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The transcription factor C/EBPγ as a novel regulator in mast cell development and function

Transkripční faktor C/EBPγ jako nový regulátor vývoje a funkce žírných buněk

Diploma thesis

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Prohlášení

Prohlašuji, že jsem diplomovou práci vypracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Contents

List of abbreviations

ACK - Ammonium-Chloride-Potassium lysing buffer

AML – acute myeloid leukemia

ATB – antibiotics

BMCP – Basophil/mast cell progenitor

BMMCs – bone marrow-derived mast cells

bZIP – basic leucine zipper domain

CMPs - common myeloid progenitors

C/EBP – CCAAT/enhancer binding protein

CPA3 – carboxypeptidase A3

CSR – class switch recombination

DCs - dendritic cells

dNTPs - deoxynucleotide triphosphates

DTT - dithiotreitol

FACS - fluorescence-activated cell sorting

FBS – fetal bovine serum

GMPs – granulocyte monocyte progenitors

HSCs – hematopoietic stem cells

IFNγ – interferon gamma

IR – immune response

JAK – Janus kinase

KO - knockout

MCP – mast cell progenitor

MCs - mast cells

ME- β-mercaptoethanol

MEPs - megakaryocyte/erythrocyte progenitors

MITF - Microphthalmia-associated transcription factor

NEAA – non-essential amino acids

NK cells- natural killer cells

PBS – phosphate bufferd saline

PCMC – peritoneal cell-derived mast cell

SCF – stem cell factor

TAD – transactivation domain

TF – transcription factor

Ubb – ubiquitin

WT – wild type

7-AAD - 7-aminoactinomycin D

Abstract

Mast cells contribute to the activities of innate and adaptive branches of the immune system. They participate in pro-inflammatory responses to a wide range of pathogens, such as parasites, bacteria, and other foreign agents. These beneficial properties are in contrast to the contribution of mast cells to certain pathologies, such as asthma, allergy, autoimmune disorders, anaphylaxis, and systemic mastocytosis. Thorough knowledge of mast cell biology in health and disease is critical for the development of new therapeutic approaches. However, molecular mechanisms that control mast cell development and function are still incompletely defined. Our preliminary data indicate that the transcription factor C/EBPγ is a key player in mast cell biology. Here, using in vitro and in vivo models, we determine how C/EBPγ regulates the commitment of hematopoietic progenitors towards mast cells, and modulates mast cells function. These efforts provide novel insights to the role of C/EBPγ in hematopoiesis, and contribute to a better understanding of the mechanisms governing mast cell biology.

Key words

Mast cells, C/EBPγ, transcription factors, bone marrow-derived mast cell cultures, mast cell development, *Cebpg* conditional knockout mice

Abstrakt

Žírné buňky jsou důležitou součástí vrozeného i adaptivního imunitního systému. Podílejí se na zánětlivých reakcích namířených proti široké škále patogenů, jako jsou parazité, bakterie a další typy cizorodých agens. Na druhou stranu žírné buňky přispívají k řadě patologických stavů, mezi něž patří například astma, autoimunitní choroby, anafylaxe nebo systémová mastocytóza. Poznání biologie žírných buněk je proto zásadní pro vývoj nových přístupů k léčbě výše zmíněných chorob. Navzdory tomu je naše znalost molekulárních mechanismů, které vývoj a funkci žírných buněk regulují, stále neúplná. Naše předběžné výsledky ukazují, že jedním z klíčových elementů v biologii žírných buněk je transkripční faktor C/EBPγ. Za použití *in vitro* a *in vivo* modelů určíme, jakým způsobem C/EBPγ reguluje diferenciaci hematopoetických progenitorů do mastocytární linie a jaký má vliv na funkci žírných buněk. Tato studie přinese nové poznatky o úloze C/EBPγ ve vývoji žírných buněk a přispěje k lepšímu pochopení mechanismů řídících biologii žírných buněk.

Klíčová slova

Žírné buňky, C/EBPγ, transkripční faktory, vývoj žírných buněk, *Cebpg* kondicionální knockout, kultury žírných buněk odvozené z kostní dřeně

1. Introduction

Mast cells (MCs) are critical effector cells in allergies and play an important role in the immune response. They belong to the innate part of immunity, even though they influence also the adaptive part (Abraham and St.John, 2010; Galli and Tsai, 2012; da Silva, Jamur and Oliver, 2014). The deregulation in MCs development can lead to MCs disorders such as cutaneous or systemic mastocytosis or MCs leukemia (Georgin-Lavialle *et al.*, 2013; Pardanani, 2016; Le *et al.*, 2017). Hence the proper understanding of MCs development can help to better understand the orchestration of immune responses (IR) and it can also help to better understand the cure of MC pathologies. The exact origin of MCs and description of the developmental pathway from hematopoietic stem cells (HSCs) through progenitor stages to mature MCs is still a question (Dahlin *et al.*, 2018). It has been reported that MCs have a common progenitor with basophils (BMCPs) and that basophil/MC fate choice decisions are strictly regulated by precise expression of many transcription factors (TFs) (Chen *et al.*, 2005; Qi *et al.*, 2013; Dahlin *et al.*, 2018; Grootens *et al.*, 2018).

There are two TFs among others which seem to have an eminent role in this process. One of them is CCAAT/enhancer binding protein α (C/EBPα) and the second one is Microphthalmia-associated transcription factor (MITF). These two TFs regulate each other expression in antagonistic manner in which one suppresses the expression of the other. High levels of C/EBPα leads the BMCPs to basophil lineage whereas high levels of MITF leads them towards MCs lineage (Kitamura *et al.*, 2001; Qi *et al.*, 2013). Importantly, it was reported that *Cebpg* is a direct C/EBPα target gene (Alberich-Jordà *et al.*, 2012). In addition, it is known that transcription factors from C/EBP family can form heterodimers with each other and by this interaction they can modulate each other's function (Ramji and Foka, 2002; Huggins *et al.*, 2013). These observations together with the fact, that *Cebpg* is highly expressed in activated mast cells, lead us to hypothesise that C/EBPγ could play a critical role in mast cell development and function. To test our hypothesis we use conditional C/EBPγ knockout (KO) mice and employ them to establish *in vitro* bone marrow-derived mast cell cultures (BMMCs). We characterize C/EBPγ KO BMMCs and compare them to WT BMMCs, profile the expression of several TFs, and design luciferase reporter constructs to investigate how C/EBPγ might regulate expression of other TFs.

2. Hematopoiesis

Hematopoiesis is the process by which all blood and immune cells are produced. This process takes place mainly in the bone marrow. HSCs, a rare population of cells located in the bone marrow, are responsible for the maintenance of hematopoiesis during our life-time. HSCs are characterized by three main abilities - multipotency, self-renewal and quiescence (G_0 phase). These abilities are influenced by other hematopoietic cells as well as by cellular components of the bone marrow niche. Together, they will determine HSCs maintenance and fate. During the development of the blood system, HSCs give rise to two main cell lineages which divide and further differentiate into the distinct cell types – myeloerythroid lineage which gives rise to granulocytes, megakaryocytes and erythrocytes, and lymphoid lineage which gives rise to B-, T- and NK cells (*Figure 1*) (Rieger and Schroeder, 2012).

In ontogeny, the first real HSCs originate from the aorta-gonado-mesonefros and placenta (Ottersbach and Dzierzak, 2005; Boisset *et al.*, 2010; Ivanovs *et al.*, 2011) and are products of hemogenic endothelium as was proved by single cell imaging (Eilken, Nishikawa and Schroeder, 2009). Around birth, mature HSCs migrate to bone marrow and nest there. The nesting and maintaining is enabled by the crosstalk between HSCs and the bone marrow niche (Sugiyama *et al.*, 2006). Specifically, adult HSCs reside mainly in highly vascularized endosteum to which they migrate due to interaction between ligand and receptor. Relevant examples of this interaction are (1) the chemokine CXCL12 (produced by stromal cells and endothelium) and CXCR4 (expressed on surface of HSCs), and (2) stem cell factor (SCF) (produced by same cells as CXCL12) and c-kit receptor on HSCs (Christensen *et al.*, 2004; Sugiyama *et al.*, 2006; Ciriza *et al.*, 2013). Even though HSCs reside in highly vascularized endosteum their access to oxygen is limited. It was shown, that oxygen saturation in BM is about 87,5% (Harrison *et al.*, 2002). In order to maintain their function HSCs gain energy mainly through anaerobic metabolism. Several studies have shown that HSCs fulfill their role better in these conditions than in aerobic ones (Cipolleschi, Dello Sbarba and Olivotto, 1993; Danet *et al.*, 2003; Hermitte *et al.*, 2006; Suda, Takubo and Semenza, 2011; Anthony and Link, 2014).

Defects during hematopoiesis can cause blood cell disorders. The most severe are lymphomas and leukemias, which are malignancies affecting distinct cell types. Lymphomas affect leukocyte and lymphoid system whereas leukemias affect leukocytes

in bone marrow (Charles and Sawyers, 1999; Saultz and Garzon, 2016; Avellino and Delwel, 2017; Sehn *et al.*, 2018). There are many laboratories worldwide studying these diseases.

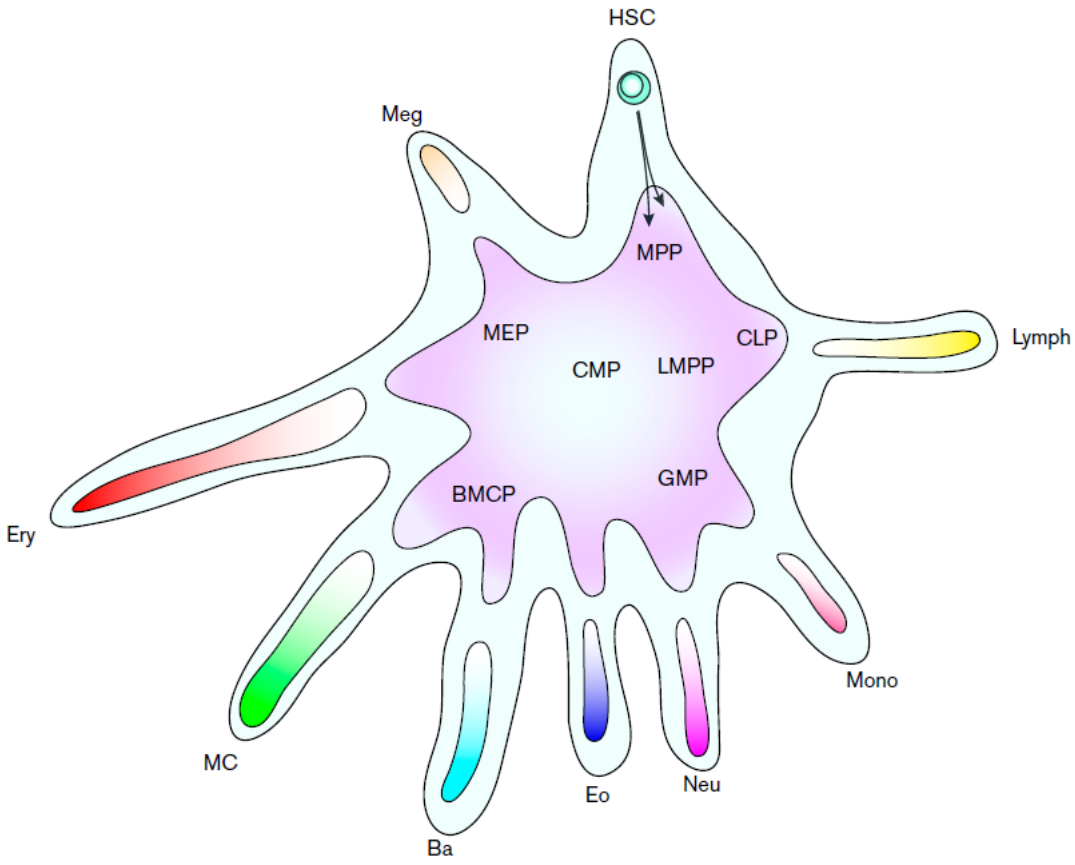


Figure 1. The new model of hematopoiesis. *HSC – hematopoietic stem cell, MPP – multipotent progenitor, CLP – common lymphoid progenitor, MEP – myelo/erythroid progenitor, CMP – common myeloid progenitor, LMPP – lymphoid multipotent progenitor, GMP – granulocyte/monocyte progenitor, BMCP – basophil/mast cell progenitor, Lymph – lymphocytes, Mono – monocytes, Neu – neutrophils, Eo – eosinophils, Ba – basophils, MC – mast cell, Ery – erythrocytes, Meg – megakaryocytes*
(Dahlin et al., 2018; Grootens et al., 2018)

3. Mast cells

MCs are immune cells belonging to the myelo-erythroid lineage. MCs contain intracellular granules with a broad spectrum of biochemical substances (interleukines, histamine, proteoglycans). These granules are metachromatic after staining, and therefore can be visualized under the microscope. They were first described by Paul Ehrlich almost

hundred and fifty years ago (Ehrlich 1878). For long time it was thought, that the role of MCs was limited to allergies and hypersensitivity (Amin 2012), and it was consequently proposed they played a negative role in immunity. Nevertheless from the evolutionary point of view, it was speculated that there had to be some benefits to keep MCs in the organism, otherwise they would have been removed during years of vertebrate development. Indeed during the last decades this point of view was supported by researchers, when more and more beneficial functions were attributed to MCs. Several examples include the role that MCs play in the protection against parasites and venoms (Mukai *et al.*, 2016; Starkl *et al.*, 2016), and in the orchestration of IR (Abraham and St.John, 2010).

There are two major subpopulations of murine MCs, which are distinct in their localization; connective tissue MCs and mucosal MCs. Mucosal MCs are localized in the intestine or the airways, whereas connective tissue MCs are localized in the skin or the peritoneal cavity. These two subpopulations are also distinct in the content of their granules. Mucosal MCs contain lower levels of histamine in their granules compared to connective tissue MCs (Nakano *et al.*, 1985; Wasserman, 1990; Galli *et al.*, 2005; Galli, Borregaard and Wynn, 2011). Similar findings have also being described in human mast cell biology. In human there are two major subpopulations of MCs distinguished by positivity for chymase and tryptase or tryptase alone. The chymase/tryptase positive MCs are similar to murine connective tissue MCs, while tryptase positive MCs are similar to murine connective tissue type MCs (Wasserman, 1990; Cildir *et al.*, 2017).

