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Maria Ekoff

Karolinska Institutet

Department of Medicine, Clinical Immunology and Allergy Unit,
Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

Mast cells are long-lived effector cells of the immune system perhaps best known for their involvement in allergic diseases. There are several acute and chronic inflammations where mast cell accumulation, activation and release of mediators are important for the initiation and perpetuation of the inflammation. Better knowledge of mechanisms regulating the number of mast cells and their activity is desirable. Mast cells do not represent a homogenous population as the surrounding microenvironment will affect their effector profile and numbers. It is known that, in contrast to many inflammatory cells, activated mast cells have the capacity to recover and thereby be activated again (Fc epsilon Receptor I (FcεRI) activation-induced survival).

In this thesis we investigated the effect of B-cell lymphoma-2 (Bcl-2) family members on mast cell survival and apoptosis in murine and human mast cells. Murine bone marrow cells were cultured in two different ways, generating mucosal-like mast cells (MLMCs) and connective tissue-like mast cells (CTLMCs). Our *in vitro*-derived MLMCs and CTLMCs were found to display similar differences in chymase expression, proliferation rate and histamine content as mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs) *in vivo*. This suggests that MLMCs and CTLMCs represent a useful *in vitro* model for committed mast cell lineages. Moreover, we found that CTLMCs, but not MLMCs, exhibit upregulation of the anti-apoptotic Bcl-2 family member *A1* and activation-induced survival upon FcεRI crosslinking. Similarly to murine mast cells, FcεRI crosslinking of *in vitro*-derived human mast cells lead to upregulation of the human homologue of A1, *bfl-1*, and by the use of *bfl-1* siRNA we demonstrate *bfl-1* to be crucial for activation-induced human mast cell survival. Furthermore, the activation-induced survival of human mast cells is sustained in response to the inhibitors ABT-737 and roscovitine which indicate a minor role for the targeted anti-apoptotic Bcl-2 family members Bcl-XL, Bcl-2, Bcl-w and Myeloid cell leukemia-1 (Mcl-1). Taken together, we provide evidence that mast cell populations differ in their ability to survive allergic reactions and identify the Bcl-2 family member A1/Bfl-1 as a potential target for treatment of allergic diseases.

The Bcl-2 homlogy 3 (BH3)-only protein Bcl-2-interacting modulator of cell death (Bim) has been found to play a role in cytokine deprivation-induced apoptosis of murine mast cells although overexpression of anti-apoptotic Bcl-2 protects mast cells more potently than loss of Bim. This indicates that other proteins, besides Bim, might be involved in this process. We describe the BH3-only protein p53 upregulated modulator of apoptosis (Puma) to be critical for the induction of mast cell apoptosis following cytokine deprivation and treatment with the DNA-damaging agent etoposide. Our data also suggest the involvement of the transcription factor Forkhead box O3A (FOXO3a) in the regulation of cytokine deprivation-induced apoptosis and the expression of Puma. Mast cells deficient for FOXO3a were markedly resistant to cytokine deprivation and overexpression of constitutively active FOXO3a caused an upregulation of Puma. We further examined the role of the two pro-apoptotic effector proteins Bcl-2-associated X protein (Bax) and Bcl-2 homologue antagonist/killer (Bak) in cytokine deprivation-induced apoptosis. Although both proteins were expressed we found a major role for Bax but not Bak in mediating mast cell apoptosis. Taken together, this identifies the pro-apoptotic Bcl-2 family members Puma and Bax to be critical for induction of apoptosis which suggest a plausible role for these pro-apoptotic proteins in the regulation of mast cell numbers *in vivo*.

LIST OF PUBLICATIONS

This thesis is based on the following articles which will be referred to in the text by their roman numerals.

I. Ekoff M, Strasser A and Nilsson G.
FcepsilonRI aggregation promotes survival of connective tissue-like mast cells but not mucosal-like mast cells. *J Immunol*. 2007, 178(7):4177-83.

II. Ekoff M and Nilsson G.
Anti-apoptotic Bfl-1 is the major effector in activation-induced human mast cell survival. *Submitted.*

III. Ekoff M, Kaufmann T, Engström M, Motoyama N, Villunger A, Jönsson JI, Strasser A and Nilsson G.
The BH3-only protein Puma plays an essential role in cytokine deprivation induced apoptosis of mast cells. *Blood* 2007, 110(9):3209-17.

