Assessing the physiological and pathological functions of mast cells by the use of novel mouse models

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1. Abbreviations

AP-1	activator protein 1
BMCP	basophil and mast cell progenitor
BMMC	bone marrow-derived mast cell
CIA	collagen-induced arthritis
cIAP	cellular inhibitor of apoptosis protein
CPA3	carboxypeptidase A3
CTMC	connective tissue mast cell
DC	dendritic cell
DTA	diphtheria toxin A
DTR	diphtheria toxin receptor
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EAE	experimental autoimmune encephalomyelitis
FcεRI	high affinity IgE receptor
GWAS	genome wide association study
HECT	homologous to E6-associated protein carboxyl terminus
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
IL-33R	IL-33 receptor
IRF	interferon regulated factor
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
IκB	inhibitor of NF-κB
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
MAP	mitogen-activated protein
MMC	mucosal mast cell
mMCP	murine mast cell protease
MS	multiple sclerosis
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
NIK	NF-κB-inducing kinase
OTU	ovarian tumor
PKC	protein kinase C
PLC	phospholipase C
RBR	RING-in-between-RING
RING	really interesting new gene

SCF	stem cell factor
SLE	systemic lupus erythematosus
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNP	single nucleotide polymorphism
TAD	transcription activation domain
Th2	T helper type 2
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFAIP	TNF α-induced protein
TNFR	TNF receptor
TRAF	TNFR-associated factor
UBAN	ubiquitin binding in ABIN and NEMO
W	white spotting
ZnF	zinc finger

2. List of Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III).

I **Heger K.***, Fierens K.*, Vahl J.C., Aszodi A., Peschke K., Schenten D., Hammad H., Beyaert R., Saur D., van Loo G., Roers A., Lambrecht B.N., Kool M. and Schmidt-Supprian M. (2014) A20-Deficient Mast Cells Exacerbate Inflammatory Responses in Vivo. *PLoS Biol* 12: e1001762. *equal contribution

II Chu Y., Vahl J.C., Kumar D., **Heger K.**, Bertossi A., Wójtowicz E., Soberon V., Schenten D., Mack B., Reutelshöfer M., Beyaert R., Amann K., van Loo G. and Schmidt-Supprian M. (2011) B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice. *Blood* 117: 2227–2236.

III **Heger K.**, Seidler B., Vahl J.C., Schwartz C., Kober M., Klein S., Voehringer D., Saur D. and Schmidt-Supprian M. (2014) CreER^{T2} expression from within the c-Kit gene locus allows efficient inducible gene targeting in and ablation of mast cells. *Eur J Immunol* 44: 296–306.

3. Summary

The immune system defends its host against various pathogens, including viruses, bacteria, fungi and parasites. In addition, it plays important roles in the protection against and avoidance of noxious substances. Although appropriate immune responses are essential for host fitness, exaggerated or misdirected reactions against self- or seemingly harmless non-self antigens (allergens) can have detrimental outcomes, as seen in inflammatory, autoimmune and allergic diseases. The latter are often characterized by the production of allergen-specific IgE immunoglobulins. Mast cells are well known key players in IgE-dependent anaphylactic responses. In addition, they have also been implicated in playing a pathological role in various inflammatory and autoimmune diseases. However, this notion is controversial as it is primarily based on studies in mice that lack mast cells due to mutations in the receptor tyrosine kinase c-Kit.

To dissect the contribution of mast cells to allergic, inflammatory and autoimmune diseases, I established the first gain-of-function mouse model for hyperactive mast cells by specifically ablating the ubiquitin-editing enzyme A20. This caused enhanced NF-κB activation downstream of TLRs, the IL-33R and the IgE:FcεRI module and thereby selectively enhanced pro-inflammatory mast cell reactions without affecting their degranulation. As a result A20-deficient mast cells exacerbated allergic lung and skin inflammation, and collagen-induced arthritis. In contrast, IgE-dependent immediate anaphylaxis reactions and experimental autoimmune encephalomyelitis were unaffected. These results demonstrate for the first time the consequences of enhanced inflammatory mast cell responses and provide evidence that mast cells can contribute to disease pathology (**Publication I**). In addition, I evaluated the biochemical consequences of A20 loss in B cells, which resulted in prolonged NF-κB activation leading to their pronounced hyperactivation (**Publication II**). Furthermore, I characterized a novel mouse strain expressing a tamoxifen-inducible version of the Cre recombinase under control of the endogenous c-Kit locus. This allowed specific and efficient inducible gene targeting in mast cells and enabled the study of mast cell differentiation and maintenance (**Publication III**).

In summary, these novel mouse models yielded important insights into mast cell biology and allowed a dissection of the pathological contribution of mast cells to inflammatory diseases.

4. Introduction

4.1. Mast Cell Biology

4.1.1. Mast Cell Development

More than 130 years ago Paul Ehrlich described mast cells in connective tissues based on the histological staining patterns of basic aniline dyes. Due to their characteristic morphology with numerous cytosolic granules (Figure 1) he named them mast cells, assuming that their granules reflect the tissue's nutritional or fattening state (in German "Mast") (Vyas and Krishnaswamy, 2006).

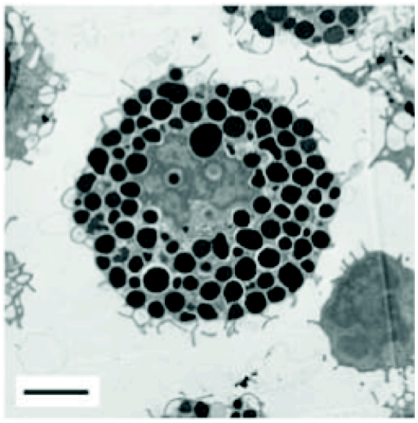

Figure 1. Mast cell ultrastructure. Transmission electron micrograph of a mouse peritoneal mast cell. Scale bar: 2 µm. Taken from Taketomi et al. (2013).

The developmental origin of mast cells remained unclear for a long time. It was initially believed that they arise from fibroblasts until bone marrow transplantation experiments hinted at their hematopoietic origin (Kitamura et al., 1978; 1977). It is now well established that mast cells are indeed innate immune cells and hence as all other cells of the immune system are derived from hematopoietic stem cells (Figure 2) (Arinobu et al., 2005; Chen et al., 2005; Franco et al., 2010; Qi et al., 2013). The exact developmental route of mast cells and their relationship to basophils still remains controversial. On the one hand, it was proposed that they share a common progenitor with neutrophils and macrophages (Suda et al., 1983) or with basophiles in the bone marrow or spleen (Arinobu et al., 2005; Qi et al., 2013). On the other hand, a mast cell-committed progenitor with a closer developmental relationship to the megakaryocyte/erythrocyte lineage has also been discussed (Chen et al., 2005; Franco et al., 2010).

Mast cells, unlike granulocytes and monocytes, do not complete their maturation in the bone marrow and do not circulate in the blood in a mature form (Gurish and Austen, 2012). This implies that their lineage-committed precursors migrate to peripheral tissues, where they undergo final differentiation under the

influence of tissue-specific growth factors and proliferate locally (Figure 2) (Tsai et al., 1991). Interestingly, these mast cell precursors peak in number in fetal blood at day 15 of gestation, seed the fetal skin and mature locally (Hayashi et al., 1985; Rodewald et al., 1996). In the adult, their precursors are rarely detectable in blood, whereas mature mast cells can be found in essentially all tissues exposed to the environment, including the skin, the serosal cavities (e.g., the pleural and peritoneal cavities), and the respiratory and gastrointestinal tracts (Gurish and Austen, 2012). In these tissues mast cells are located in close proximity to blood vessels, nerves, smooth muscle cells, glands and hair follicles (Abraham and St John, 2010).

4.1.2. Mast Cell Subsets and their Homeostasis

In peripheral tissues mast cells are not a homogenous population, rather they exhibit a considerable degree of heterogeneity. In rodents, two major subsets of mast cells have been described, based on differences in their development, localization, granular content and pharmacologically induced degranulation (Kitamura, 1989). Connective tissue mast cells (CTMCs) constitutively reside in the dermis of the skin, the serosal cavities, and the submucosa of the gastrointestinal tract (Gurish and Austen, 2012). In contrast, mucosal mast cells (MMCs) are induced during certain allergic reactions or intestinal worm infections and reside intraepithelially in the gut or

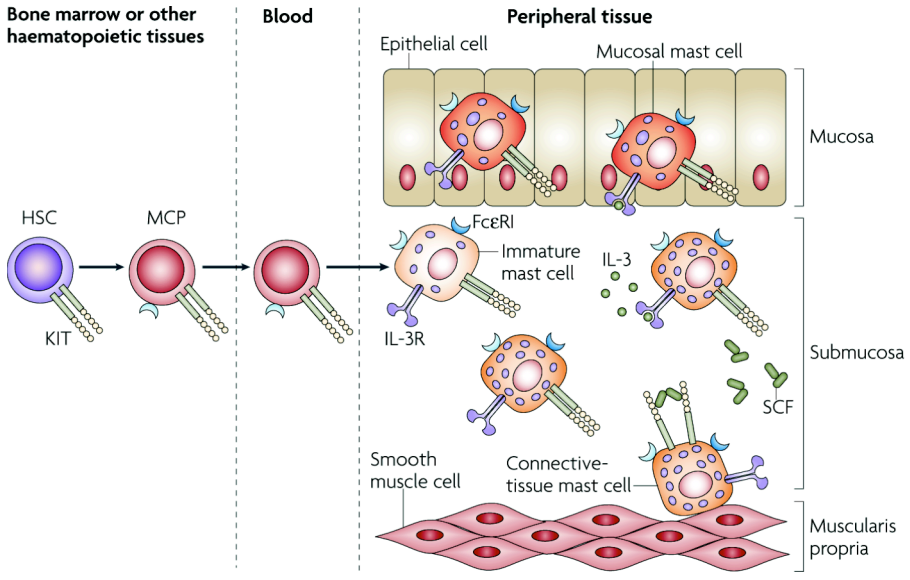

Figure 2. Mast cell development and subsets. Hematopoietic stem cells (HSC) give rise to immature blood-circulating mast cell progenitors (MCP) that enter peripheral tissues and differentiate locally. While mucosal mast cells are induced in the gut or respiratory mucosa upon allergic reactions and parasite infections, connective tissue mast cells are constitutively present in the submucosa. Modified from Galli et al. (2008).

respiratory mucosa (Figure 2) (Lantz et al., 1998; Xing et al., 2011). In their granules, mast cells store a large array of highly potent compounds including vasoactive amines, such as histamine and serotonin, proteoglycans and neutral proteases. While CTMCs express the proteoglycan heparin, the granules of MMCs mainly contain chondroitin sulfate (Gurish and Austen, 2012; Kitamura, 1989). Besides proteoglycans the main protein content of mast cell granules are neutral proteases that can be categorized into three classes, namely the metalloproteinase carboxypeptidase A3 (CPA3) and the murine serine mast cell proteases (mMCP), which have chymotryptic (chymases) or tryptic (tryptases) activities (Gurish and Austen, 2012). Murine CTMCs express CPA3, the chymases mMCP-4 and mMCP-5, and the tryptase mMCP-6 (Xing et al., 2011). In contrast, helminth-induced MMCs in the intestine express only the chymases mMCP-1 and mMCP-2 (Friend et al., 1996). Mast cells in the trachea and proximal airways provoked by allergic reactions express a broad range of proteases, which indicates a considerable degree of phenotypic plasticity (Xing et al., 2011). Hence, the protease phenotype of mast cells seems to be also regulated by the local environment and cytokine milieu.

The *in vivo* development and/or homeostasis of both CTMCs and MMCs strongly depends on signals from the receptor tyrosine kinase c-Kit, which is encoded by the *white spotting* (*W*) locus (Rodewald and Feyerabend, 2012). In the hematopoietic system c-Kit is mainly expressed by progenitors in the bone marrow (Rodewald and Feyerabend, 2012). Its expression declines in most cell types during differentiation but remains prominent through all stages of mast cell development (Arinobu et al., 2005; Rodewald et al., 1996). Hence, various compound hypomorphic c-Kit mutations result in profound CTMC and MMC deficiency (Grimbaldeston et al., 2005; Kitamura et al., 1978). In addition, mast cells are also absent in mice with mutations in the *steel* locus that encodes for the c-Kit ligand stem cell factor (SCF) (Kitamura and Go, 1979). While c-Kit transmitted signals are essential for *in vivo* development, interleukin-3 (IL-3) is sufficient for the differentiation of bone marrow-derived mast cells (BMMCs) *in vitro* (Gurish and Austen, 2012). However, IL-3 is dispensable for mast cell development *in vivo* under steady-state conditions, but drives mastocytosis upon infection with intestinal nematodes (Lantz et al., 1998). In addition, IL-4, IL-9, IL-10, IL-33, lipid mediators and immunoglobulin (Ig)E might further support the maturation and enhance the survival of mast cells in peripheral tissues (Gurish and Austen, 2012; Kashiwakura et al., 2011; Taketomi et al., 2013).