Mature MCs are connective or mucosal tissue resident cells expressing an extensive amount of receptors and surface molecules. These cells are also able to produce large amounts of chemokines, cytokines and growth factors (Mukai *et al.*, 2018). Among the surface molecules, the transmembrane tyrosine kinase c-Kit (also called CD117) and IgE receptor FcƐRI are the most prominent ones and are used as markers of maturation in flow cytometry.

C-kit act as a receptor for stem cell factor (SCF), a growth factor which is important for MCs survival *in vivo* and *in vitro*. Binding of SCF to c-kit leads to suppression of apoptosis in mature MCs (Iemura *et al.*, 1994; Mekori, Oh and Metcalfe, 1995). However SCF is dispensable for MCs progenitors (Dahlin *et al.*, 2017).

FcƐRI is high affinity IgE receptor. The complex of FcƐRI - IgE serves as a main trigger of MCs response to external stimuli. Many of these complexes have to be cross-linked to activate MCs. The crosslinking of receptors on the cell surface leads to MCs degranulation and cytokine production (Kitaura *et al.*, 2003; Mukai *et al.*, 2016; Bulfone-Paus *et al.*, 2017). The development and function of MCs are going to be described in following chapters in more detail.

3.1 Mast cell development

As previously mentioned, MCs originate in the bone marrow, more precisely from the myelo-erythroid lineage. But unlike other hematopoietic cells, they do not mature in bone marrow nor blood, but in peripheral tissues (skin, gut mucosa, lungs) (Dahlin and Hallgren, 2015). It was shown in recent years that MCs have a common progenitor with basophils (BMCP), but the whole developmental process from HSCs to mature MCs is not fully understood yet (Chen *et al.*, 2005; Qi *et al.*, 2013; Dahlin *et al.*, 2018; Grootens *et al.*, 2018).

In the murine embryogenesis, the MCPs originate from yolk sac and migrate to skin. However, during the development of embryo, the yolk sack derived MCs are replenished with MCs from bone marrow. Later on in development, these yolk sack MCs are fully replaced by definitive MCs in adult (Gentek *et al.*, 2018). However, whether definitive MCs are produced directly in bone marrow or in other tissues stays unclear (Arinobu *et al.*, 2005; Dahlin *et al.*, 2018; Grootens *et al.*, 2018).

There are mainly two distinct research lines addressing the origin of MCs. One believes that MCs origin from spleen, whereas the second believes that they origin from the bone marrow. More than ten years ago a population of BMCPs in spleen was described (Arinobu *et al*., 2005). Authors characterize the population as Lin^- $cKit^+$ $Fc\gamma RII/III^{hi}$ $\beta7^{hi}$ cells and demonstrated that these cells give rise exclusively to basophils or MCs (Arinobu *et al.*, 2005). The β7 integrin is used as a marker for MCs progenitor enrichment, because β7 integrin serves as a molecule mediating migration to peripheral tissue (Qi *et al.*, 2013). Interestingly, a population of Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ (HSCs) cells expressing β7 integrin was not found in the bone marrow (Arinobu *et al.*, 2005). However, the β7 integrin was expressed by some other populations in the bone marrow (common myeloid progenitors-CMPs, megakaryocyte/erythrocyte progenitors-MEPs, granulocyte/monocyte progenitors-GMPs). Last but not least was the identification of MCPs in intestine. The intestinal

MCPs were found in Lin$^-$ CD45$^+$ mononuclear population of cells. These cells give rise only to MCs colonies (Arinobu *et al*., 2005). These data leads to conclusion, that GMPs migrate from bone marrow to spleen or intestine where they give rise to MCPs (*Figure 2*).

On the other hand, in a same year the group of Galli identified BMCPs also in bone marrow (Chen *et al.*, 2005). The authors firstly cultured progenitor cells (Lin$^-$ Sca-1$^-$ cKit$^+$) from bone marrow to produce bone marrow derived MCs (BMMCs). After series of sorting, the authors identified MCPs as a β7$^+$ T1/ST2$^+$, because only this population gave rise exclusively to MCs *in vitro*. To proof the MCPs function and ability to produce MCs, authors transplanted these MCPs into lethally irradiated MCs deficient mice (*Kit*$^{W\text{-}sh}$/ *Kit*$^{W\text{-}sh}$). This population of MCPs was able to repopulate *Kit*$^{W\text{-}sh}$/ *Kit*$^{W\text{-}sh}$ mice with functional MCs (Chen *et al.*, 2005). Recently, similar findings that BMCPs can be found in bone marrow were also observed by other groups (Qi *et al.*, 2013; Dahlin *et al.*, 2018).

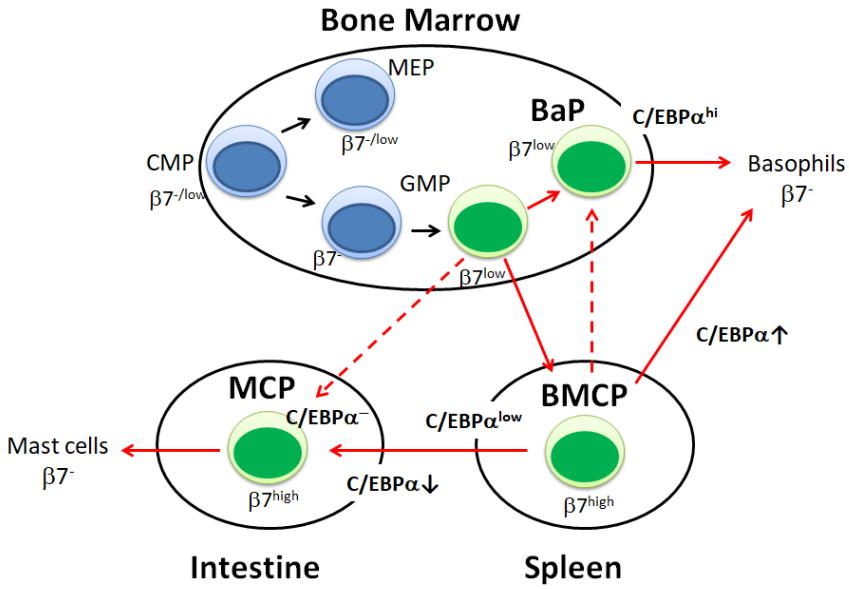


Figure 2. The proposed model of basophil/MCs development *by Arinobu et al., 2005. CMP – common myeloid progenitor, MEP – myelo/erythroid progenitor, GMP – granulocyte/monocyte progenitor, BaP – basophil progenitors, MCP – mast cell progenitor, BMCP – basophil/mast cell progenitor*

Consistent with previously mentioned results are data from a large single cell RNA sequencing study, in which the BMCPs were identified also in bone marrow. In this study, authors performed the single-cell RNA sequencing of more than forty four

thousands Lin^- $cKit^+$ and Lin^- $Sca\text{-}1^+$ $cKit^+$ cells in order to decipher the blood cells developmental entries with focus on MCs. They found bone marrow BMCPs and characterize them at a single-cell level. The bone marrow BMCPs were found in a population of cells phenotypically characterized as Lin^- $Sca\text{-}1^-$ $cKit^+$ $\beta7^{hi}$ $CD16/32^{hi}$. These cells were sorted out and cultivated in medium with myeloid cytokines in which they predominantly developed into MCs (Dahlin *et al.*, 2018).

3.2 Mast cell function

MCs belong to the innate part of immunity and play key roles in many immunological processes. Among these the role in allergies and anaphylaxis is the best known. However, there are more functions of MCs in the organism (Galli and Tsai, 2012; Mukai *et al.*, 2016; Starkl *et al.*, 2016).

To better understand the role in immunity it is important to know where MCs reside. As mentioned above, MCs are localized in peripheral tissues. These tissues can be skin, gut mucosa and mucosa in airways – in other words, sites which are exposed to outer environment (Collington, Williams and Weller, 2011). Together with dendritic cells (DCs) and neutrophils, MCs are the first cells which meet pathogens invading the organism. It means that they are the first line of defense (Uhl *et al.*, 2016). MCs can contribute to defense by regulation of vascular permeability, effector cells recruitment, and modulation of IR. This is achieved by production of cytokines and other bioactive compounds (Mazzoni *et al.*, 2006; Amaral *et al.*, 2007; Abraham and St.John, 2010). Some of the MC mediators are histamine, serotonin, heparin, proteases, VEGF, leukotriens, prostaglandins, pyrogens, Th2 cytokines, chemokines, and antibacterial peptides (Marshall, 2004; Amin, 2012; Voehringer, 2013; Joulia *et al.*, 2015). There are many more MC mediators but it is behind the scoop of this brief introduction.

As described above, the role in allergies and type I hypersensitivity is the best known. The mechanism which is responsible for these pathologies include 1) sensitization of the organism, and 2) class switch to IgE antibody. Sensitization occurs when the organism is exposed to a non-harmful antigen such as food (Valenta *et al.*, 2015), dust, or mite (Miller, 2018). In the first step the antigen is engulfed by DCs and is presented to helper T lymphocytes (Th lymphocyte). If this presentation occurs under stimulation of IL-4, IL-5, IL-9 and IL-13 cytokines it leads to Th2 phenotype of lymphocytes. In the next step, Th2 lymphocytes provide co-stimulatory signals to B-cells to produce antibodies. This cell to

cell interaction is occurring in a Th2 cytokines rich environment, which leads to class switch recombination (CSR) in B-cells and production of IgE antibodies (Siebenkotten *et al.*, 1992; Sandra Delphin, 1995; Mazzoni *et al.*, 2006; Poulsen and Hummelshoj, 2009; Galli and Tsai, 2012). After the allergen specific IgE is produced, it can binds to FcεRI on MCs and the organism is sensitized. If the organism is exposed to this allergen again, MCs will respond rapidly by releasing mediators from granules. This action is called allergic reaction.

Another role of the MCs in organism is the protection against venoms and toxins, a nice example of the interplay between innate (MCs) and adaptive immunity (IgE). As was mentioned above, MCs granules contain many different bioactive compounds such as proteases (tryptase, chymase, carboxypeptidase). Carboxypeptidase A3 (CPA3) is known for MCs specific expression and its role in protein based venoms digestion (Haas and Sasse, 1979; Wernersson and Pejler, 2014, www.ncbi.com). It was shown that IgE or FcεRI deficient mice have lower survival after exposure to Russel´s viper venom compared to healthy control (Philipp Starkl *et al.*, 2016). The protective role of MCs was shown also after exposure to scorpion, Gila monster and other snakes and honey bee venoms (Metz *et al*., 2006; Schneider *et al.*, 2007; Akahoshi *et al.*, 2011).

Last but not least, MCs provide protection against endogenous and exogenous parasites such as nematodes, protists and ticks (Pennock and Grencis, 2006; Voehringer, 2013; Lu and Huang, 2017). The defense against parasites is obtained through Th2 IR. MCs as a possible producer of IL-4 and IL-13 cytokines, help to set Th2 environment. Th2 IR leads to production of IgE and IgG1 antibodies (Siebenkotten *et al.*, 1992; Mazzoni *et al.*, 2006; Galli and Tsai, 2012). MCs are also one of the effector cells, because of their ability to bind IgE with high affinity receptors (Bulfone-Paus *et al.*, 2017). However, not every IgE molecule is parasite specific. There is controversy if this issue plays a negative role in parasite clearance or not. For example in a case of mice infected with *Trichinella spiralis,* it seems that the ratio of specific/non-specific IgE in serum is important to handle infection. In some serum levels IgE can help to clear the parasite, in others enhance its survival ability (Gurish *et al.*, 2004; Watanabe, 2014). Nevertheless, in some cases of parasite infections, the MCs can play a negative role. In a study of co-infection with non-typhoidal *Salmonella species* and *Plasmodium falciparum,* the infection is much more severe in wild type mice than in MCs deficient mice. The mechanism underlying this

phenotype is histamine release by MCs and thus higher gut mucosa permeability, which leads to severe bacteraemia (Potts *et al.*, 2016).

3.3 Mast cell disorders

The two major types of MCs disorders are mastocytosis or MC leukemias (Georgin-Lavialle *et al.*, 2013; Pardanani, 2016; Le *et al.*, 2017). Mastocytosis is a rare disorder affecting less than 200 000 people in United States (Brockow, 2014). However there are not many epidemiological studies of this disease. The only epidemiological study was done in Denmark and the prevalence of systemic mastocytosis was 1 to 10 000 people (Cohen *et al.*, 2014), thus the actual prevalence worldwide could be different. Mastocytosis can be subdivided into seven groups (*Table 1*) (Tamay and Ozceker, 2016).

Type of mastocytosis	Reference
1. Cutaneous mastocytosis	
-Maculopapular type (urticarial pigmentosa)	Jarnum and Zachariae, 1967; Brockow *et al.*, 2002
-Diffuse cutaneous mastocytosis	Selva Folch *et al.*, 2016
-Solitary mastocytosis	Cohen, 2016
2. Indolent systemic mastocytosis	Mikkelsen *et al.*, 2014
3. Systemic mastocytosis in association with a clonal hematological non-mast cell lineage disease	Wang *et al.*, 2013
4. Aggressive systemic mastocytosis	Valent *et al.*, 2003
5. Mast cell leukemia	Georgin-Lavialle *et al.*, 2013
6. Mast cell sarcoma	Monnier *et al.*, 2016
7. Extracutaneous mastocytosis	Castells, 2006

Table 1. Classification of mastocytosis, adapted from Tamay and Ozceker, 2016

Mastocytosis is characterized by distribution of abnormal neoplastic MCs into skin and organs (Le *et al.*, 2017). The distinct disease subtypes differ in prognosis, ranging from non-affecting asymptomatic to poor prognosis aggressive types (Valent *et al.*, 2003; Magliacane, Parente and Triggiani, 2014). The abnormal and clonal development of MCs in adults is, in the majority of cases, caused by a gain of function mutation in the tyrosine kinase receptor *KIT* (D816V) (Piao and Bernstein, 1996; Schumacher, Elenitoba-Johnson

and Lim, 2008). However, not every mastocytosis, especially in children, is caused by the D816V point mutation. There is evidence of other gain of function mutations in *KIT* leading to disease (Bodemer *et al.*, 2010).