IV. Karlberg M, Ekoff M, Labi V, Strasser A, Huang D CS and Nilsson G.
Pro-apoptotic Bax is the major and Bak an auxiliary effector in cytokine deprivation-induced mast cell apoptosis. *Submitted*.

Publication by the respondent not included in the thesis:

1. Ekoff M, Möller C, Xiang Z, and Nilsson G.
Co-aggregation of FcεRI with FcγRIIB inhibits degranulation but not induction of Bcl-2 family members A1 and Bim in mast cells. (*Allergy, Asthma and Clinical Immunology* 2006, 2(3):87-97.

CONTENTS

LIST OF ABBREVIATIONS

Ag	Antigen
Apaf-1	Apoptotic protease activating factor 1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 homologue antagonist/killer
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
BH3	Bcl-2 homology 3
Bid	BH3-interacting domain death agonist
Bim	Bcl-2-interacting modulator of cell death
Bmf	Bcl-2–modifying factor
Bok	Bcl-2–related ovarian killer
CBMC	Cord blood-derived mast cell
CTLMC	Connective tissue-like mast cell
CTMC	Connective tissue mast cell
FcεRI	Fc epsilon Receptor I
FOXO3a	Forkhead box O3A
Ig	Immunoglobulin
Kit	*c-kit* tyrosine kinase receptor
Mcl-1	Myeloid cell leukemia-1
MCP	Mast cell protease
MC_T	Mast cells containing tryptase
MC_{TC}	Mast cells containing tryptase and chymase
MHC	Major histocompatibility complex
MLMC	Mucosal-like mast cell
MMC	Mucosal mast cell
MOMP	Mitochondrial outer membrane permeabilization
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappa B
Puma	p53 upregulated modulator of apoptosis
RPA	RNase protection assay
SCF	Stem cell factor
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
Wt	Wild type

1 INTRODUCTION

Life requires death. Apoptosis, or programmed cell death, is vital for the development and health of a multicellular organism. Apoptosis is used during the development of an organism to sculpture organs and tissues and to ensure immunity. Later in life, apoptosis protects by executing deletion of damaged, aged or potentially dangerous cells. Thereby homoeostasis, the balance between cell proliferation and death, is maintained. The apoptotic process is tightly regulated as dysfunction or dysregulation could disrupt the balance between cell proliferation and cell death, potentially leading to pathological conditions such as a cancer, autoimmune and neurodegenerative diseases (1).

During apoptosis, cells die in a controlled, regulated way in response to various stimuli. The sensitivity of cells to apoptotic signals can vary depending on a number of factors such as the nature of the stimuli, the stage of the cell cycle and the expression of pro- and anti-apoptotic proteins. One key regulator involved in apoptosis is the Bcl-2 family of proteins. The Bcl-2 family consists of pro- and anti-apoptotic members. The balance between these members determines cellular fate via protein-protein interactions (2).

Mast cells are enigmatic cells that should be recognized as a critical component of our immune system. They are strategically localized at the host/environment interface, display a long lifespan once situated in the tissue and have the ability to produce, store and upon activation release immuno-regulatory molecules. Due to these characteristic features mast cells have been associated with the defense against pathogens in both innate and adaptive immunity (3). Still, mast cells are perhaps best known for their involvement in inflammatory diseases. Allergy and asthma are examples of such diseases where mast cells in the affected tissue accumulate and a correlation between mast cell numbers and disease severity has been reported (4, 5). Mast cells have the unique ability to survive inflammatory-induced activation and can be activated again which perpetuates inflammation. Therefore, it is of importance to identify the mechanism behind the increased number and survival of mast cells during an inflammatory reaction such as an allergic reaction. How Bcl-2 family members regulate mast cells in these settings could identify key proteins that affect the severity of inflammation. This thesis provides evidence that *in vitro*-derived mast cell subpopulations differ in their ability to survive allergic reactions and identifies the Bcl-2 family member A1/Bfl-1 as a potential target for treatment of allergic diseases (paper I and II).

