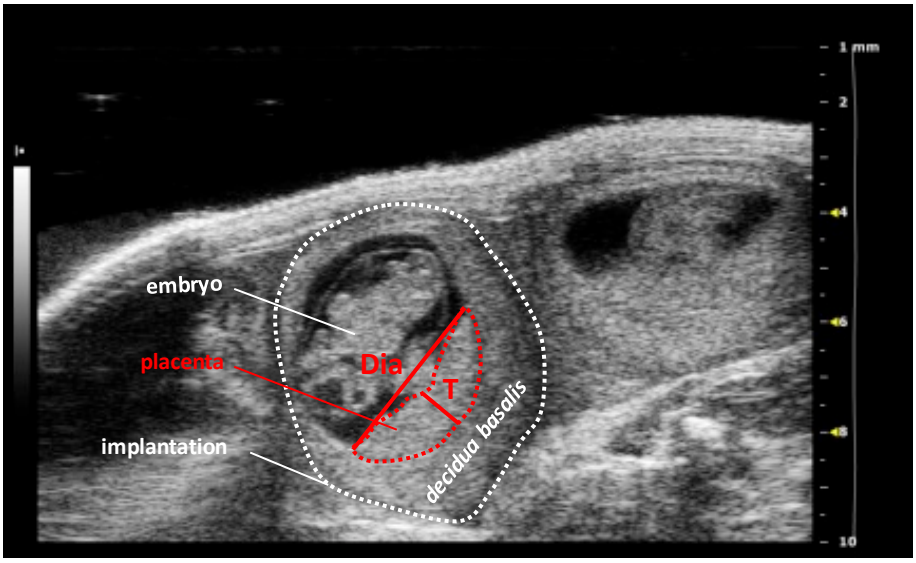


**Figure 4: Representative ultrasound image of an implantation.** Image showing the embryo, the decidua basalis, and the placenta with placenta thickness (T) and diameter (Dia).

## 2.6 Statistics

Unless otherwise indicated all data were tested for statistical significance using the unpaired Student t-test and expressed as mean ± SEM. A p-value ≤ 0.05 was considered to reflect statistical significance.

# 3 Results

## 3.1 Chm-Cre; Mcl1$^{fl/fl}$ mice exhibit comparable proliferation and differentiation rates in bone morrow-derived cultured mast cells

In mice, MCs derive from hematopoietic progenitor cell from the bone marrow. In order to determine the generation of MCs, we tested the bone marrow-derived cultured MCs (BMCMCs) of 12 week-old Chm-Cre; Mcl-1$^{fl/fl}$ mice, Chm-Cre; Mcl-1$^{+/+}$ mice were used as control. Histological analysis of BMCMCs was applied after 4 weeks *in vitro* culture. The representative light micrograph shows the comparable morphology of BMCMCs after 4 weeks' culture in IL-3–supplemented medium from Chm-Cre; Mcl1$^{+/+}$ (control) mice and Chm-Cre; Mcl1$^{fl/fl}$ mice. BMCMCs were identified morphologically on cytospin cell preparations stained with Giemsa solution (Fig. 5).

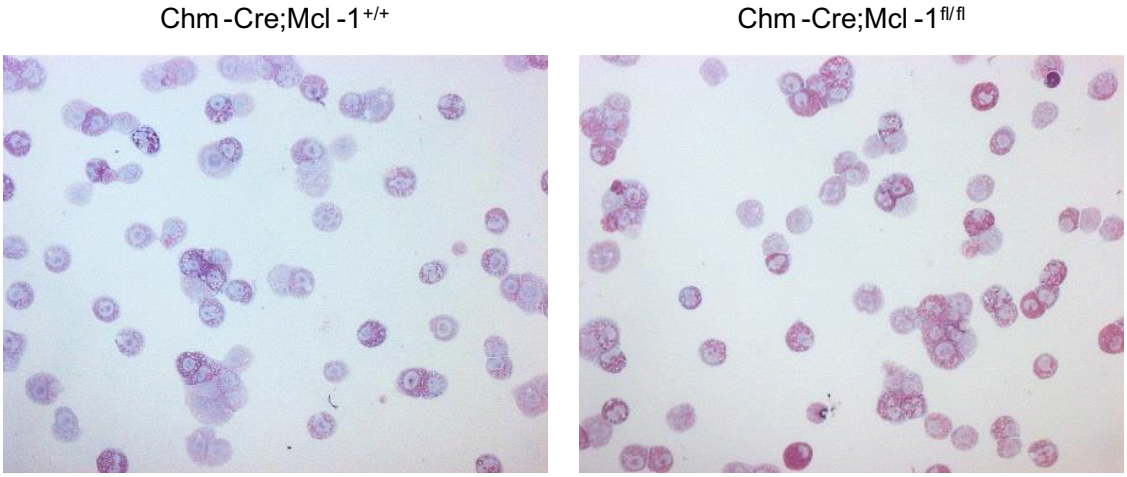


**Figure 5**: **Representative light micrograph of bone morrow-derived cultured mast cells (BMCMCs).** The representative light micrograph shows the comparable morphology of BMCMCs after 4 weeks culture in IL-3–supplemented medium from Chm-Cre; Mcl1+/+ (control) mice and Chm-Cre; Mcl1$^{fl/fl}$ mice. BMCMCs were identified morphologically on cytospin cell preparations stained with Giemsa solution. Magnification: 200X.

By further calculating the proliferation of BMCMCs (calculating the change fold of cell number compared with the cell number 7-days before at different time points: 7, 14, 21, 28 days). Data showed similar proliferation rates of BMCMCs after 7, 14, 21, and 28 days of culture from Chm-Cre; $Mcl1^{+/+}$ mice and Chm-Cre; $Mcl1^{fl/fl}$ mice. We found no significant differences in proliferation of BMCMCs generated from Chm-Cre; $Mcl\text{-}1^{fl/fl}$ on Chm-Cre; $Mcl\text{-}1^{+/+}$ mice. (Fig. 6).

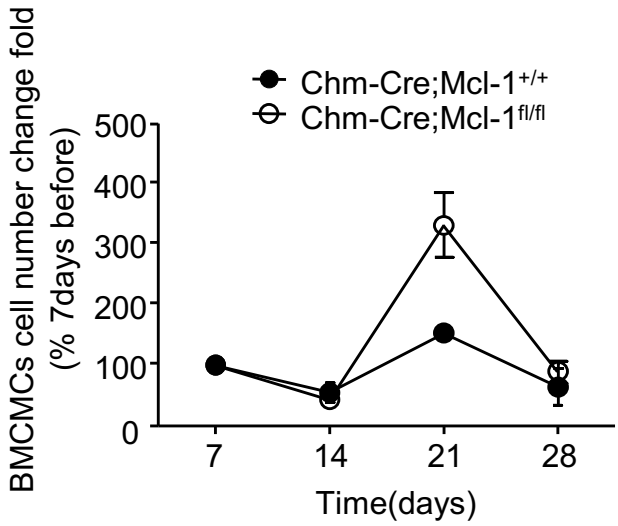


**Figure 6: Chm-Cre; $Mcl1^{fl/fl}$ mice exhibit comparable proliferation rates in bone morrow-derived cultured mast cells (BMCMCs).** The proliferation rate of BMCMCs was evaluated by calculating the change fold of cell number compared with the number 7 days before. Data showed similar proliferation of BMCMCs after 7days, 14days 21days and 28 days of culture from Chm-Cre; $Mcl1^{+/+}$ mice (n=9) and Chm-Cre; $Mcl1^{fl/fl}$ mice (n=9).