4.1.3. Mast Cell Activation and Signaling Pathways

Mast cells prominently express the high affinity IgE receptor (FcεRI) allowing them to capture circulating IgE. While priming with IgE *per se* has been shown to enhance mast cell survival, maturation, cytokine release and FcεRI expression (Kashiwakura et al., 2011), crosslinking of the IgE-bound FcεRI causes very prominent mast cell activation. The FcεRI is a heterotetrameric complex consisting of one α chain that binds IgE, one β chain and two γ chains that both possess signal transduction function through immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails (Figure 3) (Gilfillan and Tkaczyk, 2006; Kraft and Kinet, 2007). Crosslinking of the IgE-bound FcεRI leads to ITAM phosphorylation by receptor-associated Src tyrosine kinase family members (Gilfillan and Tkaczyk, 2006; Kraft and Kinet, 2007). The subsequent recruitment and activation of the kinase Syk induces various signal transduction pathways via the phosphorylation of multiple adaptor and scaffolding proteins (Gilfillan and Tkaczyk, 2006; Kraft and Kinet, 2007). These proximal signaling events lead to the activation of phosphatidylinositol 3-kinase, mitogen-activated protein (MAP) kinases (ERK, JNK, p38) and phospholipase C (PLC), which in turn control the activation of protein kinase C (PKC) isoforms through the generation of secondary messengers (1,2-diacylglycerol, inositol-1,4,5-triphosphate, and free cytosolic Ca^{2+}) (Gilfillan and Tkaczyk, 2006; Klemm et al., 2006; Kraft and Kinet, 2007). The transient increase in cytosolic Ca^{2+} from intracellular endoplasmic reticulum stores is prolonged and potentiated through store-operated calcium channels in the plasma membrane and the entry of extracellular Ca^{2+} (Vig et al., 2008). These initial events cause three major downstream effector responses in mast cells (Figure 3) (Klemm et al., 2006): (1) Active PKC and free cytosolic Ca^{2+} synergistically induce the fusion of granules with the plasma membrane and the release of granular content, a process termed compound exocytosis or degranulation (Lorentz et al., 2012); (2) Free cytosolic Ca^{2+} and phosphorylation by MAP kinases activate cytosolic phospholipase A_2 which initiate the biosynthesis of pro-inflammatory lipid mediators (including PGD_2, PGE_2, LTB_4 and LTC_4) by the release of arachidonic acid from phospholipids (Gilfillan and Tkaczyk, 2006); and (3) FcεRI crosslinking leads to the activation of various transcription factors, including activator protein 1 (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor (NF)-κB, thereby controlling late phase responses through the induction of gene expression. This results in the production of pro-

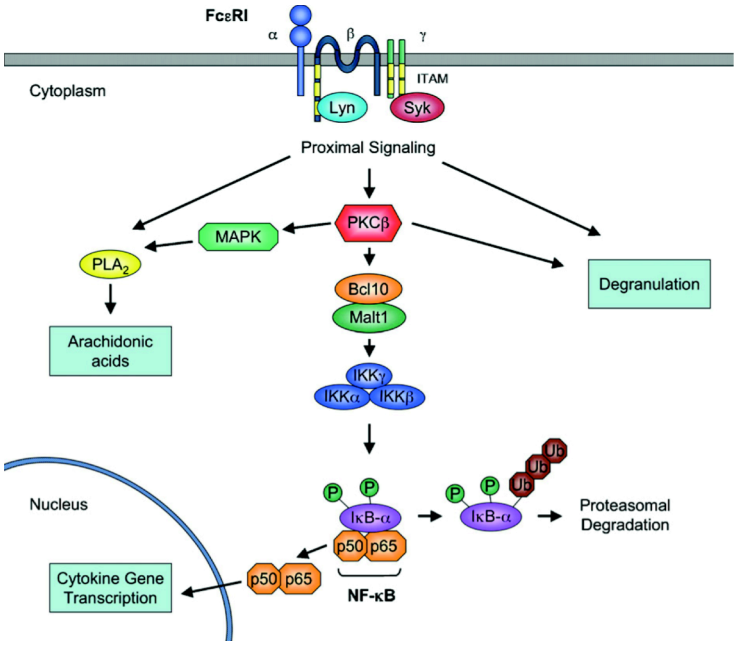

Figure 3: Model of FcεRI signaling. FcεRI aggregation triggers proximal phosphorylation events leading to the generation of various secondary messengers. These in turn activate PKC isoforms and cause three main mast cell effector responses: Biosynthesis of lipid mediators, induction of gene transcription (NF-κB activation) and degranulation. Modified from Klemm and Ruland (2006).

inflammatory cytokines and chemokines (Gilfillan and Tkaczyk, 2006). To modulate FcεRI-induced signal transduction, mast cells express several inhibitory receptors such as FcγRIIB, SIRPα and Allergin-1 (Hitomi et al., 2010; Kraft and Kinet, 2007). These share a common cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that can recruit various phosphatases and suppress activating signaling pathways (Kraft and Kinet, 2007).

Although FcεRI crosslinking causes arguably most prominent mast cell activation, other activating stimuli also shape mast cell effector responses. In agreement, FcεRI expression happens late during murine mast cell development when cells are already lineage committed (Arinobu et al., 2005; Rodewald et al., 1996) and the acquisition of FcεRI was a relatively late event in evolution that coincided with the appearance of IgE immunoglobulins in mammals (Crivellato and Ribatti, 2010). Phylogenetic studies identified potential mast cell precursors, which release histamine, heparin and tryptase upon activation indicating that they might fight microbial infections (Crivellato and Ribatti, 2010). Indeed, murine mast cells also express molecules of such ancient immune defense systems, in particular pattern recognition receptors of the Toll-like receptor (TLR) family (Matsushima et al., 2004). TLRs serve as sensors that recognize invariant pathogen-associated molecular patterns and are hence essential to combat infections (Kawai and Akira, 2010). For example TLR3, TLR4 and TLR9 recognize viral double-stranded RNA, the bacterial cell wall component lipopolysaccharide (LPS), and unmethylated viral or bacterial

CpG DNA, respectively (Kawai and Akira, 2010). In addition, mast cells can sense inflammation and tissue damage by expressing receptors recognizing IL-1 (IL-1R) and IL-33 (IL-33R), respectively (Schmitz et al., 2005). IL-33 is a recently identified member of the IL-1 family that is released upon tissue damage such as necrosis and hence seems to function as an alarmin (Moussion et al., 2008). Since mast cells express high levels of the IL-33Rα chain (ST2), they can rapidly respond to cell injury and recruit other immune cells to sustain inflammation or initiate tissue repair processes (Enoksson et al., 2011; Moritz et al., 1998). Engagement of TLRs, the IL-1R and the IL-33R triggers a signaling cascade via the recruitment of various adaptor proteins including myeloid differentiation primary response gene 88 (MyD88). This leads to the activation of MAP kinases and NF-κB, resulting in pro-inflammatory gene expression (Matsushima et al., 2004; Schmitz et al., 2005; Vallabhapurapu and Karin, 2009). Certain TLR ligands and IL-33 have also been implicated in triggering mast cell degranulation (Komai-Koma et al., 2012; Supajatura et al., 2002). Additionally, mast cells can be activated by complement components, adenosine, cytokines and chemokines (Gilfillan and Tkaczyk, 2006).

4.1.4. Genetic Mouse Models to Study Mast Cell *in Vivo* Functions

Mast cells can be studied *in vivo* by perturbing their functions using pharmacological approaches. Although this is of clinical interest and has some advantages with respect to reversibility and temporal control, systemically or locally administered agents might cause unwanted side effects that have to be taken into account when interpreting data from such studies. As an example, small molecules that inhibit the tyrosine kinases c-Kit or Syk lack mast cell specificity (Juurikivi et al., 2005; Matsubara et al., 2006) and the efficiency of the ‘mast cell stabilizer’ cromolyn in preventing degranulation (Orr and Cox, 1969) has recently been questioned (Oka et al., 2012). Genetic approaches therefore represent the method of choice to study and characterize mast cell functions *in vivo*. To date, two Kit mutant mast cell-deficient mouse strains, $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$, are most widely used (Kawakami, 2009). The W mutation results in a truncated c-Kit protein lacking the transmembrane domain, which is not expressed on the cell surface and has the characteristics of a null allele (Nocka et al., 1990); the Wv mutation encodes for dominant loss-of-function point

mutation in the c-Kit kinase domain (Nocka et al., 1990); and the W-sh mutation is an inversion within the 5' regulatory region of the c-Kit gene locus (Nigrovic et al., 2008).

Besides mast cells, c-Kit is expressed by numerous other cells of hematopoietic and non-hematopoietic origin (Rodewald and Feyerabend, 2012). Hence, $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice have several abnormalities in and outside of the hematopoietic system that are not related to mast cell deficiency. Both mouse strains lack intestinal cells of Cajal and skin melanocytes, resulting in non-pigmented white fur. Moreover, $Kit^{W/Wv}$ mice are sterile, anemic, have reduced numbers of neutrophils, basophils, megakaryocytes and intraepithelial γδ T cells, and suffer from dermatitis, gastric ulcers and dilatation of the duodenum (Grimbaldeston et al., 2005; Mancardi et al., 2011; Nigrovic et al., 2008 and references within). In contrast, $Kit^{W\text{-}sh/W\text{-}sh}$ mice are fertile, not anemic and do not suffer from spontaneous pathologies affecting the skin, stomach or duodenum. However, $Kit^{W\text{-}sh/W\text{-}sh}$ mice have histologically abnormal and enlarged spleens with neutrophilia, basophilia, megakaryocytosis and thrombocytosis (Grimbaldeston et al., 2005; Mancardi et al., 2011; Nigrovic et al., 2008). Due to a disruption in the *Corin* coding region $Kit^{W\text{-}sh/W\text{-}sh}$ mice additionally develop mild cardiomegaly (Nigrovic et al., 2008). These multiple physiological defects that are not directly related to the lack of mast cells limit the interpretation of experimental results derived from these *in vivo* models. Yet, the mast cell deficiency of $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice can be selectively repaired by the adoptive transfer of *in vitro* generated mast cells (Grimbaldeston et al., 2005; Nakano et al., 1985). These mast cell reconstituted mice, also termed 'mast cell knock-in mice', have been used for separating general Kit-dependent but mast cell-independent abnormalities from *bona fide* mast cell functions. Despite all these limitations, most of our knowledge on mast cell *in vivo* function has been gathered over the last decades from mast cell-deficient and mast cell-reconstituted $Kit^{W/Wv}$ or $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Kawakami, 2009).