The treatment of mastocytosis differs the same as the symptoms do, but in general histamine antagonist and *KIT* inhibitors are used most often (Magliacane, Parente and Triggiani, 2014; Pardanani, 2016).

One of the systemic type of mastocytosis is MC leukemia (Georgin-Lavialle *et al.*, 2013). This disease is very rare and less than 1% of all mastocytosis are MC leukemias (Lim *et al.*, 2009). MC leukemia is characterized by the same symptoms as systemic mastocytosis with addition of leukemic infiltration of abnormal MCs to BM, blood or extracutaneous organs. MC leukemia is very aggressive disease thus the prognosis is very poor with six months of median survival (Valent *et al.*, 2014). The treatment varies from using tyrosine kinase inhibitors (Longley, Reguera and Ma, 2001; Georgin-Lavialle *et al.*, 2013) to chemotherapy (Samorapoompichit *et al.*, 2003) and BM transplantation (Chen *et al.*, 2003; Georgin-Lavialle *et al.*, 2013; Bauer, Longo and Yang, 2017). However all before mentioned approaches have weak points and do not work properly in all cases (Lin, Lachmann and Nagler, 2002; Samorapoompichit *et al.*, 2003). To conclude, MC leukemia is rare and aggressive disease with poor prognosis and difficult treatment.

4. Transcription factors as regulators of mast cell development and function

MCs have a common progenitor with basophils and fate choice decisions are made under the control of many TFs. It was shown that two of the main players are C/EBPα and MITF (Arinobu *et al.*, 2005; Qi *et al.*, 2013). However there are many more TFs which have an indispensable role during mast cell development (*Table 2*). The function of these factors, which leads the way from HSCs to mature MCs, is going to be described in the following chapters (*Figure 4*).

Transcription factor	Reference (in relation to MCs)
C/EBPα	Qi *et al.*, 2013
GATA1	Migliaccio *et al.*, 2003; Inage *et al.*, 2014
GATA2	Inage *et al.*, 2014; Yapeng Li, Xiaopeng Qi, Bing Liu, 2015
GATA3	Taghon, Yui and Rothenberg, 2007
MITF	Qi *et al.*, 2013
PU.1	Inage *et al.*, 2014
STAT5	Yapeng Li, Xiaopeng Qi, Bing Liu, 2015

Table 2. ***The list of transcription factors playing key role in development of MCs***

4.1 C/EBP family

C/EBP family of TFs consist of six members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (Ramji and Foka, 2002). One of the main features of this protein family is the basic leucine rich zipper domain (bZIP) *(Figure 3)*. Homo/heterodimerization with other TFs from the C/EBP family or other bZIP containing proteins is mediated by the leucine rich zipper domain portion. The basic region itself is responsible for DNA binding. The bZIP domain is common for all the members, except C/EBPζ, which lacks the basic region. The second important structural feature in the C/EBP family is transactivation domain (TAD). This domain is present in all members except C/EBPγ (Cooper *et al.*, 1995) and C/EBPζ (Ramji and Foka, 2002) and is responsible for repression and/or activation of C/EBP target genes.

4.1.1 C/EBPα

The TF C/EBPα has an important role in granulopoiesis. It was shown that C/EBPα KO mice lack granulocytes (Radomska *et al.*, 1998; Iwasaki *et al.*, 2006; Ma *et al.*, 2014). Importantly, deregulation of C/EBPα is a frequent cause of acute myeloid leukemia (AML). The mutation in C/EBPα open reading frame (ORF) is linked to AML in 7% to 15% of human cases (van Waalwijk van Doorn-Khosrovani *et al.*, 2003; Alberich-Jordà *et al.*, 2012; Avellino and Delwel, 2017). Several other mechanisms have been described to alter C/EBPα function, but they beyond the scope of this thesis.

C/EBPα also plays an irreplaceable role in MC versus basophil differentiation. The high levels of C/EBPα lead the BMCPs towards basophils and inhibit MCs differentiation (Qi *et al.*, 2013). Enforcement reduction of C/EBPα expression in BMCPs disrupt basophil

development in C/EBPα $^{\Delta/\Delta}$ mice *(*Zhang *et al., 2004;* Iwasaki *et al.*, 2006). The function of C/EBPα in basophil/MC fate choice decision was also proved in other experimental designs (Qi *et al.*, 2013).

To conclude, although C/EBPα is a well-known TF critical for granulocytic differentiation, recent scientific reports demonstrate it has a decisive role in BMCP fate, favoring basophil production at the expenses of mast cell differentiation.

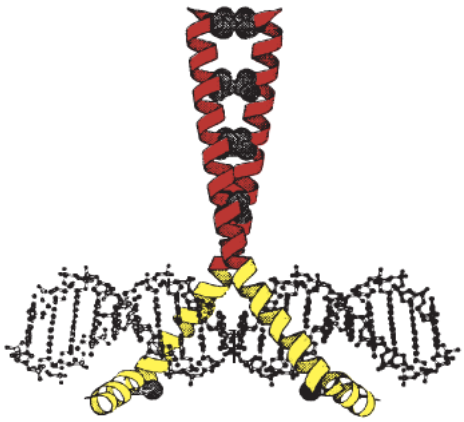


Figure 3. ***Illustration of C/EBP dimerization and DNA binding.*** *The leucine zipper part is responsible for homo/heterodimerization (red) and basic region is responsible for DNA binding (yellow).* (Johnson, 2005)

4.1.2 C/EBPγ

The TF C/EBPγ lacks transactivation domain, however C/EBPγ is able to suppress the transactivation of other TFs by alternative mechanisms. This ability is enabled by hetero-dimerization with other TFs from the C/EBP family (Cooper *et al.*, 1995; Parkin *et al.*, 2002).

C/EBPγ is ubiquitously expressed in immune cells, however, there are only few studies about its role in hematopoiesis (Huggins *et al.*, 2013). One study linked C/EBPγ function to AML. Using 526 AML patient samples, a small subset of cases with silenced *CEBPA* and up-regulated *CEBPG* was described (Alberich-Jordà *et al.*, 2012). The data showed, that *CEBPG* needs to be down-regulated in order to maintain neutrophilic differentiation and led to characterization of C/EBPγ as a protooncogene in AML (Alberich-Jordà *et al.*, 2012).

A different study was interested in *in vivo* roles of C/EBPγ. They showed that straight C/EBPγ KO mice have high neonatal mortality and die few hours after birth probably due to emphysematous changes in lungs (Kaisho *et al.*, 1999). The data also showed that C/EBPγ has an irreplaceable role in the functionality of natural killer cells (NK cells). The C/EBPγ$^{-/-}$ bone marrow chimeras had impaired splenic NK cell activity and reduce ability to produce INFγ. To conclude, the data showed an important role of C/EBPγ in NK cell function (Kaisho *et al.*, 1999). Last but not least, it was recently reported that C/EBPγ plays a role in inhibition of cellular senescence as was shown on mouse embryonic fibroblasts (Huggins *et al.*, 2013).

4.2 GATA family

The GATA family of TFs consists of six members in vertebrates (GATA1, GATA2, GATA3, GATA4, GATA5 and GATA6) and is based on the highly conserved motif by which they bind to DNA. These proteins contain one or two zinc fingers followed by a basic region (Merika and Orkin, 1993; Lowry and Atchley, 2000). The first three members of this family (GATA1, GATA2 and GATA3) have a role in the hematopoietic system and are going to be described here (Ferreira 2015) (Ferreira, Ohneda, Yamamoto, 2005). The disruptions in proper expression of GATA1 or GATA2 may lead to blood malignancies such as chronic myeloid leukemia in the case of GATA2 alterations and acute megakaryocytic leukemia in Down syndrome patients in case of GATA1 defects (Rosenbauer and Tenen, 2007; Zhang *et al.*, 2008). The GATA3 pathologies are more linked to breast cancer than to malignancies of blood (Chou, Provot and Werb, 2010).

4.2.1 GATA1

The TF GATA1 is one of the key regulators of hematopoiesis. GATA1 influences the development of different blood cells such as DCs, eosinophils, erythroid cells and megakaryocytes (Ferreira 2015, Gutiérezz 2007) (Ferreira, Ohneda, Yamamoto, 2005; Gutiérrez *et al.*, 2007). The deregulation of GATA1 can lead to Down syndrome-acute megakaryocytic leukemia (Rosenbauer and Tenen, 2007). In mice it was shown that GATA1 deficiency leads to embryonic death at day E12.5 (Takahashi *et al.*, 1997) and conditional ablation of GATA1 in adult mice leads to defects in erythropoiesis (Gutiérrez *et al.*, 2008). However the need of GATA1 in MCs development and differentiation is not clear. Experiments on conditional GATA1 KO mice showed that GATA1 ablation has a minimal effect on number and distribution of mature MCs (Ohneda *et al.*, 2014).

Nevertheless some experiments using GATA1 KO mice showed defects at all stages of MC differentiation (Migliaccio *et al.*, 2003). These discrepancies can be explained by the use of different mouse models in each study. In the first study, used mice model lacks the whole *Gata1* coding region, whereas in the second study, authors used the knock-down *Gata1* model with intact coding sequence (Ohneda *et al.*, 2014). The proper role of GATA1 in MCs development and function is still not fully understood.

4.2.2 GATA2

The TF GATA2 has an important role in development of blood progenitor cells and HSCs maintenance (Rodrigues *et al.*, 2012). For proper maturation of HSCs to distinct hematopoietic lineages the gradual reduction of GATA2 expression is crucial. However the full GATA2 ablation in mice leads to disrupted HSCs production and to embryonic lethality (Tsai and Orkin, 1997; Ling *et al.*, 2004; Vicente *et al.*, 2012). GATA2 can be transcribed from two alternative promoters IS and IG depending on cell type (Minegishi *et al.*, 1998). Based on experimental data, GATA2 seems to be more important for regulation of MC specific genes than GATA1 (Tsai and Orkin, 1997; Ohneda *et al.*, 2014). GATA2 also plays a critical role in differentiation of basophils and MCs from their common progenitor (Li, Qi, Liu, 2015). The orchestration of expression with other TFs in development seems to be critical for proper development of MCs. It was shown, that suppressed expression of C/EBPα in granulocyte monocyte progenitors (GMPs), followed by the expression of GATA2 in later stages leads the cells towards basophil/MCs lineage. On the other hand, if C/EBPα was expressed, GATA2 expression leads the cells into eosinophils (Iwasaki *et al.*, 2006). Last but not least, GATA2 is crucial for the expression of FcεRIα in either basophils or MCs and also for expression of c-Kit in MCs. GATA2 cooperates with STAT5, which is going to be described further down in this chapter (Li, Qi, Liu, 2015).

4.2.3 GATA3

The TF GATA3 has a multiple function in hematopoiesis and especially in T cell development, more precisely in Th2 lineage (Ho, Tai, Pai 2009; Wang *et al.*, 2013). Additionaly, it was suggested that GATA3 regulates HSCs maintenance and self-renewal (Ku *et al.*, 2012; Frelin *et al.*, 2013), even though the role of GATA3 in HSC self-renewal is controversial. Experiments with GATA3 conditional KO mice showed, that GATA3 has a redundant role in HSC self-renewal. The mice with ablated GATA3 showed no

significant differences in repopulation pattern of blood cells compared to controls (Buza-Vidas *et al.*, 2011). On the other hand, experiments with *Gata3*-null mutant mice revealed that the number of long term HSC were reduced to 49% compared to controls. However, these fifferences can be caused by observing animals of different age (newborns versus adults) or by usage of different murine strains (Ku *et al.*, 2012).

It is interesting that GATA3, the "master " transcriptional regulator of T lymphocytes, can lead T cells also towards MC lineage (Taghon, Yui and Rothenberg, 2007). The link between MCs and T cells was proposed long ago (Burnet, 1977). For a proper development of MCs from their progenitors it is important GATA3 cooperation with Hes-1 and up-regulation of their expression. The mechanism behind this process relies on Notch2 signaling which is another link to T cell biology (Deftos, 2000; Sakata-Yanagimoto *et al.*, 2008).

4.3 PU.1

PU.1 TF has a role in monocyte and B cell lineage development, nevertheless the role in MCs is also important (Scott *et al.*, 1994; Takemoto *et al.*, 2010; Inage *et al.*, 2014). The correctly timed and orchestrated expression of various TFs is important for MCs development, which is also true for PU.1. This TF has two distinct roles in MCs, one in development and the second one in receptor expression (Takemoto *et al.*, 2008; Takemoto *et al.*, 2010; Inage *et al.*, 2014).

The developmental role is characterized by regulation of GATA1. This regulation is mediated through direct binding of GATA1 to PU.1 (Takemoto *et al.*, 2010). The finding that PU.1 deficient cells do not express any markers of MCs differentiation is in agreement with the need of PU.1 for correct MCs development (Walsh *et al.*, 2002).

The second important process regulated by PU.1 is expression of the α chain part of FcεRI. This was addressed by experiments based on siRNA PU.1 knockdown in human MC line LAD2. After the transfection of siRNA, which downregulates PU.1, the expression of α-chain FcεRI was decreased. The expression of FcεRI is not regulated only by PU.1, since GATA1 and GATA2 knockdowns by siRNA had also an impact on receptor expression (Inage *et al.*, 2014).

4.4 STAT5

STAT5 belongs to STAT family of TFs, which consist of 7 members: STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5a and STAT5b) and STAT6 (Teglund *et al.*, 1998; Lim and Cao 2006). The STATs have a role in cytokine signaling (Teglund *et al.*, 1998; Shuai, 1999), hematopoiesis (Staerk and Constantinescu, 2012; Li, Qi, Liu, 2015; Seif *et al.*, 2017), growth hormone signaling (Herrington *et al.*, 2000) and also in oncogenesis and blood malignancies (Bromberg and Darnell, 2000; Benekli *et al.*, 2003; Clevenger, 2004). STAT proteins are localized in the cytoplasm of cells, where they mediate signal transduction from extracellular space into nucleus. The translocation of STAT proteins from the cytoplasm to the nucleus is enabled by Janus kinase (JAK)) – STAT pathway. STAT is phosphorylated and dimerized with another STAT in order to translocate to the nucleus. In the nucleus, STAT activates or represses transcription of target genes (O´Shea, Gadina and Schreiber, 2002; Rawlings, 2004).