Furthermore, in order to determine the differentiation of BMCMCs, flow cytometric analysis was assessed for expression of MC surface markers CD117 (Kit) and FcεRI in BMCMCs at different time points (7, 14, 21, 28 days). We found that the BMCMCs derived from Chm-Cre; $Mcl\text{-}1^{fl/fl}$ exhibited similar levels of surface expression of Kit and FcεRI as those from control Chm-Cre; $Mcl\text{-}1^{+/+}$ mice. In addition, the BMCMCs after 28 days' culturing from both strains exhibited more than 95% double positive cells (Fig. 7), which at a normal pace of differentiation and maturation. Accordingly, the generation

capacities of MCs myeloid origin, the bone marrow, were comparable and intact in Chm-Cre; Mcl-1$^{fl/fl}$ mice and Chm-Cre; Mcl-1$^{+/+}$ mice.

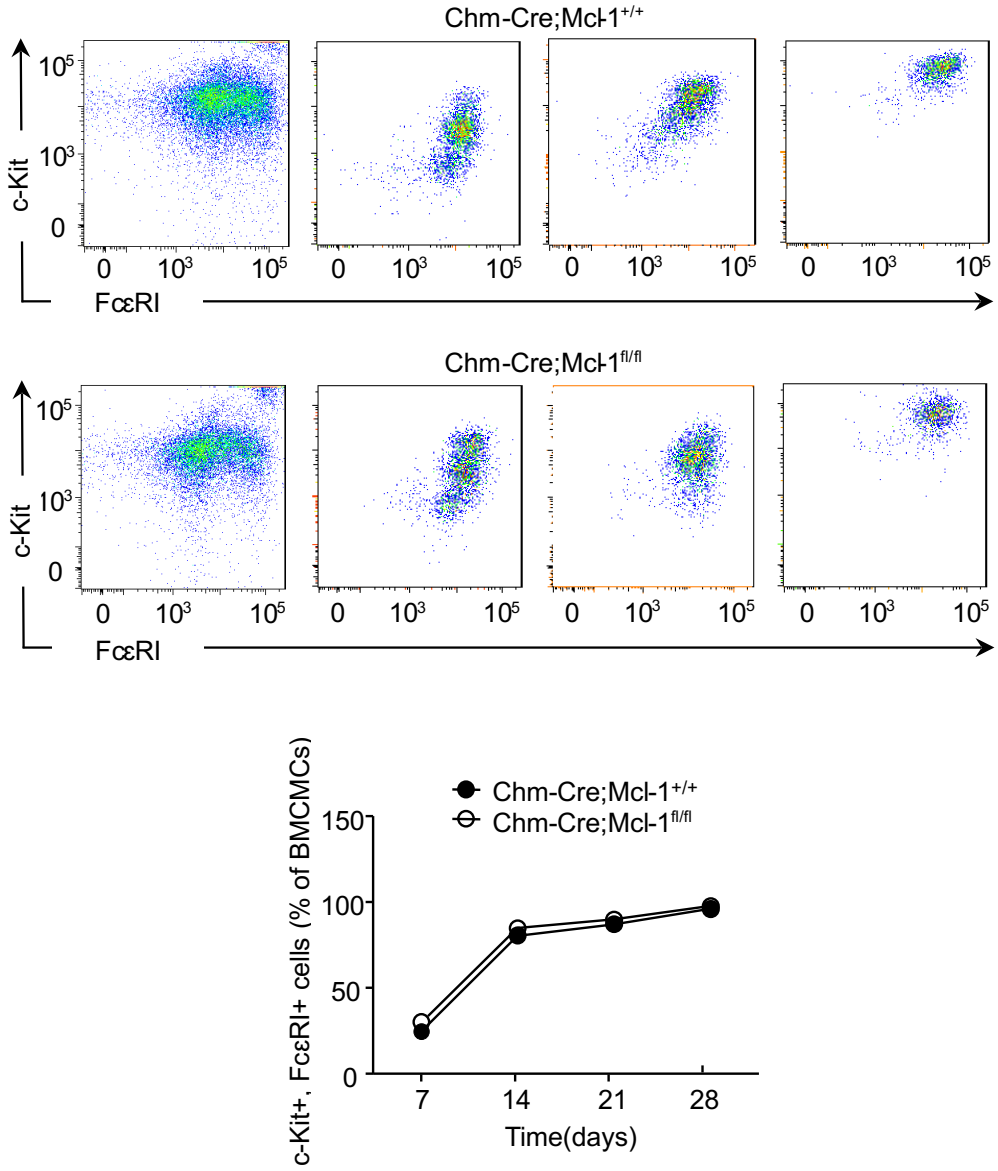


**Figure 7: Chm-Cre; Mcl1$^{fl/fl}$ mice exhibit comparable differentiation rates in bone morrow-derived cultured mast cells (BMCMCs).** Representative flow cytometry plots (up) and percentage (down) of c-Kit and FcεRI expression show a comparable differentiation rate on BMCMCs isolated from Chm-Cre; Mcl1$^{+/+}$ mice(n=9) and Chm-Cre; Mcl-1$^{fl/fl}$ mice(n=9) after 7days, 14days 21days and 28days' culture.

## 3.2 Chm-Cre; Mcl1$^{fl/fl}$ mice exhibit normal numbers of connective-tissue type mast cells (CTMCs) in peritoneum and skin

We further investigated whether Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit differences in the number of CTMCs at typical sites. Our study focused on peritoneal mast cells (PMCs) and cutaneous mast cells (CMCs), both of which are representative CTMCs (Yamada et al., 2003). By histological analysis and mast cell counting, we found PMCs detected in Chm-Cre; Mcl-1$^{fl/fl}$ mice presented similar morphology and comparable cell number as Chm-Cre; Mcl-1$^{+/+}$ mice (Fig. 8).