Recent advances in gene targeting approaches led to the development of novel tools to manipulate the genome of mast cells using the Cre/loxP technology. Several groups utilized the regulatory elements of mast cell protease genes (Feyerabend et al., 2011; Lilla et al., 2011; Müsch et al., 2008; Scholten et al., 2008) or the FcεRIβ chain (Furumoto et al., 2011) to drive constitutive expression of the Cre recombinase. With these mice mast cell-specific conditional gene manipulation has been accomplished to various degrees of efficiency and specificity. In addition, these mice were used to generate novel Kit-independent models of mast cell deficiency. Scholten et al. generated bacterial artificial chromosome transgenic mice

with Cre under the control of the *Mcpt5* gene locus (Mcpt5Cre) and hence expression is limited to mast cells expressing the chymase mMCP-5 (Scholten et al., 2008). This allowed mast cell-specific expression of the diphtheria toxin A (DTA) subunit resulting in constitutive CTMC ablation. In addition, Cre-mediated expression of the human diphtheria toxin receptor (DTR) permitted inducible selective depletion of CTMCs upon diphtheria toxin injections (Dudeck et al., 2011). Müsch et al. generated a transgenic mouse line with Cre under the control of a 600 bp baboon α-chymase promoter fragment (Chm:Cre). A LacZ reporter strain indicated that recombination was limited to MMCs (Müsch et al., 2008). Lilla et al. generated mice harboring a transgene with Cre under the control of a 780 bp Cpa3 promoter fragment (Cpa3-Cre). A florescent reporter strain revealed Cre-mediated recombination in various leucocytes in addition to mast cells (Lilla et al., 2011). To ablate mast cells, Cpa3-Cre mice were crossed to a conditional Mcl-1 allele encoding for a potent pro-survival member of the Bcl-2 family. This caused mast cell deficiency in Cpa3-Cre Mcl-$1^{F/F}$ mice but also basopenia, neutrophilia and macrocytic anemia (Lilla et al., 2011). Feyerabend et al. inserted Cre into the *Cpa3* locus by homologous recombination ($Cpa3^{Cre/+}$). Surprisingly, these mice did not allow mast cell-specific gene targeting but lacked all mast cell subsets in addition to a reduction in the number of basophils presumably due to Cre-mediated toxicity (Feyerabend et al., 2011). Similarly, Furumoto et al. used homologous recombination into the *FcεRIβ* chain locus to drive Cre expression in mast cells and basophiles with only limited efficiency ($FcεRIβ^{Cre/+}$) (Furumoto et al., 2011). Finally, Otsuka et al. generated transgenic mice, in which DTR expression is driven by IL-4 promoter and enhancer fragments allowing the inducible ablation of all mast cell subsets in addition to basophils upon diphtheria toxin injections (Mas-TRECK) (Otsuka et al., 2011). These novel Kit-independent mast cell deficient mice will allow to reassess *in vivo* mast cell functions and will permit to dissect the role of specific genes using the Cre/loxP technology.

4.1.5. Mast Cells in Pathologies

Based on correlative studies in human patients and genetic mouse models such as $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice, mast cells have been implicated in various pathologies including allergic, inflammatory and autoimmune diseases (Brown and Hatfield, 2012; Galli and Tsai, 2012).

4.1.5.1. Mast Cells in Allergic Responses

Allergic diseases, including atopic dermatitis, food allergies, allergic rhinitis and allergic asthma, are considered to be abnormal or misdirected immune responses against seemingly harmless non-self antigens (allergens) (Galli and Tsai, 2012). These reactions closely resemble type 2 immune responses, which are generated upon intestinal helminth infection, and are characterized by activation of epithelial barriers, goblet hyperplasia and mucus production, polarization of T helper type 2 (Th2) cells and increased serum levels of IgG1 and IgE, accompanied by the activation of basophils, eosinophils and mast cells (Galli and Tsai, 2012). Mast cells are well known effector cells in IgE-associated reactions. Through crosslinking of the IgE-bound FcεRI by multivalent allergens, mast cells contribute to early-phase allergic reactions (type I immediate hypersensitivity reactions) by the rapid release of preformed mediators such as vasoactive amines and the production of pro-inflammatory lipid mediators (Feyerabend et al., 2011; Klemm et al., 2006). In severe cases, exaggerated immediate allergic hypersensitivity reactions can cause life-threatening anaphylaxis in susceptible hosts (Rodewald and Feyerabend, 2012). In addition, mast cells participate in late-phase allergic reactions by the production of pro-inflammatory lipid mediators and cytokines that orchestrate the recruitment of leucocytes to substantiate inflammation (Galli and Tsai, 2012; Klemm et al., 2006). During allergic asthma the host is repeatedly exposed to allergens causing early- and late-phase reactions that result in inflammation and airway remodeling (Galli and Tsai, 2012). Mast cell numbers increase during asthma (Brightling et al., 2002) and in rodent asthma models using $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice, mast cells were shown to be essential for disease induction when sensitization to model allergens occurred under mild conditions (Nakae et al., 2007; Williams and Galli, 2000). In this context mast cells might be stimulated by IgE-dependent and -independent pathways (Galli

and Tsai, 2012; Mayr et al., 2002). In the latter scenario, they might be activated by TLR ligands or IL-33 to exacerbate disease pathology (Galli and Tsai, 2012). However, in addition to playing a pathological role, several reports also implicated mast cells in dampening allergic inflammation by protease-mediated cleavage of IgE, IL-13 or IL-33, making their exact function a matter of debate (Rauter et al., 2008; Waern et al., 2013; 2012).

Allergic reactions are generally considered to be detrimental misdirected type 2 immune responses originally designed to protect the host from macroparasites However, they could also be part of a more general defense mechanism against noxious substances, xenobiotics and irritants (Palm et al., 2012). Indeed, mast cells have been shown to degrade bee, snake, gila monster and scorpion venoms through the secretion of granule-associated proteases (Akahoshi et al., 2011; Metz et al., 2006). It remains to be determined if mast cells are directly activated by venoms (Metz et al., 2006) or if a protective IgE-dependent immune response is required and in this context mast cells are activated through the FcεRI (Marichal et al., 2013; Palm et al., 2013). In addition to the secretion of proteases mast cells rapidly release histamine upon activation. This causes vasodilatation through its effect on the endothelium, leads to bronchoconstriction and enhances peristaltic movement by acting on smooth muscle cells, and provokes itch by stimulating nerve endings (Thurmond et al., 2008). These effects could lead to the dilution of venoms, expulsion of noxious particles by sneezing, coughing, tearing, vomiting and diarrhea, and itch-mediated mechanical removal of ectoparasites such as ticks (Palm et al., 2012). Therefore, mast cell-dependent allergic reactions might not only be detrimental but could also be beneficial for the host. Along the same line, allergic anaphylaxis reactions might be overshooting responses analogous to fatal septic shock and exaggerated inflammation upon bacterial infection.

4.1.5.2. Mast Cells in Autoimmune Diseases

In addition to allergic reactions mast cells have been implicated in playing a pathological role in various autoimmune diseases. When work on this thesis commenced, prominent roles for mast cells were suggested for multiple sclerosis (MS) and rheumatoid arthritis (Brown and Hatfield, 2012; Sayed et al., 2008).

MS, a chronic inflammatory disease of the central nervous system, is characterized by a breakdown of the blood-brain barrier, mononuclear and T cell infiltration, extensive demyelination and the formation of lesions consisting of fibrous plaques (Hauser and Oksenberg, 2006). This ultimately causes impairment in various sensory, motor and other neural functions (Hauser and Oksenberg, 2006). Accumulation of mast cells or mast cell-associated transcripts have been detected in these lesions (Sayed et al., 2008). In addition, mast cells seem to be activated, as suggested by high levels of tryptase and histamine in the cerebrospinal fluid of MS patients (Sayed et al., 2008). Degranulated mast cells were also detected in the central nervous system of animals suffering from experimental autoimmune encephalomyelitis (EAE), a rodent model for MS (Brenner et al., 1994). An essential contribution to this disease was proposed based on studies using pharmacological inhibition (Dimitriadou et al., 2000) or the analysis of mast cell-deficient $Kit^{W/Wv}$ mice (Secor et al., 2000). These mice showed reduced disease incidence, delayed onset and milder symptoms in comparison to control animals, all of which could be restored upon intravenous mast cell reconstitution (Secor et al., 2000). Several mechanisms have been proposed for how mast cells influence the outcome of EAE. Mast cells are thought to degrade myelin through the release of proteases, influence permeability of the blood brain barrier by the secretion of histamine (Sayed et al., 2008) and enhance the recruitment of neutrophils and T cells through tumor necrosis factor (TNF) release (Sayed et al., 2010). However, as transferred mast cells did not repopulate the central nervous system, despite restoring susceptibility in $Kit^{W/Wv}$ mice (Tanzola et al., 2003), it was proposed that they could also exert their function in the periphery by functioning as antigen presenting cells to generate an adaptive T cells response or influence the function of dendritic cells (DCs) (Sayed et al., 2008). During EAE, mast cells might become activated by scavenger receptor-mediated detection of myelin (Medic et al., 2008) or through activating Fc receptors and TLRs (Brown et al., 2002; Sayed et al., 2008).

Rheumatoid arthritis is an autoimmune disease characterized by chronic joint inflammation, synovial hyperplasia and immune cell infiltrations leading to bone and cartilage destruction. In recent years, much progress has been made in identifying molecular players of the disease, but the exact etiology still remains incompletely understood (Eklund, 2007). Correlative studies in human patients identified mast cells at sites of cartilage erosion and their numbers correlate with disease severity (Eklund, 2007). Several rodent models are used to study arthritis pathology, including

serum transfer and active induction models (Lee et al., 2002; Pitman et al., 2011). Transfer of autoantibody-containing serum from K/BxN mice, which spontaneously develop arthritis, induces disease pathology in recipient mice in a manner that depends on the alternative complement pathway, Fc receptors, neutrophils and the pro-inflammatory cytokine IL-1 (Nigrovic and Lee, 2007). Using this model Lee and colleagues demonstrated that $Kit^{W/Wv}$ mice were completely protected from arthritis induction and susceptibility could be selectively repaired upon mast cell reconstitution (Lee et al., 2002). In this context, mast cells have been proposed to aggravate disease pathology by leukocyte recruitment through the release of LTB_4, TNF, IL-1 and tryptase, promoting vascular permeability, generating local inflammation, or the destruction of synovium and bone (Brown and Hatfield, 2012; Eklund, 2007; Nigrovic and Lee, 2007; Xu et al., 2008). During disease, mast cells might be activated by Fc or complement receptors (Nigrovic and Lee, 2007). Moreover, the recently identified pro-inflammatory alarmin IL-33 might play an important role in the induction of arthritis (Palmer and Gabay, 2011) and it has been proposed that IL-33 fulfills its function at least in part by activating mast cells (Xu et al., 2008). Collectively, our current understanding of *in vivo* mast cell functions during autoimmune diseases are derived from correlative studies in human patients and mainly one experimental system, namely mast cell-deficient $Kit^{W/Wv}$ mutant mice. However, the pathological contribution of mast cells to autoimmune diseases is not as well-defined as it might appear as several recent reports using different models of mast cell deficiency have challenged some of these initially proposed findings (Bennett et al., 2009; Li et al., 2011; Piconese et al., 2011; Zhou et al., 2007).

4.2. Regulation of NF-κB Activation by the Ubiquitin-Editing Enzyme A20

4.2.1. The Ubiquitin System

Ubiquitination, the covalent attachment of ubiquitin, is a reversible and dynamic post-translational modification involved in various cellular processes, including signal transduction, protein turnover, DNA repair, cell-cycle control, receptor endocytosis and vesicle trafficking (Dikic et al., 2009). Ubiquitination of substrate proteins is brought about by a three-step enzymatic cascade catalyzed by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) (Figure 4) (Hershko and Ciechanover, 1998; Malynn and Ma, 2010). The E1s initiate this process in an ATP-dependent reaction leading to the formation of a thioester bond between the carboxy group of the C-terminal glycin residue and a cysteine in the E1's active center. The activated ubiquitin is then transferred to an E2, which, in combination with an E3 ligase, attaches ubiquitin to a substrate protein by forming an isopeptide bond between the C-terminus of ubiquitin and, typically, an ε-amino group of an internal lysine residue (K) (Dikic et al., 2009; Hershko and Ciechanover, 1998; Malynn and Ma, 2010). In humans, there are two E1s, approximately 40 E2s and more than 600 E3s (Malynn and Ma, 2010). Substrate specificity is mediated by E3 ligases which, based on function, can be generally subdivided into two groups. One is the family comprising the really interesting new gene (RING) and the structurally related U-box proteins, which do not form a covalent thioester bond with ubiquitin but rather bring the activated E2 into proximity with a substrate and hence function as matchmakers. The other group contains homologous to E6-associated protein carboxyl terminus (HECT) E3 ligases that, in contrast, accept the activated ubiquitin and form a covalent intermediate before transfer. Similarly, a distinct set of E3s that contain a RING-in-between-RING (RBR) domain might use a HECT-like mechanism for the transfer of ubiquitin (Komander and Rape, 2012).