Mast cells are strategically situated in the tissue and their numbers are maintained by migration and by the rate of proliferation versus apoptosis. These processes are highly regulated by stem cell factor (SCF), the most important factor in mast cell biology (6). This essential growth factor promotes mast cell survival through the regulation of Bcl-2 family proteins (7). In this thesis I have identified the pro-apoptotic Bcl-2 family members Puma and Bax to be critical for induction of apoptosis when *in vitro*-derived mast cells are deprived of essential growth factors and cytokines which suggest a

plausible role for these proteins in the regulation of mast cell numbers *in vivo* (paper III and IV).

1.1 MAST CELL BIOLOGY

The discovery of this important cell type in immunological history was made by Paul Erlich. In his doctoral thesis from 1878 he describes mast cells as large, granular cells that react to aniline dyes with a characteristic staining patter and notes that mast cells are often found localised around blood vessels (8). Today we know that the strategic positioning and the secretory granules of mast cells are key features that allow them to exert their function as effector and regulatory cells of the immune system.

1.1.1 Mast cell origin, differentiation and heterogeneity

Mast cells are mainly found in peripheral tissues throughout the body but originate from haematopoietic stem cells in the bone marrow (9, 10). Attempts have been made to identify a bone marrow cell population restricted to the mast cell linage in mice but recent research on the ontogeny of mast cells suggest that the final commitment of this cell type occur in the spleen and peripheral tissues (11). Thus, unlike other haematopoietic cells that leave the bone marrow fully matured, mast cells stem from immature precursors that leave the bone marrow to circulate the blood. The recruitment of these cells into the peripheral tissues requires adhesive interactions as well as directed migration through chemotaxis. Once the cells are recruited into the peripheral tissues they can, under the influence of SCF and other locally produced cytokines, differentiate into mature mast cells (Figure 1) (6, 12). Similar developmental and recruitment mechanisms are also seen for human mast cells.

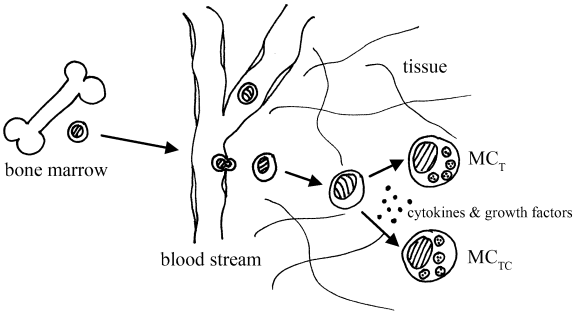

Figure 1. Mast cell differentiation and maturation.

Mature mast cells do not represent a homogenous population. The surrounding microenvironment in the tissue affect mast cell numbers as well as their protease content and effector profile so that their phenotype reflect the local milieu they are situated in (13). Mast cells are usually divided into two main subtypes. Rodent mast cells are classified, based on their sensitivity to fixatives and staining properties, into connective tissue mast cells (CTMC) and mucosal mast cells (MMC) (14) whereas human mast cells are classified, based on the expression of proteases in their granules, into mast cells containing tryptase (MC_T) and mast cells containing tryptase and chymase (MC_{TC}) (15). In terms of tissue localization human MC_T correspond to rodent MMCs, being located predominantly in mucosal tissues, whereas human MC_{TC} correspond to rodent CTMCs and are mainly found in connective tissues (16).

SCF and the receptor for SCF, the *c-kit* tyrosine kinase receptor (Kit), expressed on the surface of mast cells, are critical for mast cell functions such as differentiation and

survival. The importance is highlighted by the almost complete lack of mast cells in mice deficient in Kit or SCF (17, 18). These mice, such as the W/W^v mice, deficient for functional Kit and subsequently lacking mast cells, are important tools for mast cell researchers determining the importance of mast cells in different settings, commonly by the use of mast cell reconstitution. However, W/W^v mice are sterile and today the W^{sh}/W^{sh} mice, also lacking functional Kit and mast cells, offer an alternative mouse model (19). In addition to SCF, MMCs also depend on T-cell-derived cytokines whereas CTMCs primarily require SCF for their persistence. Mice deficient in T-cells lack the MMC subset whereas the CTMC subset have no T-cell dependence and subsequently appear at normal numbers (20). This is also consistent with the finding that humans with T-cell immunodeficiency lack MC_T in the intraepithelial compartment of their intestine, while MC_{TC} appear at normal numbers in the surrounding connective tissues (21).