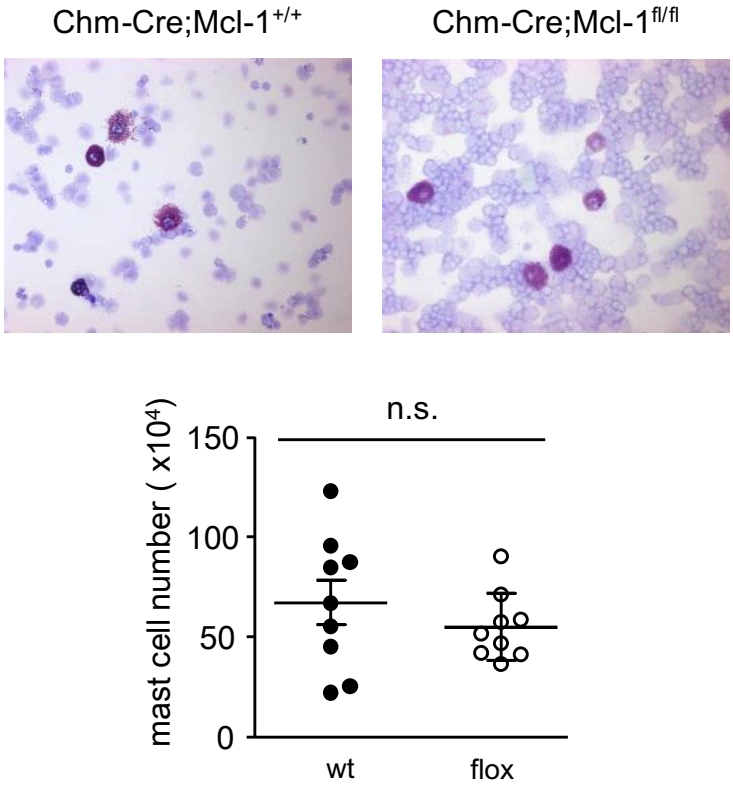


**Figure 8: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit no difference in the numbers of peritoneal mast cells (PMCs) comparing to Chm-Cre; Mcl-1$^{+/+}$ mice.** Numbers of PMCs (left) and representative light micrograph (right) showed similar cell number and morphology of PMCs isolated from peritoneal lavage fluid from Chm-Cre; Mcl-1$^{+/+}$ mice (wt, n=9) and Chm-Cre; Mcl-1$^{fl/fl}$ mice (flox, n=9). PMCs were identified morphologically on cytospin cell preparations stained with Giemsa solution. Magnification: 200X. n.s. not significant.

Further, flow cytometric analysis showed that the surface expression of Kit and FcεRI were similar in PMCs from Chm-Cre; Mcl-1$^{fl/fl}$ mice compared to those from control Chm-Cre; Mcl-1$^{+/+}$ mice (Fig. 9).

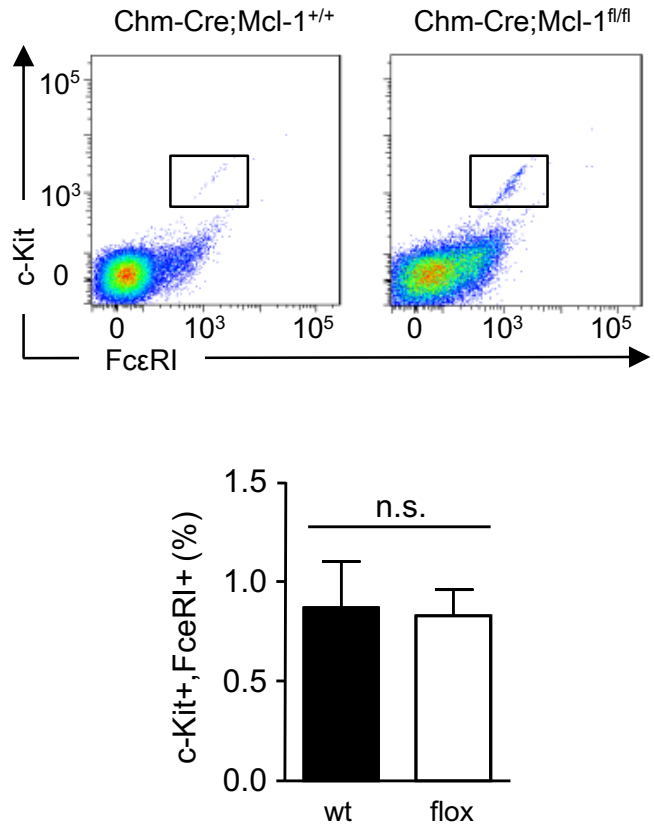


**Figure 9: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit no difference in the surface expression of peritoneal mast cells (PMCs) when compared to Chm-Cre; Mcl-1$^{+/+}$ mice.** Percentage of mast cell surface markers expression (left) and representative flow cytometry plots (right) showed comparable expression of c-Kit and FcεRI on PMCs isolated from Chm-Cre; Mcl1$^{+/+}$ mice(wt, n=9) and Chm-Cre; Mcl-1$^{fl/fl}$ mice (flox, n=9). n.s. = not significant.

Moreover, by quantitative histomorphometry analysis of dorsal skin (Fig. 10a) and ear skin we observed equal numbers of CMCs in Chm-Cre;Mcl-1$^{fl/fl}$ (Fig. 10b, dorsal skin: 10.61±0.78/HPF; ear skin: 10.1±0.85 /HPF, n=25) and Chm-Cre;Mcl-1$^{+/+}$ control mice (Fig. 10a, dorsal skin: 11.21±0.65/HPF; ear skin: 10.10±0.85 /HPF, n=25). These results revealed that the number and phenotype of CTMCs in Chm-Cre; Mcl-1$^{fl/fl}$ mice, at least the representative CTMCs, PMCs, and CMCs, exhibited no difference compared to control Chm-Cre; Mcl-1$^{+/+}$ mice.