Substrate proteins can be modified with a single ubiquitin moiety (monoubiquitination), with multiple single ubiquitin moieties (multimonoubiquitination), or with polymeric chains (polyubiquitination) (Dikic et al., 2009; Komander and Rape, 2012). The latter modification is generated through successive addition of ubiquitin to the N-terminal amino group (linear or M1-linked chains) or to one of the seven internal lysine residues (K6-, K11-, K27-, K29-, K33-, K48- or K63-linked chains) of

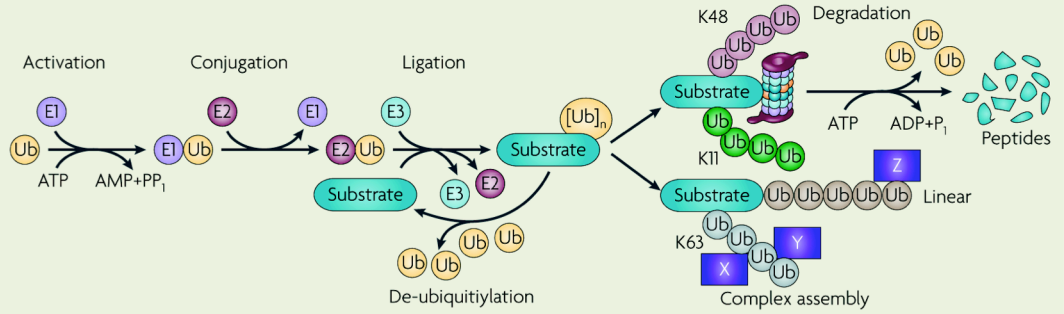

Figure 4. The ubiquitin system. Ubiquitin is conjugated to substrate proteins in a manner dependent on the serial and coordinated action of three classes of enzymes (E1s, E2s and E3s). Attachment of monoubiquitin or of polyubiquitin chains, which adopt different topologies as a result of their linkage type, marks the modified protein for diverse biological consequences. As an example, K48- and K11-linked chains target proteins for proteasomal degradation, while linear and K63-linked chains promote the assembly of complexes. Deubiquitinating enzymes can remove conjugated ubiquitin moieties. Taken from Hymowitz and Wertz (2010).

the previously attached ubiquitin (Dikic et al., 2009; Komander and Rape, 2012). These polyubiquitin chains adopt distinct conformations, which can be distinguished by linkage-specific ubiquitin-binding domains and hence fulfill different functions (Dikic et al., 2009; Komander and Rape, 2012). K11- and K48-linked chains both adopt a compact conformation and have been mainly studied in the context of targeting substrate proteins to the 26S proteasome for degradation (Figure 4) (Bremm et al., 2010; Eddins et al., 2007). In contrast, linear and K63-linked chains adopt an open topology with a high degree of conformational freedom and predominantly seem to fulfill non-proteolytic functions (Figure 4) (Komander et al., 2009b). In addition to homotypic linkage types, heterotypic mixed and branched chains have been reported (Dikic et al., 2009). HECT domain-containing E3 ligases are thought to intrinsically dictate the linkage type, whereas, in case of RING or U-box E3 ligases linkage specificity is determined by the E2 (Komander and Rape, 2012). As an example, the E2 UBC13 together with the E2 variant UEV1a and the RING E3 ligase TNF receptor associated factor 6 (TRAF6) promotes the formation of K63-linked chains (Deng et al., 2000). In contrast, the E2 UBCH5 and the RING E3 ligases cellular inhibitor of apoptosis protein 1/2 (cIAP1/2) can assemble chains with different topologies, such as K11-, K48- and K63-linked polyubiquitin chains (Dynek et al., 2010). The linkage type of linear polyubiquitin chains is thought to be intrinsically determined by the linear ubiquitin chain assembly complex (LUBAC), which consists of two RBR domain-containing proteins, HOIL-1 and HOIP, in addition to SHARPIN (Kirisako et al., 2006).

Similar to phosphorylation, ubiquitination is a reversible protein modification (Figure 4). Ubiquitin moieties can be removed by deubiquitinating enzymes (DUBs). In humans, there are approximately 79 DUBs, which are either cysteine or metalloproteases (Komander et al., 2009a). DUBs fulfill several key functions in cells: they generate free ubiquitin from ubiquitin precursors; they recycle ubiquitin by removing chains from proteins that are substrates for degradation; and they can trim, edit and remove chains to regulate intracellular signaling events or rescue proteins from degradation (Komander et al., 2009a).

4.2.2. The NF-κB Family of Transcription Factors

The NF-κB family of transcription factors regulates innate and adaptive immunity as well as inflammatory reactions. It fulfills these pleiotropic functions through the transcriptional control of various genes that regulate proliferation, survival and inflammation (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009; Wertz and Dixit, 2010). In mammals, the NF-κB family comprises five members: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), c-Rel and RelB. The family is characterized by an N-terminal Rel homology domain that allows homo- and heterodimer formation and is essential for binding to κB sites in promoters or enhancers of target genes. RelA and c-Rel largely heterodimerize with p50, whereas RelB binds p52 or its precursor p100. Only RelA, c-Rel and RelB additionally contain a C-terminal transcription activation domain (TAD) and can positively regulate transcription. As p50 and p52 lack this TAD, they might function as transcriptional repressors or activators depending on whether they bind to κB sites as homodimers or interact with TAD-containing NF-κB family members, respectively (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009).

NF-κB family members primarily interact with inhibitor of NF-κB (IκB) proteins, which consist of three groups: The classical IκB proteins, IκBα, IκBβ and IκBε, are characterized by the presence of multiple ankyrin repeats that mediate binding to NF-κB dimers and mask their nuclear localization sequence, hence sequestering them in the cytoplasm. Upon cellular activation the classical IκB proteins are rapidly degraded in a proteasome dependent fashion. This releases cytoplasmic NF-κB dimers that can now migrate into the nucleus and activate the transcription of various genes (Ghosh and Hayden, 2008; Vallabhapurapu and Karin,

2009). The second group, the precursor NF-κB proteins p105 and p100 contain ankyrin repeats and thus can also function as inhibitors. In resting conditions the majority of p105 is constantly processed to generate the NF-κB family member p50, however, its full-length form p105 can retain NF-κB proteins such as RelA and c-Rel in the cytoplasm. These are liberated upon stimulation-induced phosphorylation of p105, leading to its complete proteasomal degradation. In contrast, p100 sequesters RelB in the cytoplasm. Activation-induced phosphorylation marks p100 for partial proteasome-mediated processing that results in the generation of the p52 fragment bound to RelB (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009). The third group contains the atypical IκB proteins IκBNS, IκBζ and BCL-3, which are induced upon activation, primarily act in the nucleus and, depending on the context, might function as transcriptional repressors or activators (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009).

4.2.3. Canonical and Alternative NF-κB Activation

In general, two distinct signaling pathways mediate the activation of NF-κB transcription factors. The canonical pathway centers around the trimeric IκB kinase (IKK) complex that contains two catalytic subunits, IKK1 (also known as IKKα) and IKK2 (IKKβ), and the regulatory/scaffolding subunit NF-κB essential modulator (NEMO or IKKγ) (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009; Wertz and Dixit, 2010). Various stimuli, such as microbial products, pro-inflammatory cytokines or signals from antigen receptors, activate the IKK complex (discussed below), which subsequently phosphorylates IκBα on specific serine residues, leading to its K48-linked polyubiquitination by the $SCF^{\beta TrCP}$ E3 ubiquitin ligase complex. Ubiquitinated IκBα is then degraded by the proteasome resulting in the liberation NF-κB heterodimers (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009; Wertz and Dixit, 2010).

The alternative or non-canonical NF-κB pathway is activated by a small subset of TNF family members and their receptors (TNFR), including the lymphotoxin β receptor, B cell-activating factor receptor and CD40. This pathway depends on the labile NF-κB-inducing kinase (NIK), which in non-stimulated cells is modified with a K48-linked polyubiquitin chain and is hence constantly degraded by the proteasome (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009; Wertz and Dixit, 2010). However, upon receptor engagement, NIK is stabilized, accumulates and

phosphorylates IKK1. IKK1-mediated phosphorylation of p100 results in its processing to p52 and translocation of RelB:p52 heterodimers into the nucleus (Ghosh and Hayden, 2008; Vallabhapurapu and Karin, 2009; Wertz and Dixit, 2010).

4.2.4. Activation of the IKK Complex Is Regulated by Ubiquitination

Activation of the canonical NF-κB pathway is intimately regulated by ubiquitination (Skaug et al., 2009; Wertz and Dixit, 2010). Engagement of pattern-recognition, cytokine and antigen receptors leads to the assembly of polyubiquitin chains of various topologies including K63-linked and linear chains (Skaug et al., 2009; Wertz and Dixit, 2010). These receptor-proximal polyubiquitin chains are thought to function as molecular scaffolds and recruitment platforms for kinase complexes (Skaug et al., 2009; Wertz and Dixit, 2010). In this context, the ubiquitin-binding motif of NEMO (ubiquitin binding in ABIN and NEMO, UBAN), which shows a preference for linear chains but can interact with K11-, K48- and K63-linked polyubiquitin chains, mediates recruitment of the IKK complex (Dynek et al., 2010). Similarly, the regulatory proteins TAB2/3 contain ubiquitin-binding domains, which interact with K63-linked polyubiquitinated substrates, and bring the associated kinase TAK1 into juxtaposition with the IKK complex (Kanayama et al., 2004). This induced proximity is thought to result in the phosphorylation and activation of IKK1/2 by TAK1. Alternatively, induced proximity of IKK1/2 *per se* could result in their phosphorylation in trans or conformational changes in NEMO upon ubiquitin binding might promote IKK1/2 activation (Wertz and Dixit, 2010).

Although canonical NF-κB activation universally depends on polyubiquitin chain assembly for proximal signal transduction, each receptor recruits a distinct set of adaptor proteins, kinases and E3 ligases. In the TNFR pathway, ligand-induced receptor trimerization leads to recruitment and polyubiquitination of the adaptor protein RIP1. In this context, TRAF2 and TRAF5 together with cIAP1/2 are thought to be the E3 ligases that mediate K63-polyubiquitination (Skaug et al., 2009; Wertz and Dixit, 2010). In addition, post TNFR engagement LUBAC modifies RIP1 and NEMO with linear polyubiquitin chains (Gerlach et al., 2011; Tokunaga et al., 2009). TLRs, the IL-1R and the IL-33R contain a common cytoplasmic motif, called the Toll/IL-1 receptor (TIR) domain, and thus initiate NF-κB activation by related mechanisms. Engagement of these receptors results in the recruitment of the TIR domain-containing protein MyD88, among other proximal adaptor proteins. This promotes the

activation of the E3 ligases TRAF6 and Pellino in a manner dependent on IRAK1/4 and their subsequent K63-linked polyubiquitination (Skaug et al., 2009; Wertz and Dixit, 2010). Furthermore, canonical NF-κB activation initiated by IL-1 and certain TLRs depends on linear ubiquitination by LUBAC (Sasaki et al., 2013; Tokunaga et al., 2009). In case of ITAM-containing receptors such as antigen receptors expressed by B and T lymphocytes a proximal tyrosine phosphorylation cascade is initiated, resulting in the assembly of a complex containing CARMA1, Bcl-10 and MALT1. Recruitment and MALT1-induced oligomerization of TRAF6 has been proposed to regulate its E3 ligase activity, leading to the generation of K63-linked polyubiquitin chains (Skaug et al., 2009; Wertz and Dixit, 2010). Thus, a general scheme emerges whereby K63-linked polyubiquitin chains generated by receptor-specific E3 ligase complexes and linear chains assembled by LUBAC trigger activation of the IKK complex and canonical NF-κB signaling.

4.2.5. Termination of NF-κB Responses

Efficient and rapid NF-κB activation is essential for appropriate immune responses to combat infections. However, proper termination is needed to avoid uncontrolled, excessive or chronic inflammation as seen in septic shock or autoimmune diseases like rheumatoid arthritis (Ma and Malynn, 2012; Vereecke et al., 2009). Various regulatory mechanisms terminate NF-κB activation at all levels of signal transduction, from the modulation of receptor-proximal steps all the way to directly affecting the function of NF-κB in the nucleus (Renner and Schmitz, 2009). Classical IκB proteins such as IκBα are the main regulators of NF-κB activity and are themselves NF-κB target genes: they therefore function in a negative feedback loop (Vallabhapurapu and Karin, 2009). IκBα can interact with DNA-bound NF-κB dimers resulting in their inactivation and export from the nucleus (Vallabhapurapu and Karin, 2009). Other regulatory mechanism within the nucleus include degradation of RelA (Natoli and Chiocca, 2008) or binding of atypical IκB proteins such as IκBζ that modulate NF-κB transactivation (Vallabhapurapu and Karin, 2009). The regulation of more proximal events includes degradation of signaling components, generation of decoy receptors or dominant-negative signal transducers, and removal of activating post-translational modifications such as phosphorylation (Renner and Schmitz, 2009). Furthermore, NF-κB activation can be regulated at the level of ubiquitination. Clearly, ubiquitin-

binding proteins such as ABINs, optineurin and A20 (discussed below) and DUBs such as CYLD, Cezanne, USP21, OTULIN and A20 (discussed below) play essential roles (Harhaj and Dixit, 2011; Keusekotten et al., 2013; Verstrepen et al., 2009). As an example, the tumor suppressor CYLD can cleave linear and K63-linked polyubiquitin chains and thereby is thought to target a large set of signaling components including TRAF2, TRAF6, NEMO and RIP1 to terminate NF-κB activation (Komander et al., 2009b; Harhaj and Dixit, 2011).