The developmental role of STAT5 is linked to GATA2 (Li, Qi, Liu, 2015). STAT5 controls GATA2 expression through direct binding to its promoter and thus STAT5 can regulate the BMCP fate decision. Another supportive data to STAT5 importance in MCs development are experiments with STAT5 deficient mice (Shelburne *et al.*, 2003). These mice lack tissue MCs *in vivo* and BMMCs have an altered maturation and survival *in vitro*). STAT5 is not only linked to GATA2, but also to MITF and C/EBPα. These two TFs are downstream molecules of STAT5 signaling (Qi *et al.*, 2013). STAT5 function in MCs is not restricted only to development (Shelburne *et al.*, 2003; Dahlin and Hallgren, 2015). It is not surprising that the proper STAT5 function is needed also for correct signaling through FcεRI (Barnstein *et al.*, 2006).Taken together, STAT5 is critical for proper MCs development and function.

4.5 MITF

Microphthalmia-associated transcription factor (MITF) has at least seven isoforms in mice and five in human, each of them originates from a distinct promoter. There are two MC specific isoforms of MITF in mice (Amae *et al.*, 1998; Yasumoto *et al.*, 1998; Shibahara, 2001; Oboki *et al.*, 2002; Li *et al.*, 2010; Takemoto *et al.*, 2010). The protein is characterized by several molecular structures such as transactivation domain, basic domain, helix-loop-helix structure and leucine zipper. The expression of MITF is important for the development of melanocytes, mast cells, osteoclast and retinal pigmented epithelial cells (Oboki *et al.*, 2002; Takemoto, Yoon and Fisher, 2002; Levy,

Khaled and Fisher, 2006; Li *et al.*, 2010; Lu and Li, 2010). The mutation in MITF during embryogenesis can lead to disorders in eye development in humans as was proved in mouse model (Capowski *et al.*, 2014). Mutations in MITF can also lead to Waardenburg syndrome type II, which is also linked to eye development (Tassabehji, Newton and Read, 1994; Levy, Khaled and Fisher, 2006). MITF can also serve as an oncogene in melanomas (Hartman and Czyz, 2015).

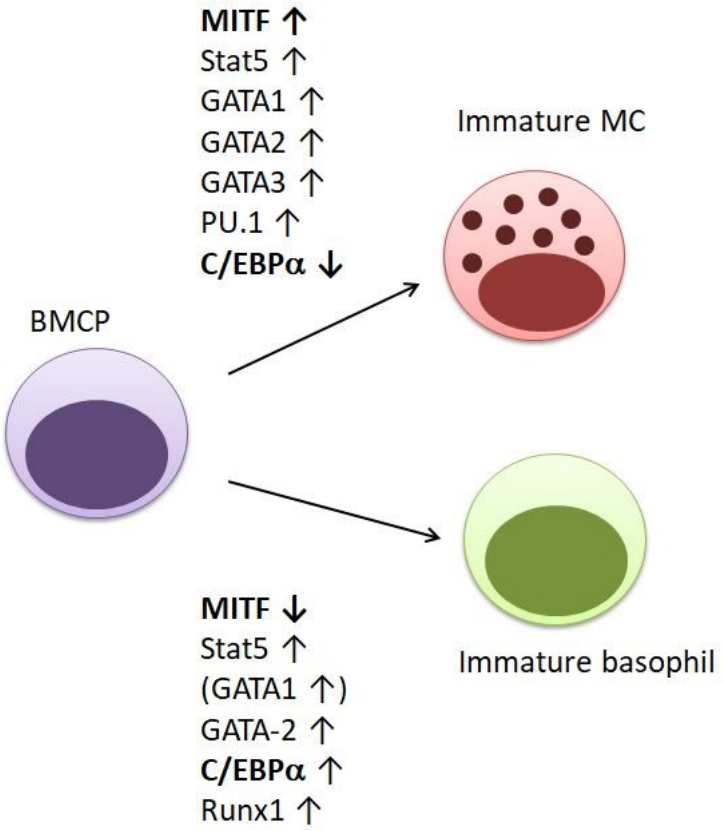


Figure 4. ***Transcription factors and their role in MCs development.*** *BMCP – basophil/mast cell progenitor, ↑-upregulation, ↓ - downregulation (Dahlin and Hallgren, 2015; Schmetzer et al., 2015)*

The role of MITF in MCs development is linked to GATA2/STAT5 axis. MITF together with C/EBPα is induced by GATA2/STAT5 expression (Qi *et al.*, 2013). In order to produce MCs from BMCPs, higher levels of MITF than C/EBPα are essential. These two TFs regulate each other in a suppressive manner, one directly suppressing the other and vice versa (Qi *et al.*, 2013; Sasaki, Kurotaki and Tamura, 2016). The importance of MITF

in MCs development can be demonstrated by mutation in MITF allele. The *mi/mi* mice, which are characterized by a *null* mutation in *MITF,* have decreased levels of MCs (Stechschulte *et al.*, 1987; Kitamura *et al.*, 2001).

5. Aim of the study

TFs form a complex network, which controls mast cell production and function (Tshori and Nechushtan, 2012; Qi *et al.*, 2013; Sasaki, Kurotaki and Tamura, 2016). To date, several TFs have been reported to regulate each other at different levels in order to orchestrate BMCP fate (*see chapter 4*). However, the complete transcriptional network regulating mast cell biology is not completely understood.

The aim of my thesis is to define the role of C/EBPγ in the development of mast cells. Thus, we generated BMMCs from C/EBPγ KO and WT mice, characterized those cultures, measured the expression of TFs involved in mast cell development, and prepared luciferase reporter constructs to determine the transcriptional control of C/EBPα and GATA2 by C/EBPγ.

6. Material and methods

Mouse strains

The *Cebpg* conditional KO mice were used (*Figure 5*) (Kardosova *et al.*, 2018). The *Cebpg* allele is floxed by loxP sites and the excision is induced by crossing animals to Vav-iCre transgenic mice (Georgiades *et al.*, 2002; Shimshek *et al.*, 2002). The excision leads to the generation of *Cebpg*$^{-/-}$ animals. In this project we employed *Cebpg*$^{f/f}$ Vav-iCre^{-} and *Cebpg*$^{f/f}$ Vav-iCre^{+} mice, from now on referred as WT and KO, respectively.

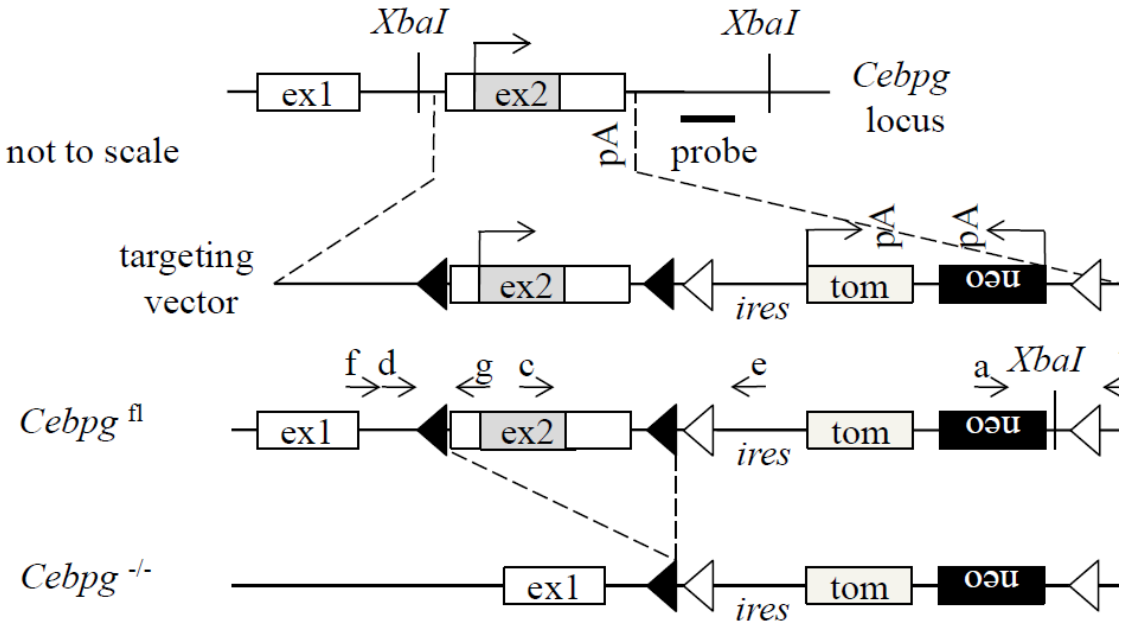


Figure 5. Generation of Cepbg conditional KO mice. *Cebpg conditional allele. The Cebpg targeting construct containing exon 2 (ex2) of Cebpg surrounded by Lox P sites (◀) and a reporter cassette flanked by Frt sites (◁). The reporter cassette contains an internal ribosome entry site-tdTomato (ires-tom) and the selection marker neomycin (neo). Arrows indicate the direction of transcription. The targeting construct was introduced into the Cebpg locus by homologous recombination to generate the Cebpg fl allele. When crossing to Vav-iCre transgenic mouse strain (Georgiades et al., 2002; Shimshek et al., 2002), exon 2 was deleted, generating Cebpg*$^{-/-}$*, referred here as KO. Letters from a to g indicate primer location for genotyping and excision. 3'probe indicates probe location for southern blot analysis. Adapted from Kardosova et al., 2018*

Mice genotyping

Genomic DNA isolation

Mouse tails (1-2 mm) were cut off from 21 days old pups and put into 1,5 ml eppendorf tubes. Next, 80 µl of 50 mM NaOH was added to each eppendorf tube. The tails were

boiled for 20 minutes at 95°C. Tubes were vortexed to dissolve the tails completely. Next, 20 µl of 1 M TrisHCl (pH=6,8) were added to eppendorf tubes to neutralize the NaOH. The tails were vortexed again and then spin at 13 000 g for 30 seconds to sediment the tail debris. The isolated DNA was stored at 4°C.

PCR reaction

The reactions and cycling was set as written in *Table 3.* Primers are listed in *Table 4*.

Vav-iCre genotyping (236 bp)			
One reaction	**Temperature**	**Time**	**Step**
1,5 µl DNA	98°C	3 minutes	1
0,5 µl forward primer (10 µM)	98°C	40 seconds	2
0,5 µl reverse primer (10 µM)	56°C	40 seconds	3
2,7 µl 5M betaine	72°C	40 seconds	4
1,55 µl water	72°C	2 minutes	5
6,75 µl 2xPPP mastermix	10°C	∞	6
Total volume 13,5 µl	Steps 2-4 were run in cycle 38 times		
***Cebpg* genotyping (wt allele = 664bp, fl allele = 280bp)**			
One reaction	**Temperature**	**Time**	**Number of cycles**
2 µl DNA	96°C	5 minutes	1
0,8 µl primer A (10 µM)	96°C	30 seconds	2
0,8 µl primer B (10 µM)	64°C	30 seconds	3
0,8 µl primer C (10 µM)	72°C	1 minute	4
4,2 µl 5 M betaine	72°C	7 minutes	5
1,9 µl water	10°C	∞	6
10,5 µl 2xPPP mastermix	Steps 2-4 were run in a cycle 35 times		
Total volume 21 µl			

Table 3. Setup of PCR reactions for mouse genotyping.

Primer	Sequence from 5´to 3´
Vav-iCre forward	AGATGCCAGGACATCAGGAACCTG
Vav-iCre reverse	ATCAGCCACACCAGACACAGAGATG
Cebpg A	CTCCAGACTGCCTTGGGAAA
Cebpg B	AAAGAGCCATTTCTGGGGAAGT
Cebpg C	AGTGTCATTCATACTCAGGCACA

Table 4. Sequences of primers used for mouse genotyping

Electrophoresis

PCR products were run in 2,5 % agarose gel at 110V 180A for 25 minutes.

Peritoneal lavage and flow cytometry staining

Mice were sacrificed by cervical dislocation andwet with 70% ethanol to prevent sample contamination by fur. The outer skin overlaying the peritoneal wall was carefully removed by scissors and 5 ml of phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) were injected into peritoneal cavity. The abdomen was massaged for 1 minute to enable release of peritoneal MCs into PBS + 2% FBS. Afterwards, approximately 3 ml of peritoneal lavage was withdrawed by injection. Subsequently, the peritoneum was carefully opened and the rest of fluid was aspirated by Pasteur pipette. Peritoneal cavity lavage was transferred into 15 ml falcon tube and kept on ice. Afterwards, the cells were spun down and resuspended in 100 µl of PBS + 2% FBS. 50 µl of cell suspension was stained for FACS analysis (*Table 5*).

The FcR Blocking Reagent was added tothe the samples and samples were incubated 5 minutes at 4°C. Afterwards, the samples were stained with appropriate antibodies conjugated to different fluorochromes (table 5, highlighted in grey) for 15 minutes at 4°C. Hoechst 33258 was added right before measurement to label and exclude death cells from the analysis. The samples were measured using FACS LSRII.

Antibody	Colour	Volume (µl)
Fcγ block	-	0,5
FcεRI	Alexa 647	0,5
c-Kit	PE/Cy 7	0,2
TER 119	Pacific blue	0,5
Hoechst 33258	-	1
Total volume per sample		2,7

Table 5. FACS staining setup for peritoneal MCs

Bone marrow cells isolation

Mice were sacrificed by cervical dislocation. The legs and the hips were removed and the bones were cleaned and placed in cold PBS + 2% FBS. Next, the bones were crunched and the supernatant was collected in 15 ml tube. The samples were spun down at 450 g for 5 minutes at 4°C and the supernatants were discarded. The pellets were resuspended in 2 ml of ammonium-chloride-potassium (ACK) lysing buffer and incubated for 5 minutes at room temperature to lyse red blood cells. During this incubation, cell suspension was filtered using a 70 μm cell strainer and transferred to a new 15 ml conical tube. Subsequently, 10 ml of PBS + 2% FBS were added to the suspension in order to neutralize ACK lysing buffer. The cell suspension was spun down at 450 g for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 400 μl of PBS + 2% FBS.