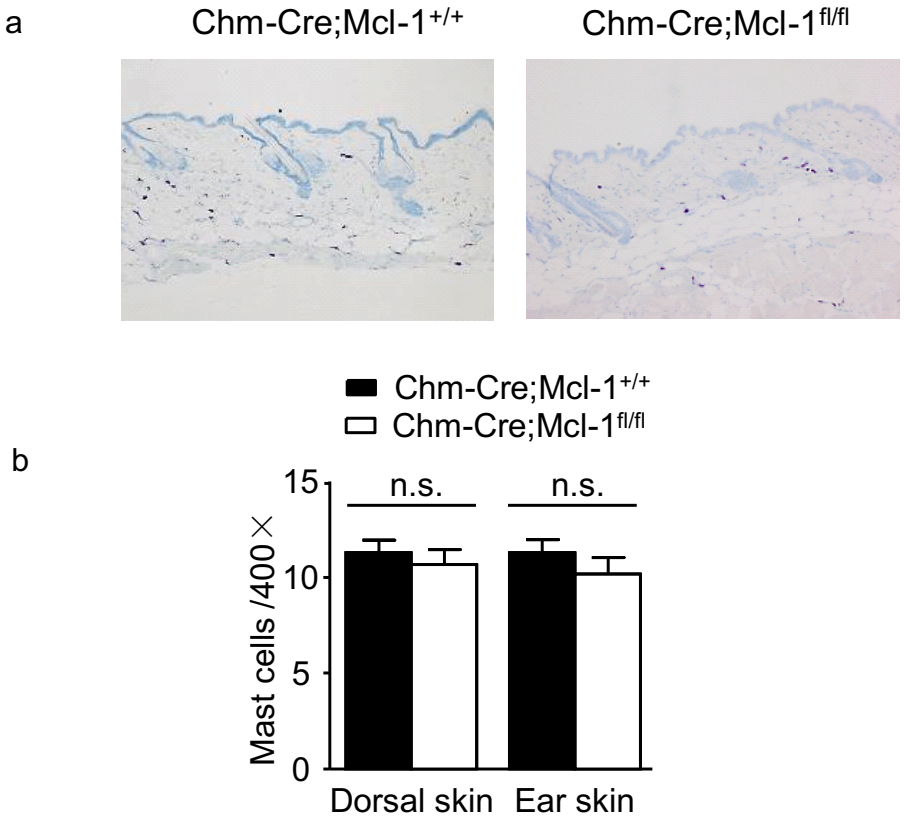


**Figure 10: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit equal numbers of peritoneal mast cells (PMCs) compared to Chm-Cre; Mcl1$^{+/+}$ mice.** (Fig 10a) Giemsa staining for mast cells in 5-µm-thick paraffin sections of dorsal skin in Chm-Cre; Mcl-1$^{+/+}$ mice (n=25) and Chm-Cre; Mcl-1$^{fl/fl}$ mice (n=25) show a comparable number of mast cells (purple). (Fig 10b) Mast cells in dorsal and ear skin tissues show a similar number of cutaneous mast cells. Magnification: 9a 200X. n.s. = not significant.

## 3.3 Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit markedly reduced numbers of mucosa type mast cells (MMCs) in representative tissues

To further study the MMCs population in Chm-Cre; Mcl-1$^{fl/fl}$ mice, we focused on the mast cells of glandular stomach and intestine tissues, which are representative tissue sites to contain MMCs. In the present study, MCs were classified according to anatomic location as previously described (Galli et al., 1987). MCs superficial to the deep border of the muscular layer of the lamina muscularis mucosae, including epithelium, lamina propria, and muscularis mucosae, were classified as MMCs. In the glandular stomach, alcian blue staining was used for detecting gastric MMCs (Fig. 11). We observed a markedly reduced number of MMCs (blue staining cells) in Chm-Cre; Mcl-1$^{fl/fl}$ mice (Fig. 13, 1.57±0.45/high power field <HPF>, n=15) compared to control Chm-Cre; Mcl1$^{+/+}$ mice (Fig. 13, 4.17±0.32/HPF, n=15).

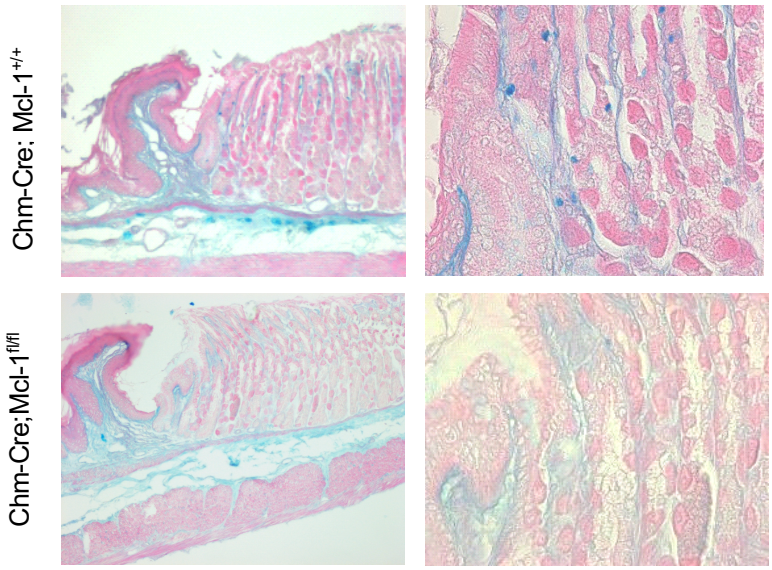


**Figure 11: Representative histological images indicating Chm-Cre; Mcl-1$^{fl/fl}$ mice have markedly reduced numbers of mucosal mast cells (MMCs) in the stomach.** Alcian blue staining for gastric mast cells in 5-µm-thick paraffin sections showed a markedly reduced number of mast cells (blue) in Chm-Cre; Mcl-1$^{fl/fl}$ mice(n=15) compared to control Chm-Cre; Mcl-1$^{+/+}$ mice(n=15). Magnification：left: 200X, right: 400X.

In the intestine, chloroacetate esterase staining was performed for visualization and examination of intestinal MMCs (Fig. 12). In contrast to the detectable representative MMCs in lamina propria of the duodenum from control Chm-Cre; Mcl-$1^{+/+}$ mice (Fig. 13, 1.33±0.13/HPF, n=15), morphological evidence indicates that Chm-Cre; Mcl-$1^{fl/fl}$ mice exhibited a reduced number of MMCs (Fig. 13, 0.56±0.10/HPF, n=15, P<0.01). Since MMCs are found in relatively low numbers in most mucosal tissues, especially in intestines (Reber et al., 2015), it is not surprising to observe some of the intestinal tissues (ileum and colon) containing barely any detectable MCs (Fig. 13). Even though a significantly reduced number of MCs was found in the duodenum, the cell numbers per high-power field were generally low. Therefore, the Chm-Cre; Mcl-$1^{fl/fl}$ mice have markedly reduced numbers of mast cells in glandular stomach and duodenum, in which locating representative MMCs.