4.2.6. A20 Is a Key Negative Regulator of Cellular Activation

A20 was initially identified in endothelial cells as a gene that is rapidly and profoundly induced by TNF treatment (Dixit et al., 1990). Thus, A20 is also referred to as tumor necrosis factor α-induced protein (TNFAIP)3. Due to two κB sites in the A20 promoter region (Krikos et al., 1992), A20 is additionally induced by other stimuli that activate NF-κB such as engagement of the IL-1R, CD40, TLRs, and B and T cell antigen receptors (Ma and Malynn, 2012; Vereecke et al., 2009). The important physiological functions of A20 were demonstrated by genetic disruption studies in mice. A20-deficient mice develop spontaneous severe multiorgan inflammation leading to perinatal lethality, which can be overcome by the elimination of MyD88-dependent signals (Lee et al., 2000; Turer et al., 2008).

A20 contains an N-terminal ovarian tumor (OTU) domain and seven C-terminal zinc finger (ZnF) motifs, which are thought to harbor two opposed ubiquitin-editing functions, namely DUB and E3 ligase activity (Wertz et al., 2004). It has been demonstrated that in the TNFR signaling pathway the OTU domain of A20 has DUB activity and removes K63-linked polyubiquitin chains from RIP1 (Figure 5) (Wertz et al., 2004). Similarly, A20 has been shown to remove K63-linked ubiquitin chains from TRAF6, RIP2 and MALT1 upon engagement of TLR4, the IL-17 receptor, NOD2 receptors or the T cell receptor (Figure 5) (Boone et al., 2004; Duwel et al., 2009; Garg et al., 2013; Hitotsumatsu et al., 2008). Although A20 appears to be specific for K63-linked chains in a cellular context, *in vitro* it preferentially hydrolyses free K48-linked polyubiquitin chains (Bosanac et al., 2010; Komander et al., 2009b; Lin et al., 2008). This suggests that other proteins such as ABINs (Mauro et al., 2006) or TAX1BP1 (Shembade et al., 2007) might direct A20 to its targets or modulate its DUB activity *in vivo*. Alternatively, A20 might not show global DUB activity but rather

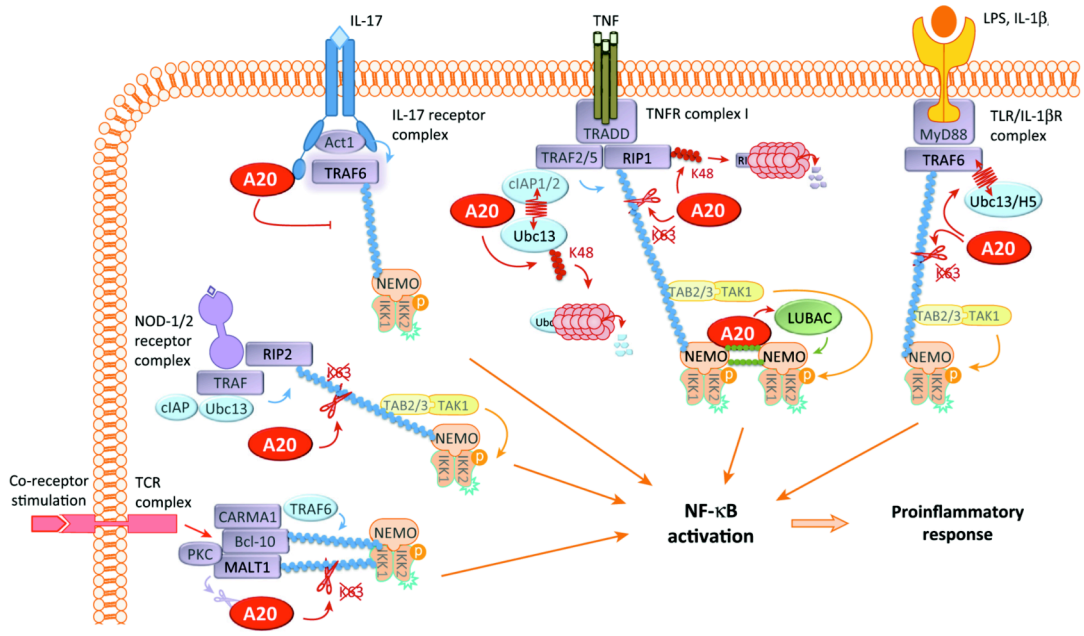

Figure 5. A20 is a central negative regulator of NF-κB activation. Upon receptor engagement proximal signaling events and the generation of polyubiquitin chains with various topologies (K48, red; K63, blue; linear, green) lead to canonical NF-κB activation. A20 restricts multiple aspects of this signal transduction by the removal of activating K63-linked chains (from MALT1, RIP1, RIP2 and TRAF6), modification of proteins with degradation-inducing K48-linked chains (RIP1), disruption of E2:E3 ligase complexes (UBC13:TRAF6, UBCH5c:TRAF6 and UBC13:cIAP1) or competitive binding to K63-linked or linear chains. Modified from Catrysse et al. (2013).

target specificity. Supporting this notion, A20 removes K63-linked chains from physiological substrates such as TRAF6. It does so by cleaving the TRAF6-ubiquitin isopeptide bond and hence seems to remove the K63-linked chain as a whole without disassembling it (Lin et al., 2008).

In addition to using its DUB activity to remove K63-linked chains from RIP1 to terminate NF-κB activation upon TNFR engagement, A20 is also thought to assemble K48-linked chains on RIP1, thereby targeting it for proteasomal degradation (Figure 5) (Wertz et al., 2004) This E3 ligase activity was ascribed to ZnF4 (Wertz et al., 2004). It was therefore proposed that A20 functions in a sequential manner: A20 first removes activating K63-linked chains from RIP1 and then adds degradation-inducing K48-linked ubiquitin chains (Wertz et al., 2004). However, it is not clear whether ZnF4 possesses intrinsic E3 ligase activity, or if the A20 interacting E3 ligases Itch and RNF11 fulfill this function (Shembade et al., 2008; 2009). To clarify the physiological contribution of A20's dual enzymatic activities, knock-in mice were generated that express mutated A20 proteins (Lu et al., 2013). Both $A20^{ZF4}$ (mutations within ZnF4) and $A20^{OTU}$ (mutation of the catalytic cysteine in the OTU domain to alanine) mice were outwardly normal, in contrast to A20-deficient

mice that show chronic inflammation and perinatal lethality (Lee et al., 2000; Lu et al., 2013). However, both $A20^{ZF4}$ and $A20^{OTU}$ mice were hypersensitive to TNF injections, indicating that both domains contribute to some extent to A20's ability to restrict inflammation (Lu et al., 2013).

Since A20 mutants lacking the catalytic cysteine in the DUB domain can still inhibit NF-κB activation both *in vitro* and *in vivo* (Evans et al., 2004; Lu et al., 2013), additional non-catalytic mechanisms have been described. For example, A20 was shown to disrupt E2:E3 ligase complexes such as UBC13:TRAF6, UBCH5c:TRAF6, and UBC13:cIAP1. This leads to K48-linked polyubiquitination of UBC13 and UBCH5c targeting the E2s for proteasomal degradation, thereby generally affecting global K63-linked polyubiquitination (Figure 5) (Shembade et al., 2010). In addition, A20 can interact with K63-linked and linear chains through ZnF4 and ZnF7. By competitively binding to these chains, A20 is thought to inhibit proper assembly of ubiquitin-dependent signaling platforms (Figure 5) (Bosanac et al., 2010; Verhelst et al., 2012). Finally, A20 has also been implicated in targeting TRAF2 for lysosomal degradation (Li et al., 2009). In conclusion, A20 regulates multiple aspects of ubiquitin-dependent signaling to NF-κB by its diverse catalytic and non-catalytic functions.

A20 was initially characterized as a protein inhibiting TNF-induced cell death (Opipari et al., 1992). However, the function of A20 in the regulation of apoptosis remains controversial and seems to be stimulus and cell type specific. On the one hand, A20 was shown to have anti-apoptotic functions in response to death receptor engagement (Jin et al., 2009; Lee et al., 2000; Opipari et al., 1992). In this context, one potential mechanism could be the regulation of K63-linked polyubiquitin and thereby activation of caspase-8 by A20 (Jin et al., 2009). On the other hand, pro-apoptotic functions of A20 seem to be predominantly mediated by inhibiting NF-κB-dependent expression of pro-survival Bcl-2 family members (Kool et al., 2011; Tavares et al., 2010). In addition, A20 has been shown to regulate the non-canonical TGFβ pathway, Wnt signaling, the antiviral interferon regulated factor (IRF)3 and IRF7 pathways and autophagy (Jung et al., 2013; Maelfait et al., 2012; Saitoh et al., 2005; Shao et al., 2013; Shi and Kehrl, 2010).

4.2.7. Cell Type-Specific Functions of A20

The multiorgan inflammation and perinatal lethality of A20-deficient mice impeded studying the role of A20 in adult mice during immune homeostasis and disease pathology (Lee et al., 2000). Hence, several conditional alleles were generated allowing A20 deletion in a temporal and lineage-specific fashion (Hövelmeyer et al., 2011; Tavares et al., 2010; Vereecke et al., 2010). Loss of A20 in macrophages and granulocytes (LysM-Cre $A20^{F/F}$) caused MyD88-dependent spontaneous polyarthritis connected to increased systemic pro-inflammatory cytokine levels such as IL-6 (Matmati et al., 2011). A20 ablation in DCs (CD11c-Cre $A20^{F/F}$) led to their spontaneous activation, excessive pro-inflammatory cytokine production and profound disturbances of immune homeostasis (Hammer et al., 2011; Kool et al., 2011). Interestingly, two groups independently generated DC-specific A20 knock-out mice with remarkable differences regarding the resulting disease pathologies. One strain spontaneously developed MyD88-dependent colitis associated with arthritis reminiscent of human inflammatory bowel disease (Hammer et al., 2011). In contrast, the other strain developed systemic lupus erythematosus (SLE)-like DNA-specific autoantibodies accompanied by glomerulonephritis (Kool et al., 2011). A20-deficient B cells (CD19-Cre $A20^{F/F}$) exhibited enhance NF-κB activation in response to various stimuli, produced higher levels of pro-inflammatory cytokines such as IL-6 and caused an autoimmune syndrome characterized by the presence of class-switched tissue-specific autoantibodies (Chu et al., 2011; Hövelmeyer et al., 2011; Tavares et al., 2010). Loss of A20 in intestinal epithelial cells (Villin-Cre $A20^{F/F}$) increased susceptibility to TNF-induced cell death and inflammation in addition to rendering mice hypersensitive to experimental colitis (Vereecke et al., 2010). Epidermis-specific A20 deletion (K14-Cre $A20^{F/F}$) did not cause spontaneous skin inflammation, however, mice showed keratinocyte hyperproliferation and ectodermal abnormalities (Lippens et al., 2011). These studies revealed the various cell type-specific functions of A20 highlighting its critical role for the prevention of inflammation and autoimmunity.

4.2.8. A20/TNFAIP3 in Human Diseases

Human genome wide association studies (GWASs) have linked germline single nucleotide polymorphisms (SNPs) in human *TNFAIP3* (the gene encoding for A20) with susceptibility to human inflammatory and autoimmune diseases, including rheumatoid arthritis, SLE, type I diabetes, Crohn's disease, coeliac disease, coronary artery disease, psoriasis and systemic sclerosis (Ma and Malynn, 2012; Vereecke et al., 2009). Most of the SNPs are located outside the coding region, suggesting that they might interfere with A20 expression. Indeed, an SLE-associated SNP within a putative 3' enhancer reduces A20 expression (Adrianto et al., 2011). So far only two SNPs within the *TNFAIP3* coding region have been reported, one of which is an SLE-associated SNP resulting in a phenylalanine-to-cysteine change within the DUB domain that reduces A20 function (Musone et al., 2008). As A20 regulates cellular activation in a gene-dose-dependent fashion, reducing its expression or function might have physiological relevance (Chu et al., 2011).