Mast cells normally survive within the tissue for several months and their numbers are kept relatively constant. However, intestinal helmith infection of rodents will generate a T_H2-type inflammatory response resulting in MMC hyperplasia which is needed for the expulsion of the worm (22). The influence of T_H2-derived cytokines can also be seen in allergic diseases of humans where they are thought to contribute to the increase of mast cell numbers in the mucosal epithelium of the nose, bronchi and gastrointestinal tracts (23), linking T_H2-type inflammatory responses with mast cell hyperplasia (24). Mast cells arising during the T_H2 immune response undergo changes in their granule architecture and their content of proteases due to the milieu of T-cell-derived cytokines (22).

1.1.2 Mast cell activation

As Erlich described, mature mast cells reside in the tissue throughout the body but are often found around blood vessels, in close contact with peripheral nerves or just beneath epithelial surfaces exposed to the external environment. This makes them likely to be one of the first cells to encounter and respond to exogenous stimuli. The best known and most extensively studied form of mast cell activation is crosslinking of the FcεRI, the high affinity receptor for immunoglobulin E (IgE). Binding of an antigen (Ag) to IgE, already bound to FcεRI, lead to mast cell degranulation and release of pro-inflammatory mediators (Figure 2).

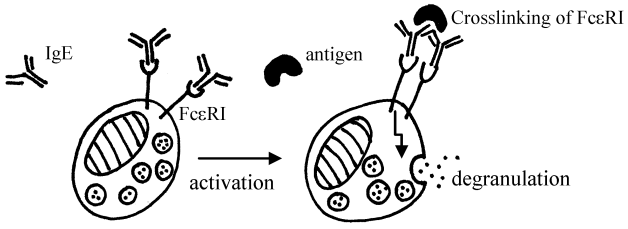

Figure 2. Mast cell activation through high affinity receptor IgE (FcεRI) crosslinking.

There have also been reports about mast cell activation using monomeric IgE (25, 26), Ig light chains (27) and IgG (28, 29). Moreover, mast cells can be activated by a wide variety of Ig-independent stimuli (reviewed in (16)) such as SCF, neuropeptides,

cytokines, chemokines, chemical agents such as compound 48/80, complement factors (C3a and C5a) and toll like receptors (TLRs) (3).

Following activation, mast cells will respond by releasing mediators differentially and selectively, with or without degranulation, depending on the nature of the stimuli. This selectivity suggests mast cells to be refined and adaptive cells, tailoring their mediator release in response to the stimuli. In general, mast cell mediators fall into three groups: preformed mediators, lipid-derived mediators and various cytokines, chemokines and growth factors (Figure 3). The preformed mediators, e.g. histamine, proteases and proteoglycans, are all stored in secretory granules and can be rapidly released upon mast cell activation. Lipid-derived mediators such as leukotrienes and prostaglandins are *de novo* synthesized within minutes following activation and subsequently secreted. These mediators and cytokines are associated with an immediate response that leads to vasodilatation, bronchial and gastrointestinal smooth muscle contraction and recruitment of leukocytes, augmenting the acute inflammatory response (16). Promoting a late phase inflammatory response, cytokines including various interleukins (ILs), tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interferons and chemokines are newly synthesized and secreted over hours following activation (30).

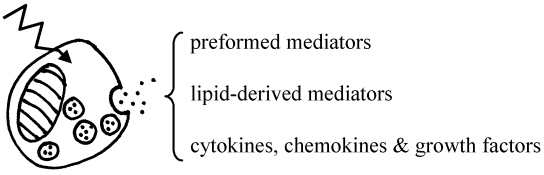

Figure 3. Mast cell activation by various stimuli leads to the production and release of mediators.

1.1.3 Mast cells in health and disease

Mast cells have primarily been regarded as effector cells in atopic diseases, releasing pro-inflammatory mediators upon FcεRI crosslinking although their strategic distribution and their capacity to secrete potent factors, indicate that they can take part in various immune responses apart from allergic inflammation. During recent years mast cells have been given a key role in both host defense against parasites and bacteria and in many different diseases, where they either can be protective or promote disease.