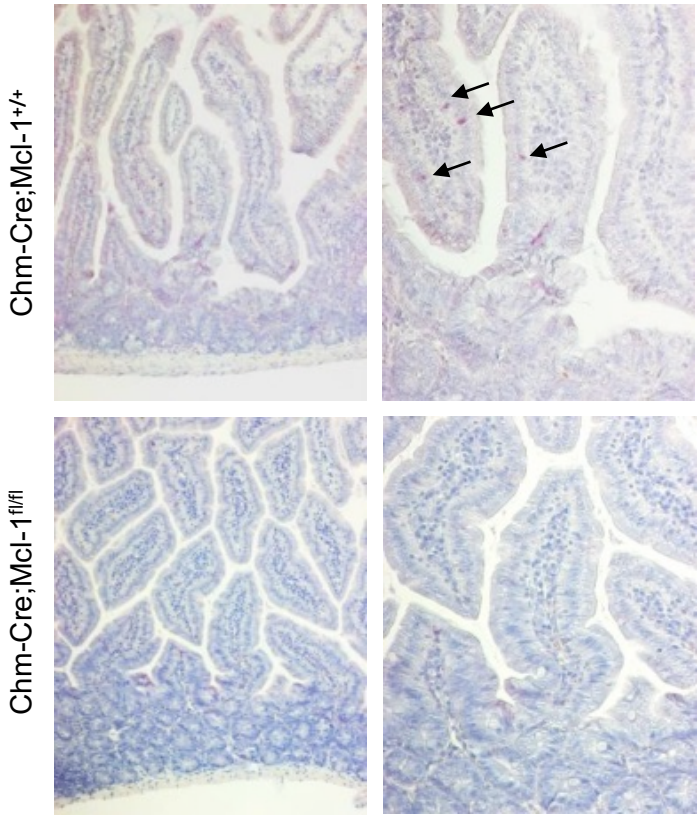


**Figure 12: Representative histological images indicating Chm-Cre; Mcl-$1^{fl/fl}$ mice have markedly reduced numbers of mucosal mast cells (MMCs) in the intestine.** Chloroacetate esterase staining for intestinal mast cells showed a decreased number of mast cells (red) in the duodenum from Chm-Cre; Mcl-$1^{fl/fl}$ mice(n=15) compared to control Chm-Cre; Mcl$1^{+/+}$ mice(n=15). Magnification：left: 200X, right: 400X.

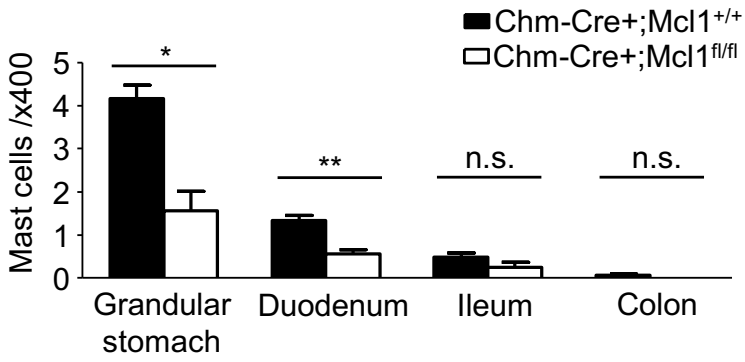


**Figure 13: Chm-Cre; Mcl-1$^{fl/fl}$ mice have markedly reduced numbers of representative mucosal mast cells (MMCs).** Numbers of mast cells from Chm-Cre; Mcl-1$^{fl/fl}$ mice(n=15) and Chm-Cre; Mcl1$^{+/+}$ mice(n=15) in different gastrointestinal tissues were measured by quantitative histomorphometry analysis. *P<0.05, **P<0.01, n.s. not significant.

## 3.4 Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of mast cells in the uterus (uMCs) and decreased placental thickness

Except for tissues containing typical MMCs or CTMCs, we also investigated tissues that consist of both MMCs and CTMCs. We harvested uterine tissue from 12-week-old Chm-Cre; Mcl-1$^{fl/fl}$ and Chm-Cre; Mcl-1$^{+/+}$ mice following histomorphometry analysis. Interestingly, we observed a significantly reduced number of MCs in the uterus of Chm; Cre; Mcl-1$^{fl/fl}$ mice (Fig. 14, 1.29±0.58/HPF, n=9) compared to Chm-Cre; Mcl-1$^{+/+}$ control mice (Fig.14, 6.34±1.04/HPF, n=9, P<0.001).

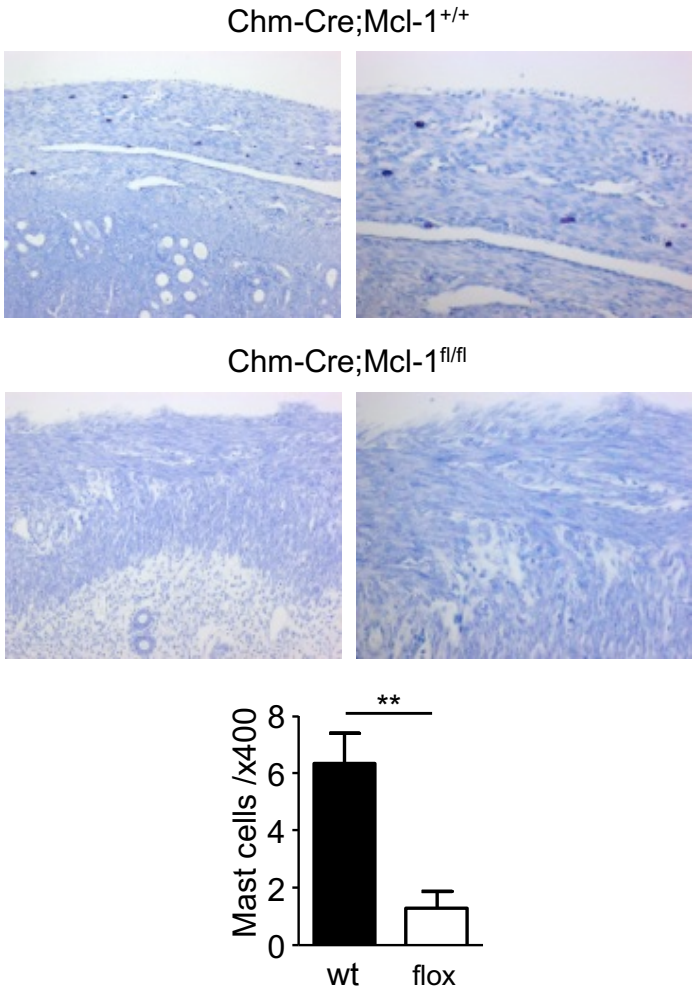


**Figure 14: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of uMCs.** Giemsa staining for uterus mast cells in 5-µm-thick paraffin sections showed a markedly reduced number of mast cells (purple) in Chm-Cre; Mcl-1$^{fl/fl}$ mice (flox, n=9) compared to control Chm-Cre; Mcl1$^{+/+}$ mice(wt, n=9).

In consideration of the difference in uterine MC (uMC) numbers during the fertile period of the estrus cycle and early gestation, we further quantified the number of uMCs/mm$^2$

from uterus of virgin Balb/c-paired female Chm-Cre;Mcl-1$^{fl/fl}$ and Chm-Cre;Mcl-1$^{+/+}$ mice in estrus or at gestation day 5 (gd5) of pregnancy. During estrus, Chm-Cre;Mcl-1$^{fl/fl}$ mice (Fig.15, 3.72±3.9/mm$^2$, n=5, P=0.017) presented significantly reduced numbers of MCs compared to Chm-Cre;Mcl-1$^{+/+}$ mice (Fig.15, 12.70±5.4/mm$^2$, n=5).