In addition, mutations and deletions in *TNFAIP3* have been identified in several human B lymphomas including Hodgkin's lymphoma, mucosa-associated lymphoid tissue-type lymphoma, diffuse large B cell lymphoma and primary mediastinal B cell lymphoma (Malynn and Ma, 2009). These mutations result in the introduction of stop codons, frame shifts or amino-acid substitutions that might affect A20 function and/or stability (Malynn and Ma, 2009). Reconstitution of A20 in in A20-null lymphoma cell lines suppresses NF-κB activation leading to the induction of apoptosis and growth arrest, indicating a tumor suppressor role (Compagno et al., 2009). Similar GWASs have identified *TNIP1* (the gene encoding for the A20 binding partner ABIN1) as a susceptibility locus for autoimmune diseases such as psoriatic arthritis and SLE (Ma and Malynn, 2012), in addition to asthma (Li et al., 2012). Furthermore, somatic mutations in *TNIP1* were identified in human lymphomas, further suggesting a functional relationship between A20 and ABIN1 (Ma and Malynn, 2012).

5. Aims of the Thesis

Mast cells have long been recognized as key effector cells in IgE-dependent anaphylactic reactions, but were also implicated in playing a central role in several aspects of inflammatory and autoimmune diseases. However, concepts on these pathological mast cell functions were primarily based on studies employing Kit mutant mast cell-deficient mice. These mice suffer from various abnormalities besides the loss of mast cells and, hence, many of the initially obtained results were challenged later on. Furthermore, the signals mast cells receive under physiological and pathological conditions are poorly defined. Additionally, mast cell-deficient mouse models are not suited to study the function of specific genes during mast cell development, homeostasis and function. Hence, novel mouse models that allow to study the role of mast cells using gain-of-function approaches and to inducibly manipulate their genome are urgently required.

In the course of this thesis, I experimentally addressed the following three critical points:

1. Dissecting the consequences of A20 deficiency on mast cell activation during physiology and pathology to generate a novel mouse model for hyperactive mast cells (Publication I).
2. Investigating the role of A20 during *in vitro* B cell activation (Publication II).
3. Characterizing the $Kit^{CreERT2}$ mouse strain as a novel genetic tool for inducible conditional gene targeting in and ablation of mast cells (Publication III).

6. Abstracts of Publications

For copyright reasons, full versions of the publications are not included in the online version of this thesis. Please follow the links below the abstracts to download the pdf files directly from the publisher's website.

6.1. Publication I

A20-Deficient Mast Cells Exacerbate Inflammatory Responses *in Vivo*.

Heger K.*, Fierens K.*, Vahl J.C., Aszodi A., Peschke K., Schenten D., Hammad H., Beyaert R., Saur D., van Loo G., Roers A., Lambrecht B.N., Kool M. and Schmidt-Supprian M. (2014) *PLoS Biol* 12: e1001762. *equal contribution

Mast cells are implicated in the pathogenesis of inflammatory and autoimmune diseases. However, this notion based on studies in mast cell-deficient mice is controversial. We therefore established an *in vivo* model for hyperactive mast cells by specifically ablating the NF-κB negative feedback regulator A20. While A20 deficiency did not affect mast cell degranulation, it resulted in amplified pro-inflammatory responses downstream of IgE/FcεRI, TLRs, IL-1R and IL-33R. As a consequence house dust mite- and IL-33-driven lung inflammation, late phase cutaneous anaphylaxis and collagen-induced arthritis were aggravated, in contrast to experimental autoimmune encephalomyelitis and immediate anaphylaxis. Our results provide *in vivo* evidence that hyperactive mast cells can exacerbate inflammatory disorders and define diseases that might benefit from therapeutic intervention with mast cell function.

Full text

6.2. Publication II

B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice.

Chu Y., Vahl J.C., Kumar D., **Heger K.**, Bertossi A., Wójtowicz E., Soberon V., Schenten D., Mack B., Reutelshöfer M., Beyaert R., Amann K., van Loo G. and Schmidt-Supprian M. (2011) *Blood* 117: 2227–2236.

The ubiquitin-editing enzyme A20/TNFAIP3 is essential for controlling signals inducing the activation of nuclear factor-κB transcription factors. Polymorphisms and mutations in the TNFAIP3 gene are linked to various human autoimmune conditions, and inactivation of A20 is a frequent event in human B-cell lymphomas characterized by constitutive nuclear factor-κB activity. Through B cell-specific ablation in the mouse, we show here that A20 is required for the normal differentiation of the marginal zone B and B1 cell subsets. However, loss of A20 in B cells lowers their activation threshold and enhances proliferation and survival in a gene-dose-dependent fashion. Through the expression of proinflammatory cytokines, most notably interleukin-6, A20-deficient B cells trigger a progressive inflammatory reaction in naive mice characterized by the expansion of myeloid cells, effector-type T cells, and regulatory T cells. This culminates in old mice in an autoimmune syndrome characterized by splenomegaly, plasma cell hyperplasia, and the presence of class-switched, tissue-specific autoantibodies.

Full text

6.3. Publication III

CreER^{T2} expression from within the c-Kit gene locus allows efficient inducible gene targeting in and ablation of mast cells.

Heger K., Seidler B., Vahl J.C., Schwartz C., Kober M., Klein S., Voehringer D., Saur D. and Schmidt-Supprian M. (2014) *Eur J Immunol* 44: 296–306.

Mast cells are abundantly situated at contact sites between the body and its environment, such as the skin and, especially during certain immune responses, at mucosal surfaces. They mediate allergic reactions and degrade toxins as well as venoms. However, their roles during innate and adaptive immune responses remain controversial and it is likely that major functions remain to be discovered. Recent developments in mast cell-specific conditional gene targeting in the mouse promise to enhance our understanding of these fascinating cells. To complete the genetic toolbox to study mast cell development, homeostasis and function, it is imperative to inducibly manipulate their gene expression. Here, we report the generation of a novel knock-in mouse line expressing a tamoxifen-inducible version of the Cre recombinase from within the endogenous c-Kit locus. We demonstrate highly efficient and specific inducible expression of a fluorescent reporter protein in mast cells both *in vivo* and *in vitro*. Furthermore, induction of diphtheria toxin A expression allowed selective and efficient ablation of mast cells at various anatomical locations, while other hematopoietic cells remain unaffected. This novel mouse strain will hence be very valuable to study mast cell homeostasis and how specific genes influence their functions in physiology and pathology.

Full text

7. Discussion

Despite their discovery more than 130 years ago and extensive research during the last decades, mast cells are still one of the most enigmatic cell types of the immune system, and their precise physiological and pathological functions remain controversial. Studies in Kit mutant mast cell-deficient mice have been instrumental in assessing their *in vivo* role. However, in the context of inflammatory and autoimmune disorders these mouse strains have yielded ambiguous and contradicting results, presumably due to additional effects of c-Kit deficiency. Moreover, the role of specific genes during mast cell development, homeostasis and function are poorly defined. Hence, novel genetic tools to evaluate the consequences of mast cell deficiency, to activate or inactivate genes in a mast cell-specific and inducible fashion are urgently required. Therefore, through mast cell-specific A20 deficiency, I established the first gain-of-function model for hyperactive mast cells with overshooting inflammatory responses. Moreover, I characterized the first mouse strain that allows inducible conditional gene targeting in and ablation of mast cells. My analyses of these novel mouse strains yielded important insights into mast cell biology and pathology and promise to enhance our understanding of these puzzling cells.

7.1. Generation of an *in Vivo* Mouse Model for Hyperactive Mast Cells

7.1.1. A20 Selectively Regulates Inflammatory But Not Anaphylactic Mast Cell Responses

When work on this study commenced, *in vivo* analyses of mast cell functions were almost entirely based on a single experimental system, namely the use of mast cell-deficient $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice and their selective reconstitution with cultured mast cells (Grimbaldeston et al., 2005; Nakano et al., 1985). However, conflicting results were obtained regarding the impact of mast cells on inflammatory and autoimmune diseases (Rodewald and Feyerabend, 2012). Hence, I reasoned that a genetic gain-of-function *in vivo* model would allow addressing these important questions from a different angle. In addition, this approach would circumvent potential

cellular compensation artifacts caused by the constitutive absence of mast cells. Moreover, enhancing mast cell functions would help to define their maximal impact in a given disease context.

Genetic studies demonstrated that the ubiquitin-editing enzyme and NF-κB negative feedback regulator A20 is crucial for the prevention of inflammation in mice (Lee et al., 2000). Moreover, polymorphisms in the *A20* gene locus were linked to several human inflammatory and autoimmune diseases (Ma and Malynn, 2012; Vereecke et al., 2009). Hence, ablation of A20 was a promising and physiologically relevant method to generate hyperactive mast cells. In addition, this approach would allow dissecting the nature of stimuli mast cells sense under steady-state conditions and how they are activated during pathologies. When I started working on this thesis, A20 had been shown to restrict signal transduction triggered by TLRs, the IL-1R, TNFRs and intracellular NOD2 receptors (Boone et al., 2004; Hitotsumatsu et al., 2008; Lee et al., 2000; Wertz et al., 2004), however, little was known about its cell type-specific functions. Hence, I first investigated the impact of A20 deficiency on mast cell functions *in vitro*. By comparing (at the time) known A20 targets to components used in mast cells for signal transduction, I hypothesized that, in addition to known pathways, A20 might also restrict NF-κB activation downstream of the IgE/FcεRI module and the IL-33R.

This hypothesis was based on the following considerations. Firstly, in mast cells, Bcl-10 and MALT1 were identified as essential signaling components mediating NF-κB activation upon FcεRI crosslinking (Klemm et al., 2006). Interestingly, in T cells MALT1 was shown to interact with TRAF6, thereby stimulating its E3 ligase activity resulting in K63-linked polyubiquitin chain assembly and activation of the IKK complex (Sun et al., 2004). Secondly, upon stimulation with IL-33, TRAF6 gets recruited to the IL-33R and is essential for signal transduction to NF-κB (Funakoshi-Tago et al., 2008; Schmitz et al., 2005). Finally, A20 was shown to terminate NF-κB responses by removing K63-linked polyubiquitin chains from TRAF6 (Boone et al., 2004; Lin et al., 2008) and from MALT1 (Duwel et al., 2009).

Confirming my initial hypothesis, A20 indeed restricted NF-κB activation in mast cells upon crosslinking of the FcεRI and stimulation with IL-33 (Publication I). As a consequence pro-inflammatory cytokine production and activation marker expression was dramatically enhanced in A20-deficient mast cells in comparison to control cells, highlighting an important and hitherto unrecognized role of A20 in these signaling pathways. In addition, I confirmed the function of A20 in the regulation of

pro-inflammatory signals triggered by IL-1β and TLR ligands (Publication I). Similarly, also in B cells A20 specifically regulated NF-κB activation and pro-inflammatory cytokine production upon engagement of the B cell receptor, CD40 and TLRs (Publication II).

Although A20 deficiency exaggerated NF-κB activation, it did not affect degranulation induced by crosslinking of the FcεRI (Publication I). The latter event is not spontaneous and requires the concerted action of several proteins (Lorentz et al., 2012). The most prominent task is fulfilled by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which form a ternary SNARE complex to bring vesicle and plasma membranes in juxtaposition (Lorentz et al., 2012). Phosphorylation of the target membrane SNARE protein SNAP-23 was shown to be critically involved in the regulation of degranulation (Hepp et al., 2005), however, which kinase fulfills this task is a matter of debate: it has been proposed that PKCβ regulates degranulation by the direct phosphorylation of SNAP-23 (Hepp et al., 2005), but later genetic studies demonstrated the essential and non-redundant function of IKK2 in this process, apart from phosphorylating IκB proteins and thus independent of NF-κB activation (Suzuki and Verma, 2008). However, NF-κB might control degranulation on a transcriptional level by regulating the expression of store-operated calcium channels, which prolong and potentiate the initial cytosolic Ca^{2+} response upon FcεRI engagement (Eylenstein et al., 2012; Vig et al., 2008).