Mast cells in innate and acquired immunity

The early innate response to many infections involves recruitment and activation of the complement system. Mast cells express on their surface several complement receptors but also pattern recognition receptors such as TLRs that recognize molecules shared by many pathogens. The interaction of these receptors with their ligands are crucial for the activation of mast cells in innate immunity and activation typically results in the production and release of cytokines, chemokines and lipidmediators with or without degranulation (31-33). In addition, mast cells can phagocytose and kill bacteria (34). Recently it was also shown that mast cells can kill some bacteria independently of phagocytosis by entrapping them in extracellular structures consisting of DNA, histones, tryptase and antimicrobial peptide (35).

An accumulation of mast cells is often observed at sites of both bacterial and viral infections or in parasitic disease. *In vivo* studies of responses to bacterial infection using mast cell deficient mice (W/W^v) demonstrate a protective role of mast cells in a model of acute septic peritonitis (36). Furthermore, the release of mast cell-derived TNF-α was shown to play a key role by early recruitment of neutrophils to the site of infection (37). During viral infection mast cells release mediators distinct from those induced by a bacterial infection and these responses selectively recruit T-cells and natural killer cells rather than neutrophils to the site of infection (3). In responses to nematodes and other parasites mast cells and in particular mast cell protease (MCP)-1 (38) is critical and have been shown to take part in the response against nematode infection. Elevated IgE levels are also seen in parasitic infections, some being specific for parasitic antigen. This IgE-associated immune response is needed for parasitic clearance in mice (39).

The role of mast cells in acquired immunity, in contrast to innate immunity, is not that well established. It has been proposed that mast cells can initiate many key events in the generation of acquired immune responses but this concept is still debated. Interestingly, animals lacking mast cells have been found to have virtually intact humoral and cellular immune functions. Still, mast cells have the capacity to phagocytose various pathogens and they express both major histocompatibility complex (MHC)-I and MHC-II (40, 41) as well as co-stimulatory molecules (42, 43). This suggests that they could function as antigen presenting cells interacting with T- and B-lymphocytes once recruited into the tissues by the release of mast cell-derived chemokines. Recently, mast cells have been demonstrated to have a crucial role in regulatory T-cell (Treg)-mediated allograft tolerance. Local reconstitution of mast cell deficient mice with mast cells extended graft survival by mechanisms dependent on the production of IL-9 by Tregs (44).

Mast cells in inflammatory diseases

At sites of tissue inflammation mast cells accumulate and orchestrate the inflammatory response. Mast cell numbers increase dramatically in several inflammatory conditions such as allergy, asthma, various skin diseases, autoimmune disease like rheumatoid arthritis and tumours. In several of these diseases a correlation between mast cell numbers and disease severity has been described (5, 45-48) Furthermore, mastocytosis is a rare inflammatory disease, characterized by abnormal accumulation of mast cells in one or several organs (49), for which there is no cure.

Mast cells are often found in association with many different tumours and studies indicate that mast cells are associated with angiogenesis in a variety of tumours and can promote growth and metastasis via the release of various mediators (50-52). These findings imply that mast cells might participate in tumourigenesis rather than providing a defense against these tumours. Mast cells have also been associated with autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA). The most compelling evidence for the involvement of mast cells in MS comes from the use of the animal model experimental allergic encephalomyelitis (EAE) where mice lacking mast cells develop less severe EAE and at a later onset (53). In RA mast cells have been suggested to contribute to the chronic inflammation and destruction of joints (54).

However, the recent finding that mast cell-derived IL-10 could limit leukocyte infiltration, inflammation, and tissue damage in a mouse model of contact dermatitis (55) caution us not to see mast cells as simply detrimental for a disease.

Mast cells in allergy and asthma

Mast cells are recognized as the critical effector cell mediating IgE-dependent allergic responses. Individuals suffering from allergy have an elevated production of IgE compared to healthy individuals and during allergy and allergic asthma the mast cell numbers are also increased (4, 5). Binding of the allergen (such as pollen, cat dander, and house dust mite) to IgE, already bound to its high affinity receptor, FcεRI, on the mast cell surface activates the mast cell. This leads to mast cell degranulation and release of pro-inflammatory mediators such as histamine (56). Allergic reactions are typically divided into early/immediate reactions and late phase reactions. During the early/immediate phase, activated mast cells release preformed mediators into the surrounding tissue causing several effects, such as vasodilation, increased vascular permeability, oedema, bronchial and smooth muscle contraction and mucous secretion (16). The affected individual typically suffers from one or several symptoms such as rhinitis, itchiness, eczema, dyspnea and anaphylaxis. These symptoms can affect the whole body or be localized to a particular body system, determined by the type of allergen, the route of administration and individual predisposition. In the worst case scenario, a systemic reaction to an allergen (anaphylaxis) can lead to an anaphylactic shock. If the shock-induced systemic vasodilation and oedema of bronchial mucosa is left untreated it might lead to death in a matter of minutes. The late phase of allergy is mediated by the production and release of *de novo* synthesized mast cell mediators and characterized by the migration of leukocytes such as neutrophils, lymphocytes, eosinophils and macrophages into the tissue where the initial reaction took place (6).