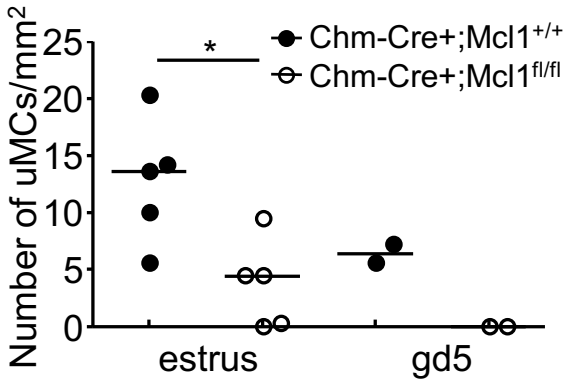


**Figure 15: Chm-Cre;Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of uMCs in estrus.** Number of uMCs/mm$^2$ quantified from uteri of virgin Balb/c-paired Chm-Cre;Mcl-1$^{+/+}$ (n=5) and Chm-Cre;Mcl-1$^{fl/fl}$ (n=5) females in estrus or at gd5 (n=2 of each group) of pregnancy. *p<0.05.

We further investigated which subpopulation of MCs contributed to the reduced MC numbers. Since anatomic location could not help to distinguish between MMCs and CTMCs in the uterus, we performed alcian blue and safranin staining for histomorphologic analysis. The control Chm-Cre;Mcl-1$^{+/+}$ mice showed CTMCs as well as MMC both in estrus and gd5, whereas Chm-Cre;Mcl-1$^{fl/fl}$ mice exhibited only CTMCs but no MMCs. Thus, these results revealed that Chm-Cre;Mcl-1$^{fl/fl}$ mice have decreased numbers of MMCs in the uterus (Fig 16 and 17). Interestingly, in the Chm-Cre;Mcl-1$^{+/+}$ mice we also observed alcian blue/safranin double positive cells (Fig 17). The presence of double stained MCs in the uterus was previously reported (Aydın et al., 1998; Brandon and Evans, 1983; Combs et al., 1965). Whether these MCs are in a premature state or in a conversion process is unclear. In fact, it has been suggested by some authors that uMCs might be able to switch their phenotype depending on the surrounding milieu.

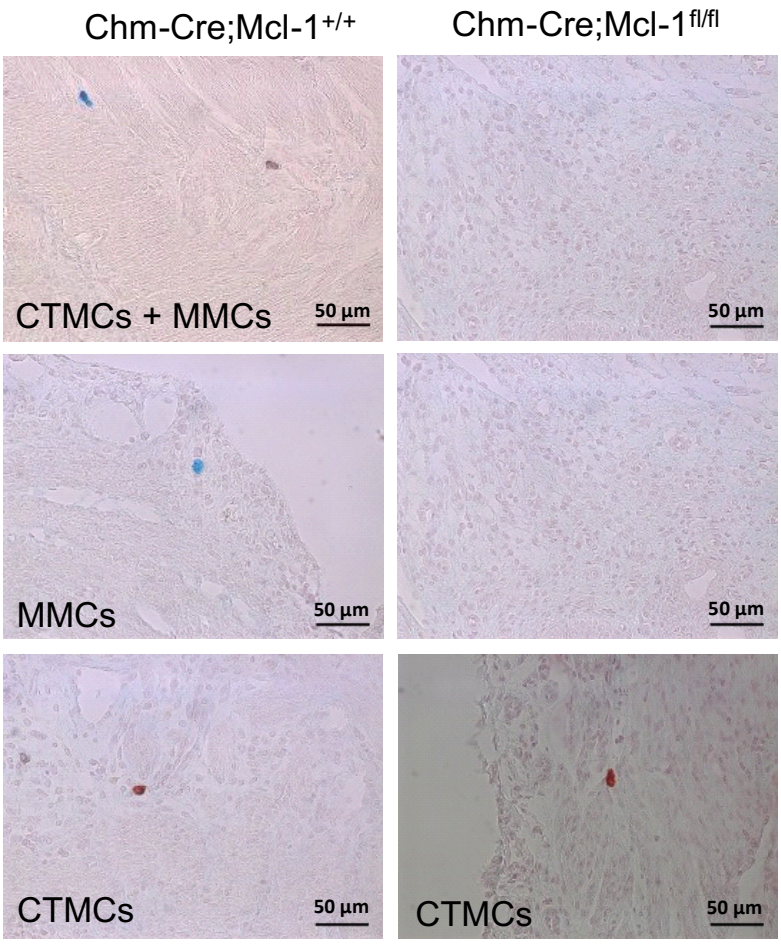


**Figure 16: Representative histological images indicating Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of MMCs in the uterus.** Representative images of alcian blue/safranin staining of the uterus from Chm-Cre; Mcl-1$^{+/+}$ (n=5) and Chm-Cre; Mcl-1$^{fl/fl}$ mice(n=5) at estrus.

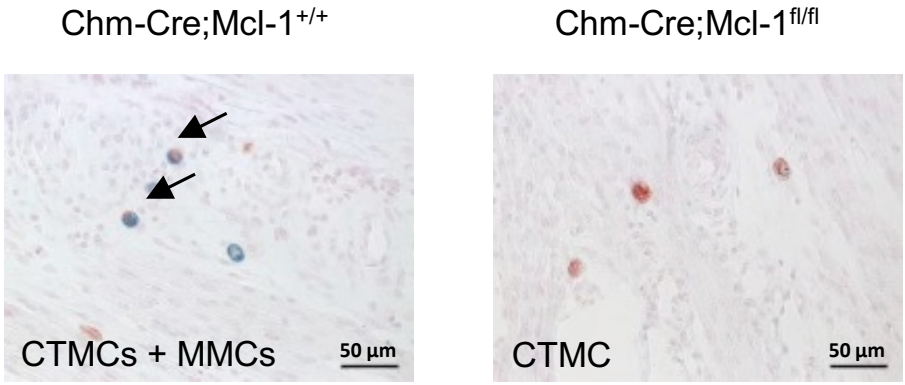


**Figure 17: Alcian blue/safranin double positive cells in the uterus**. Representative pictures of alcian blue/safranin staining of the uterus from Chm-Cre; Mcl-1 $^{+/+}$ (n=5) and Chm-Cre; Mcl-1$^{fl/fl}$ (n=5) at gd5. gd, gestation day.

We then further investigate the possible impact of MMCs on the implantation process. Placental area, placental thickness, placental diameter, and placental diameter/thickness ratio (Fig.18) from Balb/c-paired Chm-Cre;Mcl-1$^{fl/fl}$ mice (n=5, placentas n=23) and Chm-Cre;Mcl-1$^{+/+}$ mice (n=4, placentas n=22) at gd10 were studied. Interestingly, we observed significantly reduced placental thickness in Chm-Cre;Mcl-1$^{fl/fl}$ mice, however, the implantation area was comparable in Chm-Cre;Mcl-1$^{fl/fl}$ and Chm-Cre;Mcl-1$^{+/+}$ mice at gd5 and gd10 (Fig.19). These results indicated that the decreased number of uterus MMCs in Chm-Cre;Mcl-1$^{fl/fl}$ mice results in decreased placental thickness at gd10 mice, but not in an impairment of implantation. Thus, the functional role of MMCs in uterine physiology needs to be further investigated (Fig. 19).