A20 was thought to regulate receptor proximal ubiquitination events upstream of the IKK complex and hence NF-κB transcription factor activation (Wertz and Dixit, 2010). Therefore, it was possible that A20-deficieny would have an impact on degranulation by both NF-κB-independent and NF-κB–dependent mechanisms. I demonstrated that loss of A20 specifically in mast cells did not affect immediate local or systemic anaphylaxis events (Publication I) that are dependent on mast cell degranulation (Feyerabend et al., 2011; Klemm et al., 2006; Sawaguchi et al., 2012). *In vitro* studies confirmed that the loss of A20 had no impact on mast cell degranulation (Publication I). This indicated that A20 regulates signaling events after bifurcation of the pathway leading to either degranulation or NF-κB activation, presumably at the level or downstream of Bcl-10, MALT1 or TRAF6. This is in line with recent observations demonstrating that degranulation was unaffected in Bcl-10-, MALT1- and TRAF6-deficient mast cells, whereas NF-κB activation was severely compromised (Klemm et al., 2006; Yang et al., 2008).

At this stage, I can only speculate why IKK2 deficiency affected degranulation, although loss of the further-upstream signaling components A20, Bcl-10, MALT1 and TRAF6 did not (Publication I) (Klemm et al., 2006; Suzuki and Verma, 2008; Yang et al., 2008). Suzuki and Verma generated mast cells from the fetal liver in order to circumvent the early lethality of IKK2-deficient mice (Suzuki and Verma, 2008). Hence, both differentiation and expansion of fetal liver-derived mast cells proceeded in the complete absence of IKK2. This could affect their functional maturation and thereby indirectly influenced their degranulation response. Fittingly, pharmacological IKK2 inhibition in mature mast cells had no effect on degranulation (Peng et al., 2005). In order to avoid such potential biases through effects of gene deficiency during mast cell development *in vitro*, I employed the novel $Kit^{CreERT2}$ transgene (Publication III) to delete conditional A20 and MyD88 alleles in mature mast cells after differentiation (Publication I). Future similar studies using conditional or inducible ablation of IKK2 in mature mast cells will be essential to further clarify the role of IKK2 during degranulation and in anaphylactic responses.

Collectively, my results indicate that A20 selectively terminates NF-κB activation and thus pro-inflammatory mast cell responses without affecting degranulation or anaphylactic reactions. This feature has important implications for mast cell biology, as it allows a full-blown anaphylactic response to antigen in a mast cell whose pro-inflammatory signaling is impaired by high levels of A20 due to previous recognition of pathogens or alarmins. It furthermore indicates that, in a clinical setting, anaphylactic responses can neither be aggravated nor treated by modulating A20 function.

7.1.2. Despite Their Pronounced Hyperactivity, A20-Deficient Mast Cells Do Not Cause Overt Spontaneous Inflammation

The potent anti-inflammatory functions of A20 were clearly demonstrated by the generation of mice completely deficient for A20, which suffer from severe multiorgan inflammation and cachexia leading to perinatal lethality (Lee et al., 2000). Subsequent studies indicated that this phenotype is caused by exacerbated MyD88-dependent TLR signals in response to commensal bacteria, which trigger spontaneous detrimental myeloid cell activation (Turer et al., 2008). Fittingly, loss of A20 specifically in myeloid and dendritic cells disturbed immune homeostasis and resulted in spontaneous MyD88-dependent pathologies (Hammer et al., 2011;

Matmati et al., 2011). Similar to many tissue-resident myeloid cell populations, mast cells express various TLRs and are located at the host-environment border where commensals are frequently encountered (Abraham and St John, 2010). In addition, overall mast cell numbers are estimated to be equal to the total number of splenocytes (Sayed et al., 2008). Hence, it was surprising to observe that mice containing A20-deficient mast cells (Mcpt5Cre $A20^{F/F}$) did not suffer from overt spontaneous inflammation (Publication I). This indicates that mast cells might have developed additional control mechanisms, which are not shared with other myeloid cells, and hence might be intrinsically less prone to initiate inflammation. Alternatively, although A20 deficiency caused dramatically enhanced NF-κB responses to several physiologically relevant stimuli *in vitro* (Publication I), the signals mast cells receive *in vivo* might be rather weak.

Intriguingly, mast cells cannot be fully activated by TLR4-dependet stimuli. This is due to a defect in TRIF signaling that reduces pro-inflammatory cytokine production and precludes an anti-viral interferon response (Keck et al., 2011). Moreover, mast cells lack expression of the TLR4 coreceptor CD14 and hence depend on the presence of soluble CD14 to efficiently respond to the bacterial cell wall component LPS (McCurdy et al., 2001). Fittingly, high LPS concentrations had to be used *in vitro* to activate mast cells (Publication I). As commensal bacteria under steady-state conditions are recognized by TLRs (Rakoff-Nahoum et al., 2004), diminished TLR-dependent mast cell activation could at least in part account for the lack of overt spontaneous inflammation in mice with mast cell-specific A20 deficiency. These observations suggest that functional inactivation of A20 has the most dramatic consequences in cells capable of high affinity LPS recognition, such as myeloid and dendritic cells (Hammer et al., 2011; Kool et al., 2011; Matmati et al., 2011), and has less impact in cells with low-affinity LPS receptors such as B cells and mast cells (Publication I and II) (Tavares et al., 2010). This implies that, of the many MyD88-dependent receptors, TLR4 might be the main receptor driving spontaneous inflammation caused by A20-deficiency. Given the tremendous impact of diminished A20 function in human inflammatory and autoimmune diseases, this hypothesis warrants further investigation. It furthermore suggests that interference with TLR4 pattern recognition might allow translation into clinical applications to treat inflammatory diseases that are linked to SNPs in the A20 gene locus.

In addition, my *in vitro* experiments showed that mast cells are more efficiently activated by IL-33 than LPS (Publication I). Mast cells can sense IL-33 by

constitutively expressing the IL-33R α-chain (ST2) (Moritz et al., 1998). In fact, under steady-state conditions mast cells are the main cell type in the peritoneal and pleural cavities showing high ST2 cell surface levels (K. Heger and M. Schmidt-Supprian unpublished observations) (Enoksson et al., 2013). IL-33 is expressed by structural cells and is thought to be released upon cell injury, trauma or infection (Lüthi et al., 2009; Moussion et al., 2008). Indeed, mast cells can be activated *in vitro* by necrotic cells and, therefore, are considered to be the link between cell injury and the initiation of inflammation (Enoksson et al., 2011; Lunderius-Andersson et al., 2012). However, although IL-33-deficient mice have recently been reported it is unclear to what extent IL-33 is released under steady-state conditions and, hence, could be sensed by mast cells (Oboki et al., 2010). A20-deficient mast cells showed dramatically enhanced NF-κB activation and pro-inflammatory cytokine production upon IL-33 stimulation *in vitro* (Publication I). Thus, the absence of overt spontaneous mast cell activation and inflammation *in vivo* might indicate that only small amounts of IL-33 are released under physiological conditions. This might be of particular importance as upon liberation IL-33 can potently induce inflammation in addition to Th2 responses (Palm et al., 2013; Schmitz et al., 2005), which could cause sensitization towards seemingly harmless non-self antigens leading to detrimental allergic diseases (Galli and Tsai, 2012).

Although A20-deficient mast cells did not cause overt inflammation *in vivo* they displayed a pre-activated or poised state as indicated by the enhanced production of pro-inflammatory cytokines (Publication I). Moreover, in Mcpt5Cre A20$^{F/F}$ mice, mast cell numbers were enhanced due to an overall increase in peritoneal cellularity, and mice developed minor splenomegaly with mild expansions of myeloid cell subsets and T cells with an effector-like phenotype (Publication I and K. Heger and M. Schmidt-Supprian unpublished observations). All of these processes depended on the adapter MyD88 (Publication I), suggesting that mast cells - similarly to other immune cells - constantly receive some, but apparently mild signals via one or more MyD88-dependent receptors that are held in check by A20, such as TLRs, the IL-1R, the IL-18R or the IL-33R (Hammer et al., 2011; Matmati et al., 2011). Future crosses of Mcpt5Cre A20$^{F/F}$ mice onto an IL-33-deficient background or treatment with broad-spectrum antibiotics would clarify the contribution of IL-33 or the commensal microbiota to these processes and define the nature of stimuli that mast cells sense under physiological conditions.

Collectively, my results indicate that, although *in vitro* A20 deficiency causes dramatically enhanced NF-κB activation in response to various physiologically relevant stimuli, under steady-state conditions mast cell-specific A20 deficiency does not cause overt spontaneous inflammation but induces a pre-activated or poised state.

7.1.3. Dissecting the Role of Mast Cells in Mouse Models for Human Inflammatory and Autoimmune Diseases through an *in Vivo* Gain-of-Function Approach

Mast cells have been implicated as important effector cells in diverse physiological and pathological conditions, including in mouse models of asthma, multiple sclerosis and rheumatoid arthritis (Lee et al., 2002; Secor et al., 2000; Williams and Galli, 2000). However, this notion was based on mast cell-deficient $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice, which suffer from various additional defects within and outside of the immune system (Rodewald and Feyerabend, 2012). Moreover, using these mice represents a loss-of-function approach that describes the consequences of absent function, which is not always inversely correlated with excessive function. This is particularly evident when looking at DCs, as both their ablation and their persistent activation resulted in spontaneous autoimmune pathologies (Hammer et al., 2011; Kool et al., 2011; Ohnmacht et al., 2009). I found that A20 selectively regulated pro-inflammatory NF-κB-dependent but not anaphylactic reactions and caused pronounced mast cell hyperactivation (Publication I). Hence, I sought to reassess their innate inflammatory properties in the context of allergic, inflammatory and autoimmune diseases by using a novel gain-of function approach - inactivation of A20 specifically in mast cells. Loss of A20 in mast cells in Mcpt5Cre $A20^{F/F}$ mice exacerbated innate forms of asthma elicited by the administration of house dust mite extracts or following sensitization to the model allergen ovalbumin under mild conditions (Publication I). These results correlate with existing data from various mast cell-deficient mouse models that showed reduced symptoms and point to a crucial role of mast cells in asthma pathogenesis (Nakae et al., 2007; Sawaguchi et al., 2012; Williams and Galli, 2000). Mcpt5Cre $A20^{F/F}$ mice additionally showed early onset and exacerbated disease symptoms during collagen-induced arthritis (CIA), a mouse model for rheumatoid arthritis (Publication I).

In this context, it is interesting to note that various studies have suggested a pathogenic role for IL-33 in both asthma and rheumatoid arthritis (Ohno et al., 2012; Palmer and Gabay, 2011). Additionally, it was proposed that during the course of arthritis, IL-33 could promote joint inflammation at least in part by the activation of mast cells (Xu et al., 2008). This is in line with my findings that loss of A20 in mast cells exacerbates IL-33-induced NF-κB activation and pro-inflammatory cytokine production (Publication I). Hence, augmented responses to IL-33 might lead to locally restricted inflammation during arthritis pathology. In this scenario, enhanced IL-33-dependent pro-inflammatory cytokine production by A20-deficient mast cells might in turn cause greater IL-33 expression in synovial fibroblasts. Its subsequent release could signal back to mast cells leading to paracrine auto-amplification and a detrimental feed-forward loop. This scenario is supported by the fact that mast cell-specific MyD88 deficiency ameliorates arthritis onset as well as disease symptoms (Publication I).

In addition to arthritis, IL-33-dependent paracrine feed-forward loops might also play a central role in various other inflammatory diseases that have an IL-33 component and are associated with polymorphism in the A20 gene locus, such as psoriasis, systemic sclerosis and inflammatory bowel disease (Ma and Malynn, 2012; Palmer and Gabay, 2011; Vereecke et al., 2009). Interestingly, mast cells have also been implicated in these pathologies (Chichlowski et al., 2010; Eklund, 2007; Harvima et al., 2008). Hence, dissecting mast cell-specific A20-deficieny in relevant mouse models might yield important insights into the etiology and progression of these diseases.

My results are in apparent discrepancy to a study showing that the loss of mast cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice did not significantly alter CIA disease outcome (Pitman et al., 2011). However, studying the function of mast cells by their absence in $Kit^{W\text{-}sh/W\text{-}sh}$ mice bears the inherent caveat that other cell types could functionally compensate for their loss. This has already been demonstrated during the early innate response to haptens, in which neutrophils take over the function of mast cells in the context of the prominent myelodysplasia seen in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Dudeck et al., 2011). Hence, the depletion of neutrophils would clarify their contribution in $Kit^{W\text{-}sh/W\text{-}sh}$ mice during CIA.