Spleen cells isolation

Mice were sacrificed by cervical dislocation. The spleens were taken out and placed in PBS + 2% FBS. The piece offine nylon mesh was placed in the cap of 50 ml conical tube and 1 ml of PBS + 2% FBS was added into the cap together with the spleen. Spleen was mashed by the plunger end of the syringe against the mesh in order to protect the cells from bursting by pressure. The spleen was mashed until there were only cells in PBS + 2% FBS and no bigger particles. Next, cell suspension was transferred into 15 ml conical tube through another mesh at the top of new conical tube. The cell suspension was spun down at 450 g for 5 minutes at 4°C and the supernatants were discarded. The pellets were resuspended in 2 ml of ACK lysing buffer and incubated for 5 minutes at room temperature. During this incubation, cell suspension was filtered using a 70 μm cell strainer and transferred to a new 15 ml conical tube. Subsequently, 10 ml of PBS + 2% FBS were added in order to neutralize ACK lysing buffer. Conicaltubes were spun down at 450 g for 5 minutes at 4°C. The supernatants were discarded and the cells were resuspended in 400 μl of PBS + 2% FBS.

Ki67 staining

$5x10^5$ cells were washed twice with PBS and spun down at 350g for 5 minutes. The pellet was resuspended by vortexing and 3 ml of cold 70% ethanol were added drop by drop. After that, the samples were vortexed for another 30 seconds and incubated at -20°C for 1 hour. After incubation, samples were washed three times with PBS + 2% FBS and spun

down at 1300g for 3 minutes. Cells were resuspended in 100 µl of PBS + 2% FBS + 2 µl of Ki67. Cells were left incubating 30 minutes in the dark at room temperature. After the incubation, cells were washed three times with PBS + 2% FBS and analyzed using FACS LSRII.

Annexin V (BD Pharmingen^{TM}) staining

100 µl of 10 x binding buffer per sample were prepared. 10 x binding buffer consist of 0,1 M Hepes (pH 7,4), 1,4 M NaCl and 25 mM $CaCl_2$. The samples were transferred to small FACS tubes and washed with 1 ml PBS. Next, the samples were washed with 500 µl of binding buffer and resuspended in 50 µl of 1x binding buffer and 5 µl of Annexin V. The samples were left in the dark for 15 minutes at room temperature to enable proper staining. Next, the samples were washed by 500 µl of 1x binding buffer and then resuspended in 20 µl of 7-aminoactinomycin D (7-AAD). Afterwards,the samples were analyzed by using FACS LSR II.

Bone marrow-derived mast cell cultures (BMMCs)

Mice were sacrificed by cervical dislocation (day 1). The legs and the hips were removed and the bones were cleaned from the tissue (tibia + fibula, femur and pelvis). 3 ml of RPMI medium supplemented for MCs cultivation according to Kawakami (*Table 6*) was prepared in injection with 30 G needle. The ends of the bones were cut off on both sides. The needle was inserted in the bone and the bone marrow was flushed out from both sides into 40 ml cultivation flasks. Bone marrow cultures were cultivated at 37°C for 2 days. After 2 days (day 3) the media with the cells was placed into a new 75 ml cultivation flask with tempered RPMI media supplemented for MC cultivation into final volume of 30 ml. The cells were cultivated at 37°C for 3-4 days (day 7 or 8). At day 7 or 8, cells were transferred to a 50 ml conical tube, the walls of flask were washed for four times to ensure transfer of all cells. The cells were spun down at 450 g for 5 min and the supernatant was discarded. The cells were resuspended and transferred to a new 75 ml flask. Fresh RPMI media supplemented for MC cultivation was added to the cells to final concentration $2x10^6$ cells /ml. The media was changed every 7 days.

Supplement	Concentration	Volume (ml)
RPMI – 1640 Sigma	-	395
FBS (inactivated at 56°C for 30´)	10%	50
sodium pyruvate	1,1%	3,5
glucose	25%	4,5
Glutamine	3%	6
NEAA	0.7%	3,5
ATB (Streptomycin + Penicilin)	0.5%	2,5
β-mercaptoethanol	0.04%	200 μl
SCF (supernatant from CHO)	-	15
IL-3 (supernatant from WEHI)	-	20
Total volume of media		500

Table 6. RPMI medium for bone marrow mast cells according to Kawakami

RNA isolation

The bone marrow mast cells (BMMCs) were spun down at 450 g for 5 minutes and the supernatant was discarded. 1 ml of TRI reagent was added to 5- 10x 10^6 cells and the cells were lysed by repetitive pipetting. The homogenate was stored for 5 minutes at room temperature. 200 μl of chloroform were added and the sample was shaken vigorously for 15 seconds. The mixture was stored at room temperature for 2-15 minutes and then centrifuged at 12 000g for 15 minutes at 4°C. After the centrifugation, the upper aqueous phase was transferred into new 1,5 ml tubes. 0,5 ml of isopropanol was added to aqueous phase and the sample was mixed. Subsequently, the sample was stored at room temperature for 5-10 minutes and centrifuged at 12 000g for 8 minutes at 4°C. The supernatant was removed with clean tip and the RNA pellet was washed by vortexing in 1 ml of 75% ethanol and centrifuged at 7500 g for 5 minutes at 4°C. The washing step was repeated. After the second wash, the pellet was sedimented by fast spin at 12 000g for 30 seconds. The ethanol was removed by pipette and the pellet was left to air-dry for 3-5 minutes. After drying, the RNA pellet was resuspended in 15 μl of RNAse-free water.

Reverse transcription

The cDNA was transcribed from 3 μg of RNA. The RNA samples were diluted to final concentration of 3 μg per 8,5 μl. 1 μl of 10x reaction buffer with $MgCl_2$ and 0,5 μl of

RNAse-free DNAse I were added to the samples and the samples were incubated for 30 minutes at 37°C. Next, 1 µl of 50 mM EDTA was added to bind ions followed by 10 minutes long incubation at 75°C in order to deactivate DNAse I. After that, 1 µl of random primers (concentration 200 ng/µl) and 1 µl of the mixture of deoxynucleotide triphosphates (dNTPs) (dATP, dTTP, dCTP and dGTP, 10 mM each) was added. The samples were incubated at 65°C for 5 minutes and then chilled on ice followed by a brief spin. In the next step, 4 µl of 5x first strand buffer and 2 µl of 0,1 M dithiotreitol (DTT) were added. The samples were stored at 25°C for 2 minutes. In the last step, 1µl of the reverse transcriptase SuperScript II was added and the samples were incubated at 25°C for 10 minutes followed by 42°C for 50 minutes, followed by 70°C for 15 minutes. Generated cDNA was stored at -20°C.

Quantitative real-time PCR (q RT-PCR)

The cDNA obtained from reverse transcription was at least five times diluted. The reaction was set as written in *Table 7*, primers are listed in *Table 8*. All samples were analyzed in duplicates. The geometric mean of GAPDH and ubiquitin CT values was used as a control for normalization of measured CT values (Kozera and Rapacz, 2013). The qPCR was run in real time PCR cycler from Roche.

Reagent per one reaction	Volume (µl)
2x SYBR Green Master Mix	2,5
Oligo F (10 µM)	0,25
Oligo R (10 µM)	0,25
cDNA (10ng)	2
Total volume	5

Table 7. Setup of qPCR reaction

Primer	Sequence from 5´to 3´
mGAPDH forward	CCAGCCTCGTCCCGTAGAC
mGAPDH reverse	CCCTTGACTGTGCCGTTG
mC/EBPγ forward	GCGCAGAGAGCGGAACAA
mC/EBPγ reverse	GTATCTTGAGCTTTCTGCTTGCT
mUbb forward	ATGTGAAGGCCAAGATCCAG
mUbb reverse	TAATAGCCACCCCTCAGACG
mGATA2 forward	GCAGAGAAGCAAGGCTCGC
mGATA2 reverse	CGGCCCTCACACAGTTGAC
mMITF forward	AACCGACAGAAGAAGCTGGA
mMITF reverse	TGATGATCCGATTCACCAGA
mC/EBPα forward	GACCATTAGCCTTGTGTGTACTGTATG
mC/EBPα reverse	TGGATCGATTGTGCTTCAAGTT
mPU.1 forward	CCTCCATCGGATGACTTG
mPU.1 reverse	GTGTGCGGAGAAATCCCA

Table 8. Sequences of primers used for qPCR. *Ubb - ubiquitin*

Genomic DNA isolation

Genomic DNA for cloning was isolated with genomic DNA isolation kit from Zymo Research according to manufacturer's protocol.

Cloning

Inserts and vector preparation

The proximal promoter region of *Cebpa* and *Gata2* were identified and The Alggen-Promo online software (alggen.lsi.upc.es) was used to determine C/EBPγ binding sites inside of *Gata2* and *Cebpa* promoter regions. *Gata2* has two alternative promoter regions, one is IG and the second is IS. Primers (*Table 9*) were designed to amplify the proximal promoter of each TF.

Promoter regions were amplified from genomic DNA isolated from BMMC WT cultures. The amplification was done with Q5 polymerase and the reaction reagents and used conditions are described in *Table 10*.

Primer	Sequence from 5´to 3´
Gata2 IG forward	TCCCAAGCTTTAGGGGTTTCGGTCCCTGCAA
Gata2 IG reverse	ACCGCTCGAGATTCACGGGATAGGGGTGGGG
Gata2 IS forward	ACCCAAGCTTGCAGAGACATTCACCCAGTGCC
Gata2 IS reverse	ACCGCTCGAGGCAGGCAGCCGCTTTTGTCC
Cebpa forward	TCCCAAGCTTGGAGGGTGAACGAGACGCCA
Cebpa reverse	ACCGCTCGAGCCAGTGCCCCAACTGGCTCG

Table 9. Sequences of primers used for colony screening

Amplified inserts were run on 1 % agarose gel at 110V 180 A for 20 minutes. Subsequently, the bands with proper size (cca 1 kbp for *Gata2 IG*, *Gata2 IS* and *Cebpa* and 6 kpb for pxp2) were cut out from the gel and DNA was purified with Zymoclean Gel DNA Recovery Kit from Zymo Research according to manufacturer's protocol. 10 µl of purified product were digested with 0,4 µl XhoI and 0,4 µL HindIII restriction enzymes, 2 µl of 10x buffer R (Thermofischer Scientific) and 7,2 µl of nuclease-free water (total volume of reaction 20 µl) for 1 hour at 37°C. At the same time, 3 µl of pxp2 luciferase vector (concentration 300 ng/µl) were digested with 0,4 µl XhoI and 0,4 µl HindIII restriction enzymes, 2 µl of 10x buffer R (Thermofischer Scientific) and 14,2 µl of nuclease-free water (total volume of reaction 20µl) for 37°C 1 hour. After 1 hour, enzymes were deactivated by heating the tubes to 70°C for 5 minutes. Then pxp2 vector was dephosphorylated with fastAP phosphatase to prevent self-ligation of cutted vector. 1 µl of fastAP, 2 µl of fast green digest buffer and 8µl of nuclease-free water were added to reaction volume (20 µl) and sample was incubated 10 minutes at 37°C. The dephosphorylation was stopped at 75°C for 5 minutes. The digested PCR products and dephosphorylated vector were run on 1% agarose gel at 110 A and 180 V for 20 minutes. The bands with proper size (cca 1 kbp for *Gata2 IG*, *Gata2 IS* and *Cebpa* and 6 kbp for pxp2) were cut out from the gel and put into 1,5 ml tubes. DNA was purified with Zymoclean Gel DNA Recovery Kit from Zymo Research according to manufacturer's protocol.

Amplification of GATA2 IG			
Setup for one reaction	**Temperature**	**Time**	**Step**
1 µl DNA	98°C	30 seconds	1
5 µl 5x Q5 buffer	98°C	10 seconds	2
0,5 µl dNTPs	60°C	30 seconds	3
1,25 µl oligo F (10 µM)	72°C	1 minute	4
1,25 µl oligo R (10 µM)	72°C	2 minutes	5
0,25 µl Q5 polymerase	10°C	∞	6
5 µl High GC enhancer	Steps 2-4 were run in a cycle 35 times		
5,75 µl water			
Total volume 20 µl			
Amplification of GATA2 IS			
Setup for one reaction	**Temperature**	**Time**	**Step**
1 µl DNA	98°C	30 seconds	1
5 µl 5x Q5 buffer	98°C	10 seconds	2
0,5 µl dNTP	60°C	30 seconds	3
1,25 µl oligo F (10 µM)	72°C	1 minute	4
1,25 µl oligo R (10 µM)	72°C	2 minutes	5
0,25 µl Q5 polymerase	10°C	∞	6
5 µl High GC enhancer	Steps 2-4 were run in a cycle 35 times		
5,75 µl water			
Total volume 20 µl			
Amplifictaion of C/EBPα			
Setup for one reaction	**Temperature**	**Time**	**Step**
1 µl DNA	98°C	30 seconds	1
5 µl 5x Q5 buffer	98°C	10 seconds	2
0,5 µl dNTP	60°C	30 seconds	3
1,25 µl oligo F (10 µM)	72°C	1 minute	4
1,25 µl oligo R (10 µM)	72°C	2 minutes	5
0,25 µl Q5 polymerase	10°C	∞	6
5 µl High GC enhancer	Steps 2-4 were run in a cycle 35 times		
5,75 µl water			
Total volume 20 µl			

Table 10. Setup of PCR reactions for amplification of diverse inserts

Ligations

The vector (pxp2) and inserts (*Gata2 IG*, *Gata2 IS*, *Cebpa*) were ligated in 1,5 ml tubes with T4 DNA ligase at 16°C over-night. The setup and cycling parameters of reaction are in *Table 11*.

Ligase (μl)	Buffer (μl)	Vector (μl)	Insert (μl)	Water (μl)	Total volume (μl)
0,25	2	1	8	8,75	20

Table 11. Setup of ligations

Transformation of TOP 10 E. coli

TOP 10 competent cells were defrosted on ice and 1,5 ml tube for each ligated product was pre-cooled on ice. 45 μl of competent cells were placed into each tube and 8 μl of ligated product was added. The tubes with bacteria and ligated product were left on ice for 20 minutes. After 20 minutes, the tubes were heat-shocked at 42°C for 45 seconds and then immediately chilled on ice for 5 minutes. Next, 500ml of LB media were added to tubes and were shaken for 1 hour at 37°C at 650 rpm. After that, the TOP10 cells were spun down (2000 g/3 minutes) and plated on ampicillin plates tempered to room temperature. The plates were cultivated at 37°C over-night.