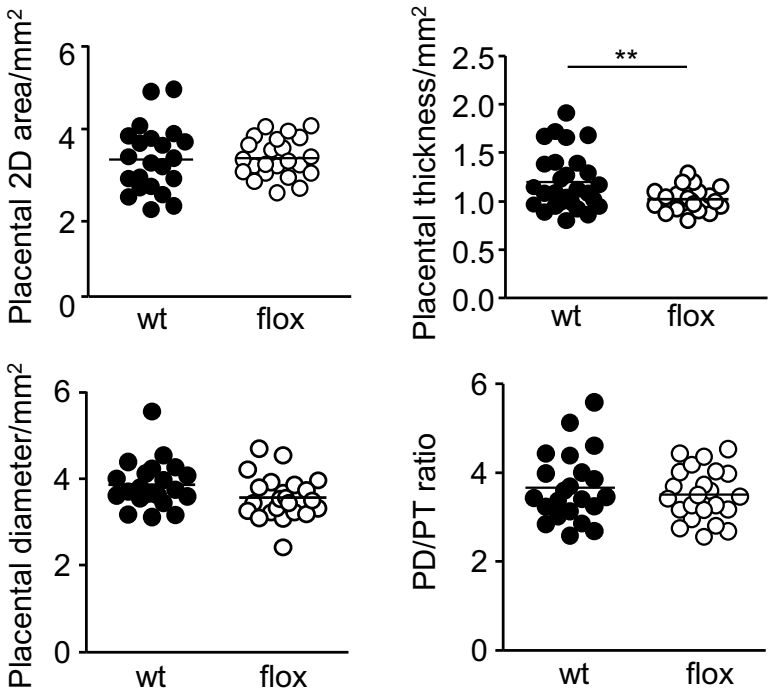


**Figure 18: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit decreased placental thickness at gd10.** Placental area, placental diameter, and placental diameter/thickness ratio from Balb/c-paired Chm-Cre; Mcl-1$^{+/+}$ (wt, mice n=4, placentas n=22) and Chm-Cre;Mcl-1$^{fl/fl}$ mice (flox, mice n=5, placentas n=23) at gd10.

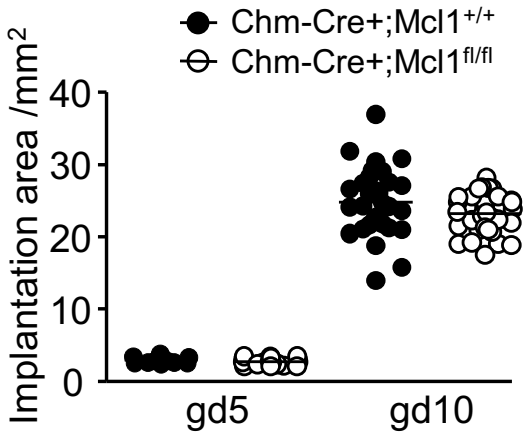


**Figure 19: Chm-Cre; Mcl-1$^{fl/fl}$ mice exhibit comparable implantation areas.** Implantation areas from Balb/c-paired Chm-Cre;Mcl1$^{+/+}$ (mice n=4, implantations n=15-32 per day) and Chm-Cre;Mcl-1$^{fl/fl}$ females (mice n=5, implantations n=21-36 per day) at gd5 and gd10.

# 4 Discussion

In the present study, we examined and characterized different types of MCs (CTMCs and MMCs) in the newly-developed mouse strain Chm-Cre;Mcl-1$^{fl/fl}$. Our data support the conclusion that Chm-Cre;Mcl-1$^{fl/fl}$ exhibit a marked reduction in the number of MMCs in representative mucosal tissues (glandular stomach and intestine) and tissue containing both types of MCs (uterus), without affecting CTMCs in representative connective tissues (dorsal skin, ear skin, or peritoneal cavity).

MC secretory granules contain abundant proteases, through the release of which MCs participate in several physiological and pathological responses. Tryptases, chymases and carboxypeptidase A are the major proteases stored and secreted by MCs. Chymases are a group of closely related MC neutral proteases, which are involved in diverse functions, such as peptide hormone processing, modulation of inflammatory response, and parasite expulsion. There are two isoenzyme groups of mammalian chymases, α and β-chymase (Chandrasekharan et al., 1996). α-chymase is encoded by a single chymase gene. Human chymase, baboon chymase, dog chymase, and mouse mast cell protease-5 are all classified as belonging to the α-chymase group (Chandrasekharan et al., 1996). In contrast, β-chymases are species-specific. Mice, rat and gerbil respectively contain four, two and one β-chymase-encoding genes. α- and β-chymases have different specificities and there are more subtle substrate specificity differences between different α-chymases (Liao et al., 1997). In order to study chymase function *in vivo*, Liao et al. generated a 571-bp domain of the baboon α-chymase promoter transgene that directs expression of the transgenes to mouse mast cells (Liao et al., 1997). By using the Cre-LacZ technique, they found that tissue expression of this promoter is distributed in lung, heart, spleen, intestine and stomach. Furthermore, by comparing with a mouse mast cell line that endogenously expresses mouse mast cell protease-5 (JKras mast cells), they concluded that the tissue expression of the transgene is similar to the expression of the endogenous mouse α-chymase mouse mast cell protease-5. In previous studies, mouse mast cell protease-5 was considered as α-chymase that is expressed mainly in the populations of mouse mast cells containing heparin sulfate proteoglycans (CTMCs), but not in chondroitin sulfate-containing mast cells(MMCs) (Caughey, 1995). This means that mMCP-5 is expressed mainly in CTMCs

rather than in MMCs. Studies of MC-deficient mouse models, i.e. Mcpt5-Cre iDTR and Mcpt5-Cre R-DTA mice, also support that mMCP-5 is a CTMC-specific chymase, by which the inducible or constitutive depletion of MCs is limited to CTMCs (Dudeck et al., 2011).

In contrast, in our study, we observed an opposite situation. Our Chm-Cre; Mcl-$1^{fl/fl}$ mice exhibited reduced MC numbers in mucosal tissues but not in connective tissues such as skin, indicating that Chm-Cre is expressed in mucosal MCs but not in connective tissues MCs (at least in the tissues that we have examined). How could this be? Is the baboon α-chymase indeed a homologue to the endogenous mMCP-5?