In contrast to airway and joint inflammation, A20-deficient mast cells did not worsen EAE symptoms (Publication I), possibly because initiation and progression of this disease is IL-33 independent (Oboki et al., 2010). These results do not

completely exclude a role for mast cells in this model. Firstly, deletion of A20 is limited to CTMCs while MMCs remain unaffected and, secondly, A20 deficiency selectively influences pro-inflammatory NF-κB-driven responses without affecting degranulation. Hence, general activation of mucosal in addition to connective tissue mast cells might have different effects than seen in Mcpt5Cre $A20^{F/F}$ mice. Nonetheless, my results clearly show that enhancing the pro-inflammatory reactions of CTMCs has no influence on MOG peptide-induced central nervous system inflammation. These results are in contrast to the apparently well-documented role of mast cells in EAE pathology (Sayed et al., 2008; Secor et al., 2000). However, this notion is primarily based on a single genetic tool, namely the use of mast cell-deficient $Kit^{W/Wv}$ mice. In recent years the use of mast-cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice has become more and more popular as this strain shows milder Kit-dependent developmental abnormalities than $Kit^{W/Wv}$ mice (Grimbaldeston et al., 2005).

Comparing mast cell-deficient $Kit^{W/Wv}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice yielded conflicting data regarding their contribution to autoimmune pathologies. Surprisingly, $Kit^{W\text{-}sh/W\text{-}sh}$ mice showed even earlier onset and developed exacerbated EAE symptoms (Li et al., 2011; Piconese et al., 2011). Furthermore, depending on the immunization protocol, EAE disease outcome can be modulated in $Kit^{W/Wv}$ mice from protective to even exacerbating (Piconese et al., 2011) and two recent reports failed to confirm that $Kit^{W/Wv}$ mice are protected from EAE (Bennett et al., 2009; Feyerabend et al., 2011). It is worth noting that all of these results were obtained in the absence of mast cells and fail to clarify whether or not mast cells participate in EAE disease pathology. Moreover, my results using a gain-of-function approach by mast cell-specific ablation of A20 (Publication I) and novel Kit-independent mast cell-deficient mice have failed to corroborate a contribution of mast cells to EAE (Feyerabend et al., 2011). Collectively, these results do not support a major function of mast cells in the pathogenesis of EAE.

In addition to playing central pathological roles in autoimmune diseases, many reports assign to mast cells various additional effector and regulatory functions in immune responses against viral (Aoki et al., 2013), bacterial (Echtenacher et al., 1996; Malaviya et al., 1996), and parasitic infections (Ha et al., 1983), in cardiovascular disorders (Sun et al., 2007), obesity and diabetes (Liu et al., 2009), in malignant diseases and angiogenesis (Coussens et al., 1999), in contact hypersensitivity in the skin (Grimbaldeston et al., 2007) and the recognition of cell injury (Enoksson et al., 2011; 2013). However, most *in vivo* evidence for these mast

cell functions is also based on Kit mutant mast cell-deficient mice. Similar to discrepant results on the role of mast cells in autoimmune diseases, studies using novel Kit-independent strains failed to confirm some of the initial results regarding contact hypersensitivity (Dudeck et al., 2011; Otsuka et al., 2011), wound healing (Antsiferova et al., 2013) and skin carcinogenesis (Antsiferova et al., 2013). These fundamental discrepancies indicate that using Kit mutant mice and the mast cell reconstitution approaches can generate misleading results and might lead to false interpretations. Therefore, it will be essential to readdress central mast cell findings using novel genetic mouse models. My analysis on the consequences of A20-deficiency in mast cells yielded specific results with regard to different disease models, specifically, exacerbation of innate forms of asthma and CIA in contrast to no effect on EAE. This shows that the Mcpt5Cre $A20^{F/F}$ strain will be a valuable and informative gain-of-function tool to complement loss-of-function studies. This should be especially true under conditions where mast cells are presumably activated through their IL-33R or TLRs such as tumor development, the initiation of Th2 responses, and the detection of tissue damage or microbial infections.

In summary, my data demonstrate that loss of A20 specifically in mast cells provides a novel genetic model system to study their pro-inflammatory properties in a gain-of-function approach. These hyperactive mast cells exacerbated allergic airway and skin responses, as well as autoimmune joint inflammation pointing to an important contribution of mast cells in these pathologies.

7.2. Inducible Conditional Gene Targeting in Mast Cells Allows Studying their Differentiation and Cellular Maintenance

When I started my thesis, mast cell-specific conditional gene targeting was in its early stages and the functions of specific genes were studied by the reconstitution of Kit mutant mice with cultured mast cells derived from mutant or transgenic bone marrow (Tsai et al., 2005). However, depending on the route of administration, reconstitution may not reach physiological mast cell levels or their number might sometimes even be higher than normal (Grimbaldeston et al., 2005; Nakano et al., 1985). In addition, the anatomical distribution and phenotype of the adoptively transferred mast cells might not be identical to their natural counterparts (Rodewald and Feyerabend,

2012). Therefore, using this mast cell reconstitution approach does not allow faithful investigation of the function of specific genes during *bona fide* mast cell development, homeostasis and function. Recent advances in gene targeting and transgenesis led to the generation of novel mouse strains that constitutively express the Cre recombinase and, thus, allow mast-cell-specific conditional gene targeting to varying degrees of efficiency and specificity (Feyerabend et al., 2011; Furumoto et al., 2011; Müsch et al., 2008; Scholten et al., 2008). However, if the loss of specific genes impedes or affects mast cell development, temporal control of Cre activity is required to delete genes in mature mast cells. Furthermore, acute gene deletion is essential for the validation of potential drug targets and the avoidance of molecular and cellular compensation mechanism. Similarly, acute gene modification is required for *in vivo* cell-fate tracking, a powerful genetic approach to study cellular differentiation.

The group of Dr. Dieter Saur has generated a knock-in mouse strain expressing a tamoxifen-inducible version of the Cre recombinase from within the endogenous c-Kit locus (Klein et al., 2013). Since Cre efficiency is relative to its expression strength (Araki et al., 1997; Schmidt-Supprian et al., 2007) and mast cells within the hematopoietic system exhibit very high c-Kit expression (Publication III), I reasoned that this mouse strain should allow efficient recombination of conditional (loxP-flanked) alleles in mast cells. Indeed, the use of a fluorescent Cre recombinase activity reporter (R26-Stop^{F}YFP) revealed high mast cell specificity and efficiency and gave important insights into mast cell development.

Mast cells are thought to share a common progenitor with basophils in the spleen (basophil and mast cell progenitor, BMCP) (Arinobu et al., 2005). However, although more than 90% of mature dermal and peritoneal mast cells, and nearly 16% of BMCPs in tamoxifen-fed Kit$^{CreERT2/+}$R26-Stop^{F}YFP animals expressed YFP, recombination in basophils was minimal (Publication III). This indicates that, under physiological conditions in the steady-state, YFP-positive BMCPs do not significantly differentiate into basophils. My results hence challenge the validity of BMCPs as authentic bipotential basophil and mast cell progenitors. This is in line with two recent publications demonstrating a greater tendency of BMCPs to develop into mast cells *in vitro* (Mukai et al., 2012; Qi et al., 2013). Further pulse-labeling experiments demonstrated that 8 weeks after cessations of tamoxifen, still more then 90% of mast cells expressed YFP (Publication III). These results indicate a very slow turnover of mature mast cells and regeneration from unaffected progenitors. To the best of my knowledge, this is the first direct genetic evidence that mature mast cells are indeed

very long-lived. Previous attempts to study mast cell turnover by radioactive thymidine labeling provided half-life estimates ranging from days up to years (Kiernan, 1979; Walker, 1961). Although the exact reasons for mast cell longevity are presently unclear, it has been speculated that mast cells might be able to modulate the content of their granules in response to certain stimuli, a process that could be considered to be a form of immunological memory (Abraham and St John, 2010).

Moreover, I found that 4 weeks after ablation of mast cells by DTA expression ($Kit^{CreERT2/+}$R26-GFP$Stop^{F}$DTA), very few mast cells had regenerated from unaffected progenitors (Publication III). These results are in full agreement with a recent study by Dudeck and colleagues who demonstrated little mast cell recurrence within 3 weeks after their selective ablation using a different genetic system (Dudeck et al., 2011). Both results corroborate the long-standing concept that, under steady-state conditions, mature connective tissue mast cells are rarely replaced by progenitors from the bone marrow (Kitamura et al., 1977). This is reminiscent of microglia in the central nervous system, Langerhans cells in the epidermis, Kupffer cells in the liver and macrophages in the serosal cavities (Gomez Perdiguero and Geissmann, 2013). These cells all originate from the yolk sac and their precursors migrate out into the periphery predominantly during embryonic development (Gomez Perdiguero and Geissmann, 2013). Similarly, mast cell colonies can be generated from yolk sac cells (Sonoda et al., 1983) and mast cell precursors peak in fetal blood around day 15 of gestation and subsequently seed the fetal skin (Hayashi et al., 1985; Rodewald et al., 1996). However, it is presently unknown to what extent mast cells present in the adult are derived from this embryonic wave. Administration of 4-hydroxytamoxifen to pregnant R26-$Stop^{F}$YFP females crossed to $Kit^{CreERT2/+}$ males might allow mast cells and their c-Kit^{+} precursors to be pulse-labelled during embryogenesis and, hence, might give important insights into the developmental origin of mature mast cells.

In summary, the novel $Kit^{CreERT2}$ mouse strain will be very useful to define the role of specific genes during physiological and pathological mast cell functions. The inducible nature of the Cre recombinase will furthermore facilitate studies of mast cell development, homeostasis and turnover.

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9. Declaration of Individual Contribution

I **Heger K.***, Fierens K.*, Vahl J.C., Aszodi A., Peschke K., Schenten D., Hammad H., Beyaert R., Saur D., van Loo G., Roers A., Lambrecht B.N., Kool M. and Schmidt-Supprian M. (2014) A20-Deficient Mast Cells Exacerbate Inflammatory Responses in Vivo. *PLoS Biol* 12: e1001762. *equal contribution

Klaus Heger performed the majority of experiments (Figure 1, 2, 3, 6, 7, 8, S1, S2, S4A, C and D, S5B-G and S6), analyzed and interpreted data, and wrote most of the manuscript. Kaat Fierens performed all airway and lung experiments (Figures 4, 5 and S3), analyzed and interpreted data, and wrote airway and lung-related parts of the manuscript. J. Christoph Vahl helped with FACS experiments (Figures 2F, S2B and S4D), Attila Aszodi conducted and analyzed joint histology (Figure S4B), Katrin Peschke performed passive systemic anaphylaxis experiments by monitoring dorsal skin temperature (Figure S5A), and Hamida Hammad helped with assessing the extravasation of fluorescently labeled microbeads (Figure 5C).

II Chu Y., Vahl J.C., Kumar D., **Heger K.**, Bertossi A., Wójtowicz E., Soberon V., Schenten D., Mack B., Reutelshöfer M., Beyaert R., Amann K., van Loo G. and Schmidt-Supprian M. (2011) B cells lacking the tumor suppressor TNFAIP3/A20 display impaired differentiation and hyperactivation and cause inflammation and autoimmunity in aged mice. *Blood* 117: 2227–2236.

Klaus Heger performed IL-6 and TNF ELISAs (Figure 4E and F) and Western blots (Figure 5A and B).

III **Heger K.**, Seidler B., Vahl J.C., Schwartz C., Kober M., Klein S., Voehringer D., Saur D. and Schmidt-Supprian M. (2014) CreER^{T2} expression from within the c-Kit gene locus allows efficient inducible gene targeting in and ablation of mast cells. *Eur J Immunol* 44: 296–306.

Klaus Heger performed most experiments (Figure 1, 2, 3, 4, 5A-C, S1, S2, S3, S4, S5, S6 and S7), analyzed and interpreted data and wrote the manuscript. Barbara Seidler and Sabine Klein generated the Kit^{CreERT2} mouse line, J. Christoph Vahl helped with FACS experiments (Figures 4A and C), Christian Schwartz did Mcpt1 stainings (Figure 5D) and Maike Kober analyzed Mcpt1 stainings (Figure 5D).

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Klaus-Dieter Heger Prof. Dr. Stefan Jentsch

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11. Curriculum Vitae

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Publications

Heger K.*, Fierens K.*, Vahl J.C., Aszodi A., Peschke K., Schenten D., Hammad H., Beyaert R., Saur D., van Loo G., Roers A., Lambrecht B.N., Kool M. and Schmidt-Supprian M. (2014) A20-deficient mast cells exacerbate inflammatory responses in vivo. PLoS Biol 12: e1001762. doi:10.1371/journal.pbio.1001762. *equal contribution

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