Colony screening

Next day six colonies from each plate were picked up and placed into 100 μl of LB media with ampicillin (concentration 1 μl/ml) in 1,5 ml tube. The tubes were shaken for 1 hour at 37°C 650 rpm. The colony screening was set up as described in *Table 12*, for primer sequences see *Table 13*. The PCR products were run on 1% agarose gel at 110V and 180A for 20 minutes.

Setup for one reaction	Temperature	Time	Step
1 μl of bacteria	94°C	1 minute	1
1 μl oligo F (10 μM)	94°C	15 seconds	2
1 μl oligo R (10 μM)	52°C	15 seconds	3
12,5 μl 2x PPP mastermix	72°C	1 minute	4
9,5 μl water	72°C	7 minutes	5
Total volume 25 ml	10°C	∞	6

Table 12. Colony screening set up *– steps 2-4 were run in cycle 30 times*

Primer	Sequence from 5´to 3´
Colony screening primer Forward	CACTGCATTCTAGTTGTG
Colony screening primer Reverse	TTACCAACAGTACCGGAA

Table 13. Sequences of primers used for colony screening

Bacteria cultures preparation for MIDI Prep

The positive colonies from colony screening were used to set up bacteria cultures (100 ml). 99µl of bacteria from colony screening tubes were put into Erlenmeyer flasks with 100 ml of LB media with ampicillin (1 µl of ampicilin per 1ml of media) and cultivated in thermal shaker at 37°C at 650 rpm over-night.

DNA isolation using MIDI preps

DNA from bacterial cultures was isolated using MIDI Prep (NucleoBond Xtra MIDI – Macherey-Nagel) according to manufacturer's protocol. The isolated plasmid DNA was stored at -20°C.

Sequencing of the constructs

Constructs were sequenced to validate proper position of the inserted sequence into pxp2 luciferase vector. Primers were designed to amplify region between digestion sites (Hind III – Xho I). Primers used for sequencing are listed in *Table 14*.

Primer	Sequence from 5´to 3´
pxp2_insert forward	CACTGCATTCTAGTTGTG
pxp2_insert reverse	TTACCAACAGTACCGGAA

Table 14. Sequences of sequencing primers

7. Results

7.1 Genotyping

Mice were genotyped in order to enable selection of KO and WT animals for experiments (*Figure 6*). Mice were genotyped by PCR as written in part 6 – Mice genotyping. Figure 6 demonstrate a representative PCR genotyping using genomic DNA isolated from tails.

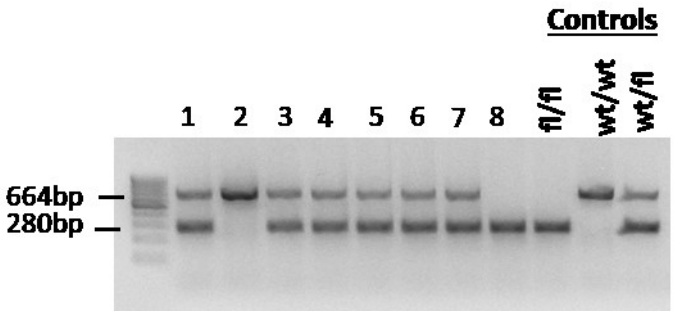


Figure 6. Conditional deletion of Cebpg in mice. *Numbers from 1 to 8 indicate samples. 664 bp band indicates amplification of wild type allele, 280 bp band indicates amplification of floxed allele. Sample number 2 is example of WT. Sample number 8 is example of KO. The rest of samples are heterozygotes.*

7.2 Characterization of BMMCs

To study the role of the TF C/EBPγ in MCs development we employed C/EBPγ KO mice and generated BMMCs. MCs are characterized by expression of c-Kit and FcεRI. To determine the effect of *Cebpg* deletion on expression of these markers, we performed FACS analysis of BMMCs cultures and assessed proliferation curves (*Figure 7*). The expression of MC markers was similar in WT and KO BMMCs (*Figure 7 A-B*). Strikingly, there was major difference in the ability to grow and produce mast cells (*Figure 7 C*). These results suggest that C/EBPγ plays an important role in regulation of the production of mature mast cells, although it is dispensable for expression of c-Kit and FcεRI.

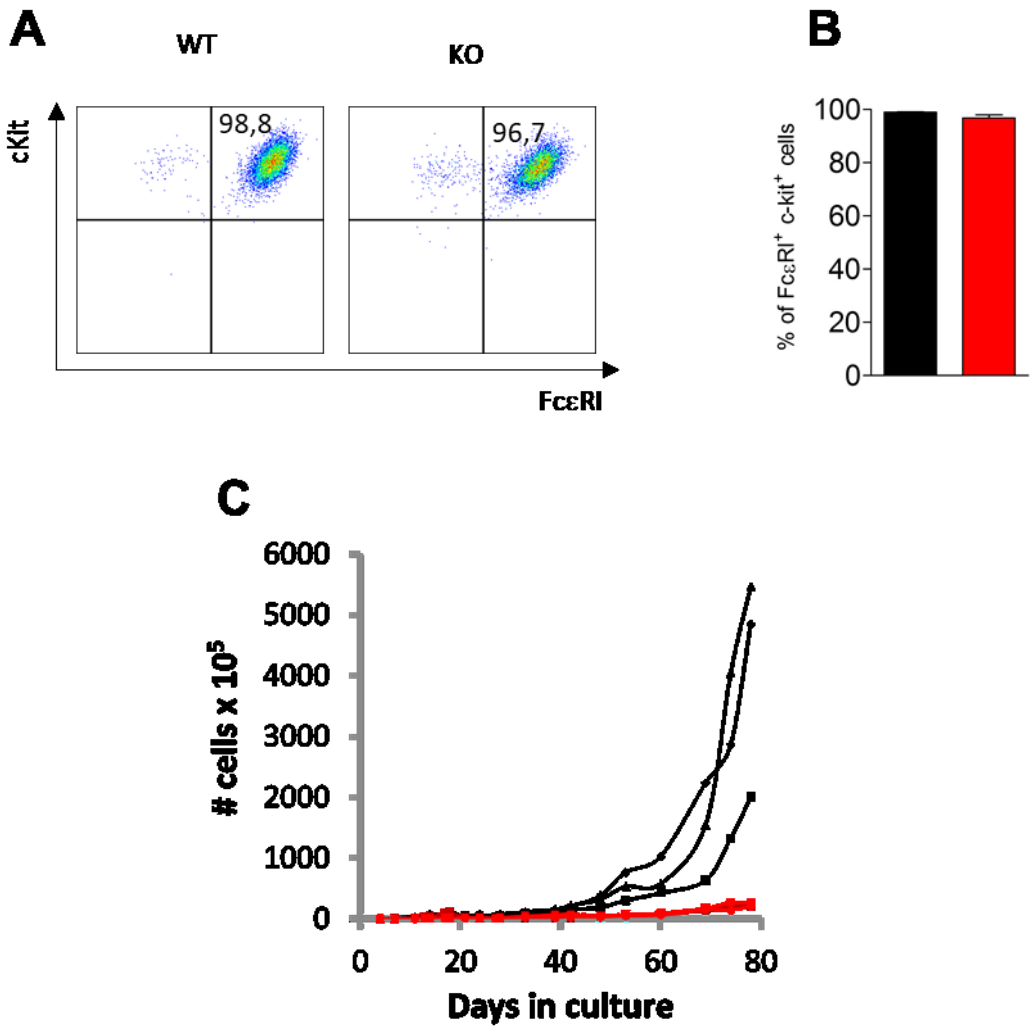


Figure 7. Effect of Cebpg deletion on BMMCs cultures. *A) Representative FACS plots showing that WT and KO BMMCs do not differ in the expression of c-Kit and FcεRI (day 23 of cultivation) B) Quantification of the expression of surface markers of MCs. Y axis indicates percentage of FcεRI$^+$ and c-Kit$^+$ cells in BMMCs cultures. C) Reduced capacity of KO BM to produce BMMCs. Y axis indicates number of BMMCs in the culture., black – WT, red - KO*

7.3 Apoptosis and proliferation of BMMCs

Since C/EBPγ KO BMMCs presented a limited ability to grow, we next investigated whether *Cebpg* ablation affected cell survival. We performed Annexin V staining in order to decipher this question. The analysis showed that C/EBPγ KO BMMCs are more prone to apoptosis than WT (*Figure 8*). In the next step, we tested the proliferation of BMMCs by Ki67 (*Figure 9*). Analysis revealed gradual changes in the expression of Ki67. To be able to assess Ki67 positive population, we used cultures from peritoneal cells-derived mast cell cultures (PCMCs), which presented clear $Ki67^-$ and $Ki67^+$ expression. We observed that there is no statistically significant difference in proliferation between WT and KO BMMCs. Altogether, we concluded that C/EBPγ controls processes linked to cell survival rather than processes linked to cell proliferation.

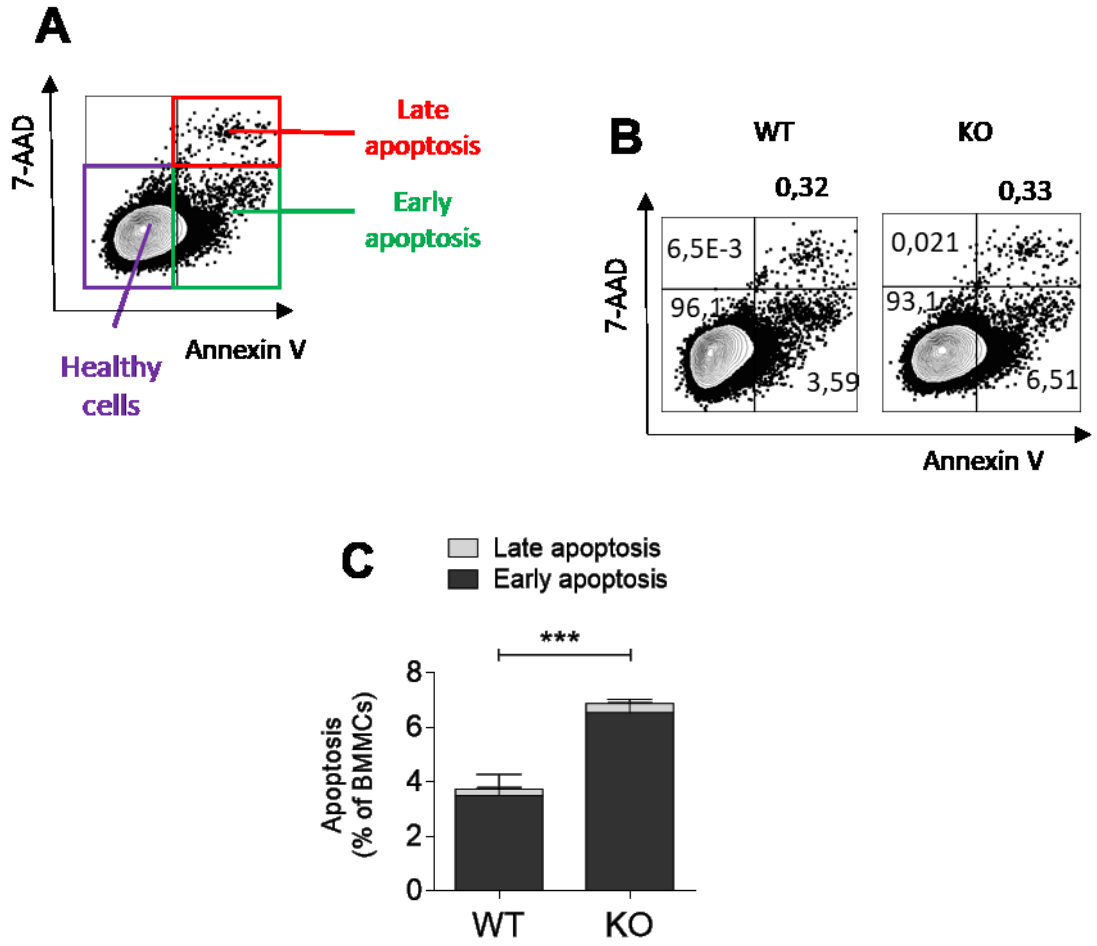


Figure 8. KO BMMCs are more prone to apoptosis compared to WT. *A) The scheme of the detection of healthy cells, early apoptotic and late apoptotic cells by using 7-AAD and Annexin Vstaining. B) Representative FACS plots showing early and late apoptosis in WT and KO BMMCs. C)Quantification of the experiment. Two-tailed Student's t-test was used to assess statistical significance, (***P<0.001)*