In the literature primate chymasee such as human and baboon chymase, which are encoded by a single chymase gene, are reported to belong to the α-family of chymase. In addition, mMCP-5 has been reported as being the only α-chymase in mouse, therefore believed to be the murine counterpart of primate α-chymase. However, a closer look into the structure and protease activity showed that mMCP-5 expresses a different amino acid in position 216 as compared to primate chymase and presents with elastase-like specificity rather than chymotrypsin-like activity (Kunori et al., 2002). These differences may suggest that mMCP-5 is not the murine homologue to primate α-chymase. Moreover, the chymotrypsin chymases, mMCP-1 (Knight et al., 2000) and mMCP-4 (Tchougounova et al., 2003), are more likely to be the functional counterpart of primate α-chymase. Interestingly, the murine chymotrypsin chymase mMCP-1 is a MMC-specific chymase, which is expressed particularly by MMCs and plays an important role in mucosal immune regulation (Knight et al., 2000). Taken together, our data suggest that the baboon α-chymase (at least in Chy-Cre; Mcl-1 transgenic mice), might be functionally more similar to the MMC-specific chymase mMCP-1. Thus, the ablation of the targeted population results in a reduced number of MMCs in Chm-Cre; Mcl-$1^{fl/fl}$ mice.

In mice, MCs originating from bone marrow precursor cells, by which MMCs and CTMCs share a common precursor and differentiate according to the local microenvironment. BMCMCs are used as an *in vitro* model for studying MC function, and have been considered as a mucosal-like population of MCs because they share some characteristics with MMCs. The generation of BMCMCs from Chm-Cre;Mcl-$1^{fl/fl}$ mice,

however, revealed differences between MMCs and BMCMCs, suggesting that BMCMCs are immature MCs that are phenotypically distinct from either MMCs or CTMCs (Yamada et al., 2003). We found BMCMCs from Chm-Cre;Mcl-1$^{fl/fl}$ mice to have the same ability in proliferation and maturation as compared to control mice (Fig 5-6), indicating that the hematopoietic precursor cell capacities for proliferation and maturation into MCs are retained. These results are consistent with earlier findings that the BMCMC population from Chm:Cre mice has no specific Chm:Cre activity. Thus, the Cre-expression driven by the baboon α-chymase promotor only affects mature MMCs (Müsch et al., 2008).

After their egress from bone marrow, MC progenitors undergo differentiation and maturation in their peripheral target tissues, in which they have settled after their migration to vascularized tissues or serosal cavities. The progenitors will give rise to distinct MC phenotypes depending on the tissues' microenvironment. In rodents, MCs are classified into CTMCs and MMCs. CMCs and PMCs represent CTMCs, while MMC are present in mucosal epithelium and lamina propria. The most important finding in our study is that Chm-Cre;Mcl-1$^{fl/fl}$ mice exhibit a profound MMCs reduction in mucosal tissues (glandular stomach, intestine) (Fig. 10-12), without affecting the number of CTMCs in connective tissues (dorsal and ear skin, peritoneal cavity) (Fig. 8-9). Although our data indicate that the Chm-Cre;Mcl-1$^{fl/fl}$ mice exhibit a significantly reduced number of intestinal MMCs, the physiological number of MMCs in wild-type mice was extremely low (Fig. 9) (Grimbaldeston et al., 2005). However, an accumulation of the expansion of MMCs can be induced under certain conditions, such as parasitic infections (Hepworth et al., 2012; McDermott et al., 2003; Ruitenberg and Elgersma, 1976). Further studies will elaborate the behavior of intestine MMCs in Chm-Cre;Mcl-1$^{fl/fl}$ mice, and explore their role and relevance for a Th2-driven immune response against intestinal infections.

Interestingly, besides the mucosal tissues, we also observed reduced MMC numbers in the uterus of Chm-Cre;Mcl-1$^{fl/fl}$ mice (Fig. 13). Since earlier studies revealed that uMCs have a positive influence on placentation, remodeling of spiral arteries (SAs) as well as placenta size and fetal growth (Meyer et al., 2017b; Woidacki et al., 2015), (Meyer et al., 2017a; Woidacki et al., 2014b), we further examined the uterus function of Chm-Cre;Mcl-1$^{fl/fl}$ mice. Interestingly, no problem for placental or fetal development, at least not at the analyzed time points, have been found, except for a decreased placental thickness. It was recently demonstrated that the absence of both, CTMCs and MMCs,

leads to several reproductive impairments, as MC-deficient *Kit*$^{w\text{-}sh}$/*Kit*$^{w\text{-}sh}$ mice and selective depletion of Mcpt5$^{+}$ CTMCs exhibit a negative impact on fetal and placental development (Meyer et al., 2017a). Our study suggests that the selective absence of MMCs has no impact on fetal or placental development. It has been shown that uterine natural killer cells (uNKs) are able to overcome the absence of uMCs by counterbalancing their effects at the feto-maternal interface to promote SA remodeling and placentation (Meyer et al., 2017b). In addition, regulatory T cells (Treg) are known as key regulators of placental implantation. Tregs transferred into abortion-prone mice, which present with insufficient numbers of uMCs, restore SA remodeling and placental development by promoting the expansion of uMCs (Woidacki et al., 2015). Further studies will elaborate on the function of uterus NK cells and regulartoy T cells in Chm-Cre;Mcl-1$^{fl/fl}$ mice, because both of which may rescue the phenotype in Chm-Cre;Mcl-1$^{fl/fl}$ mice and thereby leading to normal implantation and placental development.

Although mammalian MCs were first described more than a century ago, their detailed functions remain to be elucidated. Today, mast cells are considered to be multifunctional immune cells implicated in several physiological and disease states. However, knowledge about the biological functions of MCs subtypes, especially MMCs is limited due to the lack of suitable models to investigate MMCs *in vivo*. Therefore, one of the most important challenges for therapeutic applications is the precise characterization and modulation of MCs in order to strengthen their physiological functions and to inhibit pathological influences.

In conclusion, our data provide a phenotypic characterization of the new mouse strain Chm-Cre;Mcl-1$^{fl/fl}$ mice, which is the first mouse strain that exhibits a specific reduction of MMCs, may represent a useful tool for mast cell research, and, especially, for analyzing MMCs functions *in vivo*.

# 5 List of figures

# 6 List of tables

# 7 References


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# 9 Eidesstattliche Versicherung

„Ich, Ying Luo, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: *Chymase-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of mucosal mast cells* selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

**Anteilserklärung an etwaigen erfolgten Publikationen**

Ying Luo hatte folgenden Anteil an der folgende Publikation:

Y. Luo, C. Zimmermann, S. Heydrich, J. Scheffel, O. Schmetzer, M. Metz, M. Maurer, F. Siebenhaar. Chymase-Cre; Mcl-1$^{fl/fl}$ mice exhibit reduced numbers of mucosal mast cells. Abstract. *44th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF) Göttingen, Germany, March 9–11, 2017*.

Beitrag im Einzelnen: Ying Luo performed the experiments (including animal tissues obtaining, cell culture, histological, flow cytometric experiments), collected the data (including histological and flow cytometric analysis) and wrote the draft in the above-mentioned publication.

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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