Asthma is a complex inflammatory disease associated with airway smooth muscle hyper-reactivity and remodeling, increased mucus production and collagen deposition, bronchoconstriction and infiltration of lymphocytes, neutrophils and eosinophils (30). It is known that human airway smooth muscle cells can produce SCF and chemokines to recruit and retain mast cells at the site of inflammation and mast cell numbers have been found to increase within the airway smooth muscle of asthmatic patients (57, 58). Mast cells are thought to facilitate hyper-reactivity by mediator release and/or cell to cell contact. The mediators released by mast cells will initiate and/or sustain chronic inflammation, induce contraction of airway smooth muscle cells and mucus secretion (59). Many studies have tried to elucidate the role of mast cells in asthma using murine models and a variety of responses and pathways have been reported.

Today, allergic diseases are commonly treated with drugs aiming to suppress the symptoms of disease or prevent the inflammatory process. Since mast cells are a potent source of mediators involved in the initiation and perpetuation of allergic disease most drugs and therapies on the market or in development are aimed at inhibiting these mediators. Thus, inhibitors against histamine and leukotrienes and cromoglycate-based drugs (sodium cromoglycate) which stabilize mast cells, preventing degranulation, have been found to be useful (Figure 4). Still, developing new ways of treating allergy is of importance. A potential way of doing this is through the regulation of mast cell

numbers and their activity within in the tissue, thereby affecting the initiation, perpetuation and thus the severity of inflammation.

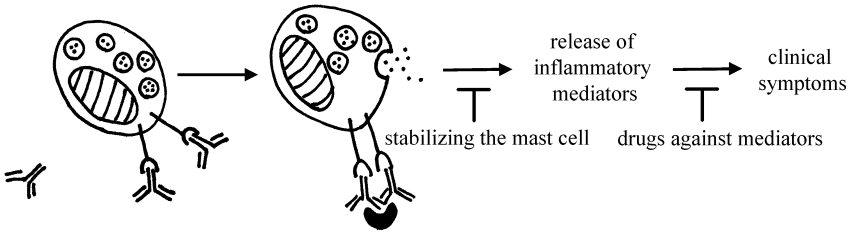

Figure 4. Mast cell mediators are often targeted in order to suppress the symptoms of allergic disease. Regulation of mast cell numbers and activity in the tissue could offer an attractive alternative.

1.2 CELL DEATH

Cell death is an essential feature of embryogenesis and later in the maintenance of cellular homeostasis. Depending on the stimuli and the state of the cell, death can be executed by necrosis, apoptosis or autophagy (60). Cell death induced by physical damage is called necrosis. Necrosis is accompanied by a breakdown of the plasma membrane and vacuolation of the cytoplasm which lead to an inflammatory response. Apoptosis is cell death regulated by the activation of a family of cysteine proteases (caspases), in which the cells die without plasma fragmentation and induction of an inflammatory response. Autophagy, sometimes defined as programmed necrosis, is a mechanism by which cells produce energy by gradually targeting their organelles and cytoplasmic elements to lysosomes for digestion. Normally autophagy protects the cell by reducing metabolic stress but excessive autophagy may result in cell death. This will not generate an inflammatory response under normal circumstances although dysfunction of the autophagic process is often associated with an inflammatory response.