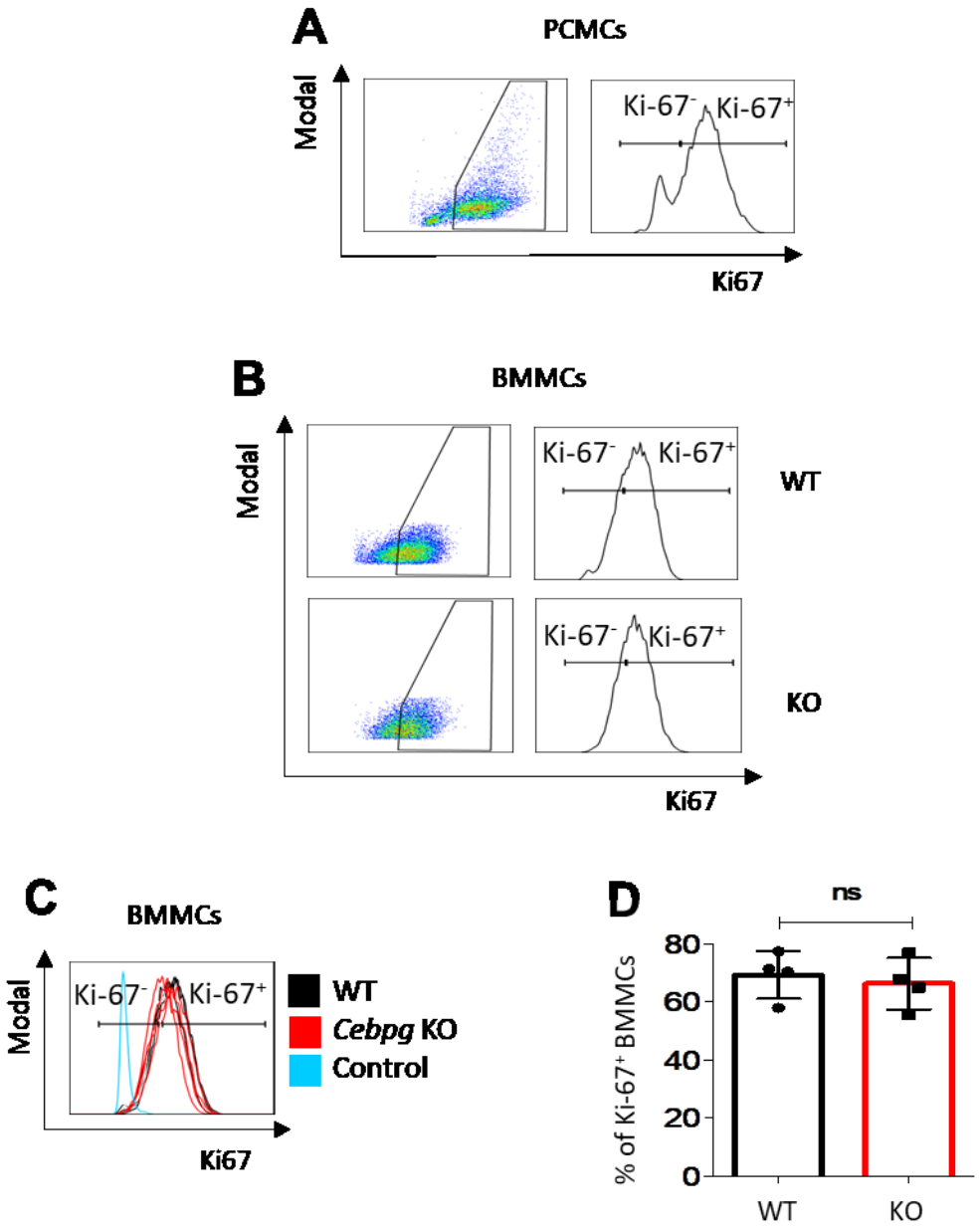


Figure 9. Proliferation of BMMCs. *A) FACS plot and histogram of control PCMCs with clear negative and positive population. B) Representative FACS plots and histograms (upper for WT and lower for KO) of the xpression of Ki-67 in population of BMMCs at day 31 of cultivation. C) Comparison of the expression of Ki67 between WT (black) and KO (red) BMMCs. Blue histogram indicates BMMC, which were not stained. D) Quantification and statistical analysis of Ki67 expression. Y axis indicates the percentage of Ki-67$^+$ cells. Two-tailed Student's t-test was used to assess statistical significance (ns: non-significant, P≥0.05), Ki-67$^+$ - positive population, Ki-67$^-$ - negative population, blue – unstained control, black – WT, red - KO*

7.4 Peritoneal lavage

One of the characterization processes of MCs in our animal model was comparing WT and KO peritoneal MCs percentages *in vivo*. To address this we have done FACS analysis of peritoneal MCs, staining setup is described in *Table 5*. Gating strategy and representative FACS plots are shown in *Figure 10*. We observed that the percentage of peritoneal MCs in WT and *Cebpg* KO mice was similar, and concluded that there is no difference between WT and KO animals.

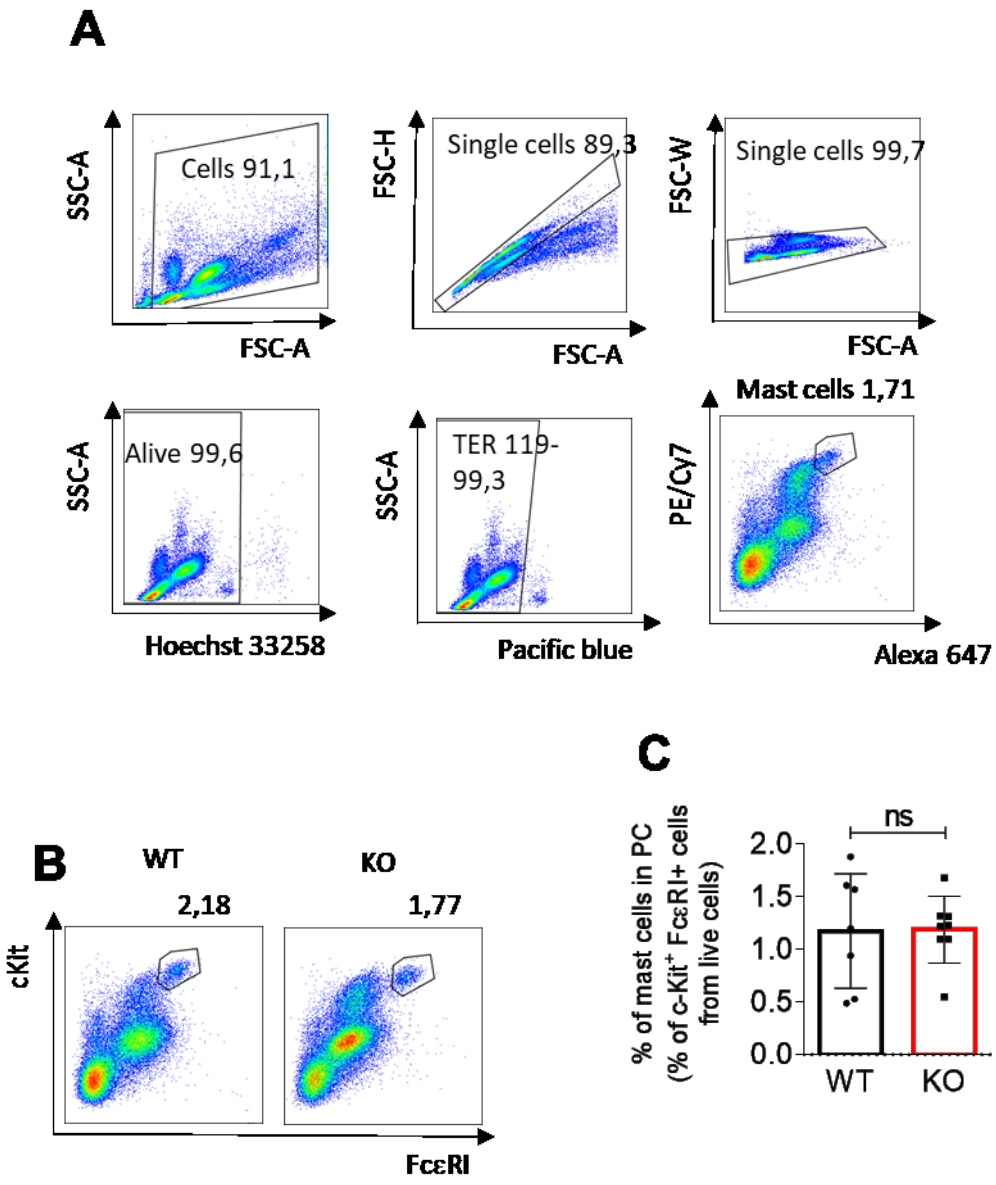
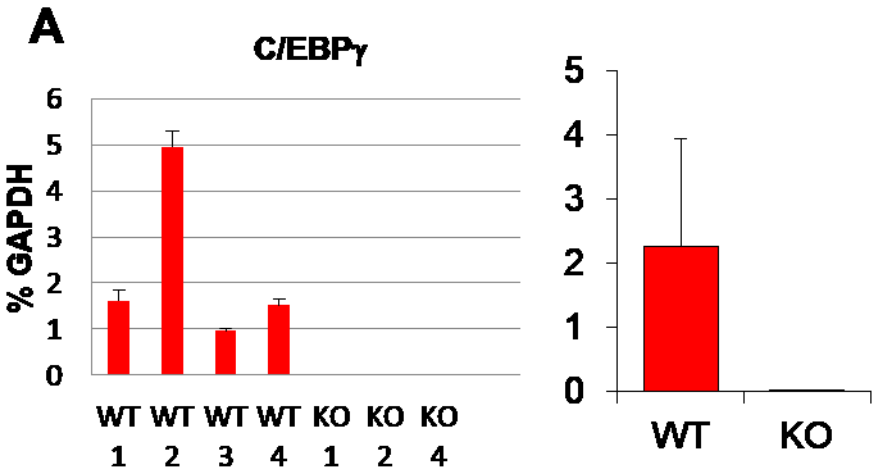
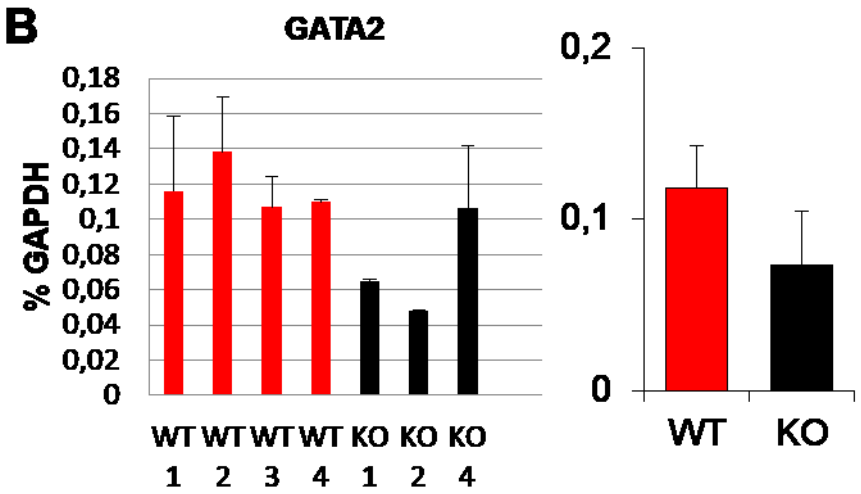


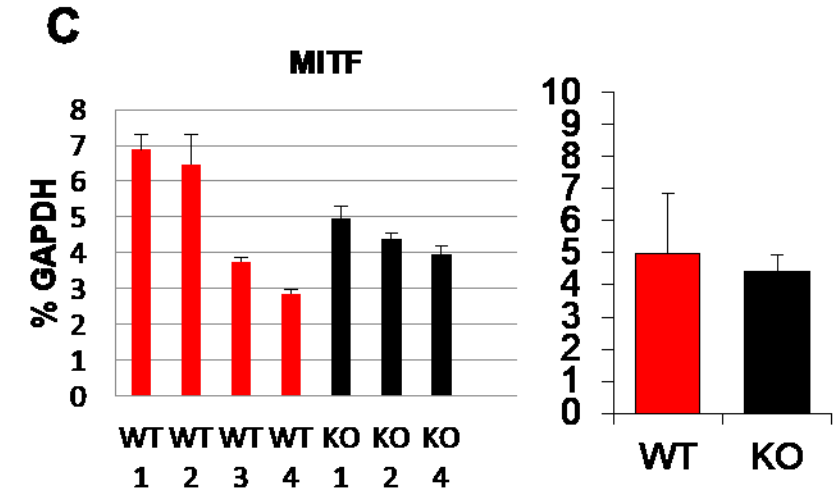
Figure 10. FACS analysis of peritoneal mast cells. *A) Gating strategy. B) Representative FACS plots showing expression of c-Kit and FcεRI in WT (left plot) and KO (right plot) mouse peritoneal cavity. C) Quantification of the experiment. Y axis indicates percentage of c-Kit$^+$ FcεRI$^+$ cells in peritoneal lavage of WT (black) and KO (red) mouse. Two-tailed Student's t-test was used to assess statistical significance (ns: non-significant, P≥0.05)*

7.5 RT - qPCR

Since the number of cells produced in WT and *Cebpg* KO BMMCs was statistically different, we next investigated the expression of some TFs involved in MCs development. We performed quantitative RT- PCR in WT and *Cebpg* KO BMMCs and determined expression of *Cebpa*, *Gata2*, *Mitf*, and *Pu*.1, (*Figure 11*). Ablation of *Cebpg* in the KO cultures was also verified by RT-PCR. We observed that *Cebpg* ablation resulted in increased expression of *Cebpa* and reduced levels of *Gata2*.






C
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6
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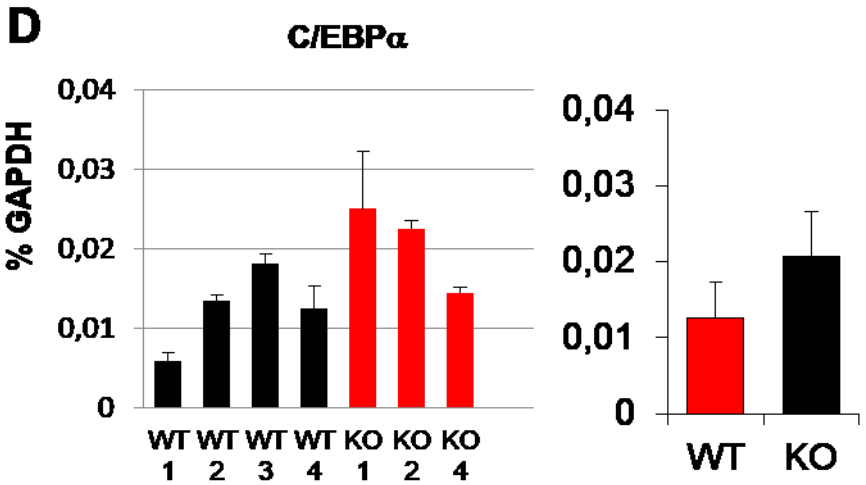
WT
KO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% GAPDH
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KO

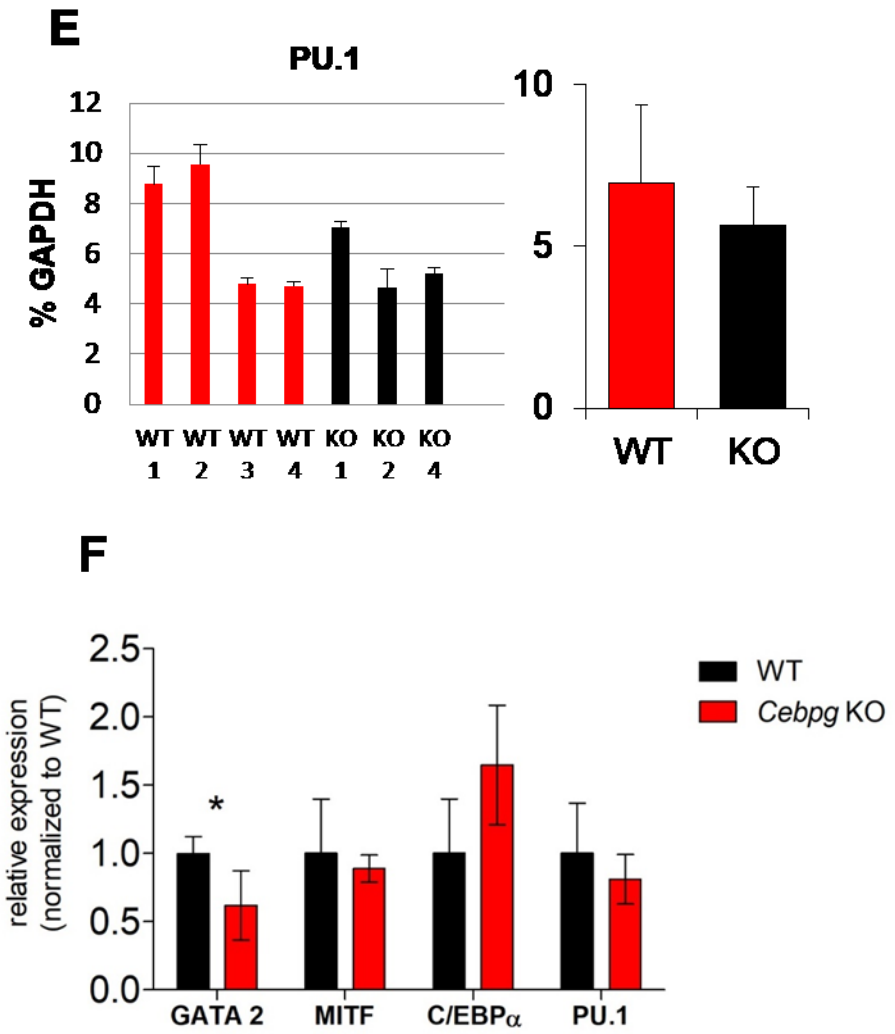

Figure 11. Different expression of MCs transcription factors between WT and KO animals involved in their development. *Expression of Cebpg (A,) Gata2 (B), Mitf (C), Cebpa (D) and Pu.1 (E) in BMMCs at 23rd day of cultivation (3 days after culture). Right graph shows average values of all WT and all KO for selected gene. F) Relative expression of selected transcription factors normalized to WT. (% of normalization control was counted as a geometric mean of GAPDH and Ubiquitin values) black – WT, red – KO.*

7.6 Cloning – Preparation of luciferase reporter constructs

Luciferase reporter vectors were generated in order to test if C/EBPγ binds directly to *Gata2* and *Cebpa* promoter regions and thus regulate their expression.

The pxp2 luciferase vector was digested by Xho I and Hind III restriction enzymesin order to prepare sticky ends for insert addition. Inserts were proximal promoter regions of *Cebpa*, *Gata2 IG* and *Gata2 IS,* which were amplified by PCR (*Figure 12*). After purification from gel, *Cebpa*, *Gata2 IG*, *Gata2 IS* were digested with same enzymes as pxp2. Pxp2 was dephosphorylated by FastAP (*Figure 13*). After ligation and transformation, the colonies were screened by PCR (*Figure 14*). Selected colonies from screening were used to set up bacterial cultures, which served as a source for DNA isolation with MIDI preps. Constructs were checked by digestion (*Figure 15*).

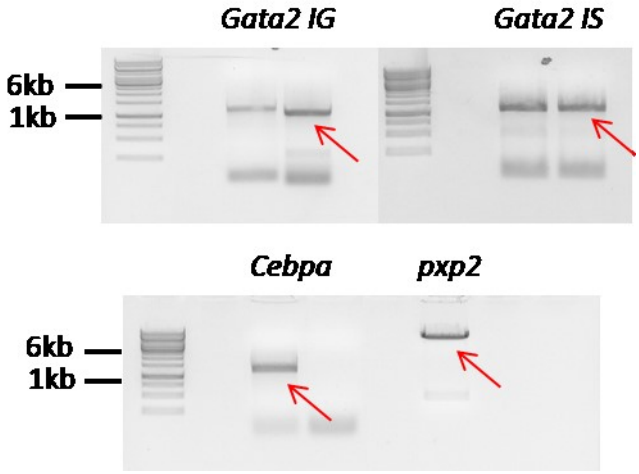

Figure 12. Amplification of proximal promoters for Luciferase assay and pxp2 digestion.
The red arrows point to bands which were cut out of gel and purified for following steps.

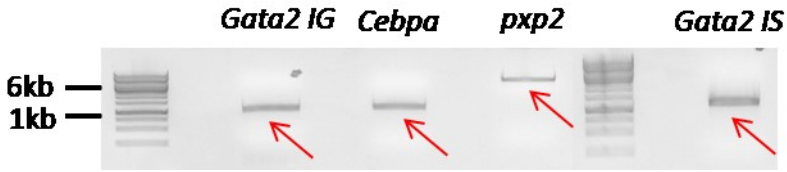

Figure 13. Digested proximal promoter parts and dephosphorylated vector.
The red arrows point to bands which were cut out of gel and purified for following steps.

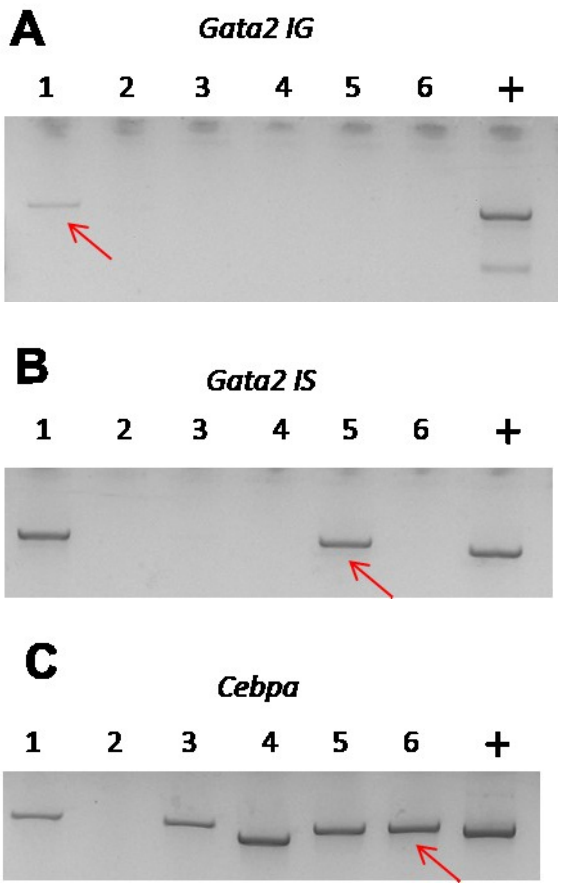

Figure 14. PCR screening of colonies after transformation. *A) Colonies with Gata2 IG construct. B) colonies with Gata2 IS construct. C) colonies with Cebpa construct. Numbers from 1 to 6 indicates single colonies picked up from plates. + - positive control, red arrow indicates which colony proceed to MIDI prep*

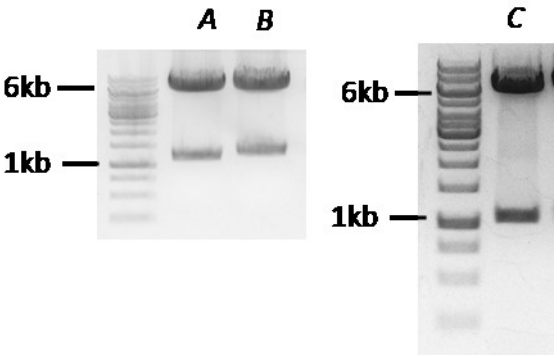

Figure 15. Control digestion after MIDI prep plasmid isolation. *A – Gata2 IS, B – Cebpa, C- Gata2 IG*

8. Discussion

MCs development is still not a fully understood process, even though interesting and innovative data has been discovered in recent years (Gentek *et al.*, 2018; Grootens *et al.*, 2018). It is known that the developmental pathway from HSCs to mature MCs is regulated by many TFs (*see chapter 4*). Among them, MITF, GATA2 and C/EBPα seem to play a prominent role (Qi *et al.*, 2013; Li *et al.*, 2015). On one hand, the expression of these TFs has to be precisely regulated, because expression of MITF leads the way towards MCs, while expression of C/EBPα leads the way towards basophils (Arinobu *et al.*, 2005; Qi *et al.*, 2013; Kasakura *et al.*, 2014). On the other hand, GATA2 expression is important for proper development of both basophils and MCs (Li *et al.*, 2015).

C/EBPγ is a TF that is highly expressed in activated MCs (www.biogps.com), however whether and how C/EBPγ plays a role in mast cell development and function is unclear. Interestingly, C/EBPγ does not have transactivation domain, therefore it was sugsested that it acts rather as dominant negative regulator of other C/EBP transcription factors (Cooper *et al.*, 1995; Parkin *et al.*, 2002). Moreover, it is known that C/EBP family of TFs can form heterodimers with each other and other bZIP domain containing proteins, and thus regulate activation or repression of their target genes (Ramji and Foka, 2002; Newman and Keating, 2003; Huggins *et al.*, 2013). Further, it was reported that C/EBPα, which suppresses mast cell production and promotes basophil formation, regulates C/EBPγ expression in myeloid cells (Alberich-Jordà *et al.*, 2012). In this thesis we hypothesize that C/EBPγ could work upstream of C/EBPα and regulate some TFs involved in MCs development. To address this question we have done several *in vivo* and *in vitro* experiments using conditional C/EBPγ KO mice.

Mast cells are difficult to study *in vivo* since they are tissue resident cells and almost impossible to isolate from murine models in order to perform experiments. Alternatively, BMMCs are employed as an *in vitro* system to study mast cell development and functionality. We established BMMCs from WT and *Cebpg* KO mice. At first we have done FACS characterization of BMMC. We have seen no differences between WT and *Cebpg* KO BMMCs regarding expression of MCs surface markers c-Kit and FcεRI (*Figure 7 A,B*). However we observed a significant difference in ability to produce new cells (*Figure 7 C*). After the finding that *Cepbg* KO BMMCs has limited ability to produce large numbers of mast cells, we analyzed proliferation and apoptosis. We found a

statistically significant difference between WT and *Cebpg* KO BMMCs in apoptosis (*Figure 8*) as *Cebpg* KO BMMCs were twice more prone to be apoptotic than WT BMMCs. However, the proliferation capacity was not altered as determined by Ki67 staining and flow cytometric analysis (*Figure 9*). In addition to BMMCs, we analyzed mast cells in the peritoneal cavity of WT and *Cebpg* KO mice. Nevertheless we did not observe differences in percentages of peritoneal MCs in our group of animals (*Figure 10*). The distinct results obtained with BMMCs and peritoneal cavity mast cells could be due to difference between *in vitro* and *in vivo* experimental systems. These differences could be generated by stress factors present during *ex vivo* cultures which are not present in healthy mice.

Our BMMCs suggested that C/EBPγ is required for proper mast cell development *in vitro*. To gain knowledge on the mechanisms that might impair the growth of the *Cebpg* KO BMMCs we investigated expression of TFs which are important for mast cell development. Our results demonstrated that absence of C/EBPγ leads to upregulation of *Cebpa* and downregulation of *Gata2*. The fact that *Cebpa* is upregulated and *Gata2* is downregulated in the absence of *Cebpg* might explain why the *Cebpg* KO cultures presented impaired growth. Of note, *Cebpa* expression in progenitor cells promotes differentiation of basophils at the expense of mast cell production. On the other hand, *Gata2* has been suggested to be important for mast cell development (Li *et al.*, 2015). Thus, our expression data suggests that *Cebpg* controls expression of *Cebpa* and *Gata2*, two critical factors important for mast cell growth.

Since our data suggest that *Cebpg* might control the expression of *Cebpa* and *Gata2*, we proceeded to perform luciferase assays. We have prepared luciferase assay vectors containing proximal promoter regions of *Cebpa*, *Gata2 IG* and *Gata2 IS* (*Figures 12 to 15*). Validation of properly inserted sequences into pxp2 luciferase assay vector needs to be done using sequencing. The luciferase assays have not been done yet. Nevertheless, we expect to see impaired transactivation of *Cebpa* promoter by C/EBPγ and transactivation of *Gata2* promoters by C/EBPγ. If the luciferase assays demonstrate that C/EBPγ controls transactivation of *Cebpa* and *Gata2*, we will advance to site-directed mutagenesis to find the proper binding site of C/EBPγ in proximal promoter regions of selected genes. But it is also possible that the interplay between C/EBPγ and *Cebpa* or *Gata2* is indirect. Alternatively, C/EBPγ could modulate mast cell development by controlling the activity

of other TFs important in this process. Thus future experiments will be performed to decipher the role of C/EBPγ in this process.

9. Conclusion

MCs are critical effector cells of the innate and adaptive immune system (Abraham and St.John, 2010; Galli and Tsai, 2012; da Silva, Jamur and Oliver, 2014). Proper MC production needs to be tightly control in order to balance host defense and prevent disease. TFs are key modulators of MC production (Arinobu *et al.*, 2005; Qi *et al.*, 2013). Despite the fact that C/EBPγ is highly expressed in activated MCs whether and how these TFs plays a role in MC production and/or function remains elusive. Here, we aimed to investigate the role of C/EBPγ in these processes. Using BMMCs derived from WT and *Cebpg* KO mice, we observed that C/EBPγ is important for proper MC development and production, and that this TF promotes survival of these cells. Further, we analyzed TFs expression in our system, and showed altered expression of *Cebpa* and *Gata2*, two factors regulating MC development. Finally, we generated luciferase reporter constructs to determine whether C/EBPγ controls transactivation of *Cebpa* and *Gata2*. To conclude it all, our aim to determine the role of C/EBPγ in MCs development is still not finished. However the interesting findings about C/EBPγ KO BMMCs and prepared luciferase assay vectors will help us in elucidating this extraordinary process.

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