1.2.1 Apoptosis

Apoptosis, also known as programmed cell death or cell suicide, is a highly regulated process. It can be induced by a number of stimuli such as growth factor deprivation, DNA damage, developmental signals, cellular stress and cytotoxic drugs. Upon apoptosis cells undergo biochemical as well as morphological changes including DNA cleavage, nuclear condensation and fragmentation and the formation of apoptotic bodies (61). The apoptotic bodies dissolve the cellular structure without plasma membrane fragmentation and in the absence of an inflammatory response. Dysregulation of apoptosis can lead to either an excess or deficit in cell death and is associated with a variety of diseases (1). In cancer and autoimmune diseases cells commonly escape apoptosis by overexpressing anti-apoptotic proteins (62, 63) thus making inhibition of these proteins an attractive strategy for therapy.

1.2.2 Apoptotic pathways

Apoptosis is regulated via two parallel but converging pathways, the extrinsic and intrinsic pathway (Figure 5). Both pathways require caspase activation and lead to

targeted degradation of cellular structures and formation of apoptotic bodies (64). The extrinsic pathway is stimulated via external signals from the environment outside the cell (65). The signal is transmitted through death receptors, belonging to the TNF receptor family, located on the surface of the cell and lead to the activation of the caspase cascade via caspase-8 (caspase-10 in humans). The intrinsic or mitochondrial pathway responds to stress factors such as growth factor deprivation, DNA damage, and other stimuli that originate from the inside of the cell (66, 67). The Bcl-2 family of proteins plays a key role in the intrinsic pathway of apoptosis. Their primary site of action is at the mitochondrial membrane where the relative balance of Bcl-2 family members determines if permeabilization of the mitochondrial outer membrane (MOMP) will occur. Following MOMP, apoptogenic factors such as cytochrome c will be released. This lead to the activation of Apoptotic protease activating factor 1 (Apaf-1) and the formation of the apoptosome; consisting of cytochrome c, Apaf-1 and ATP. The apoptosome activates caspase-9 which in turn induces the activation of effector caspases that cause proteolytic degradation and subsequent cell death (68).

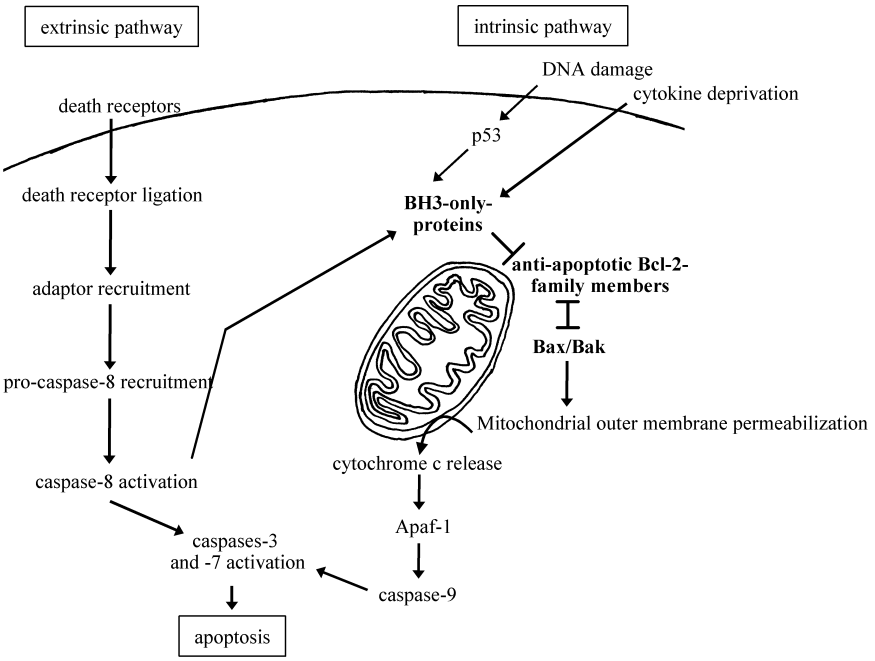

Figure 5. Apoptosis is regulated via the extrinsic and intrinsic pathway.

Since caspases are the final effectors of both the extrinsic and intrinsic pathways their activity need to be strictly regulated. The Inhibitor of Apoptosis Proteins (IAP) family interacts with caspases and as a consequence inhibits their proteolytic activity (69). IAP members in turn are regulated by the Smac/DIABLO protein which is released from mitochondria during the apoptotic process (70). Although the two pathways are independent from each other (71) crosstalk do occur. This is mediated via Bid, a member of the Bcl-2 family, which can be activated by caspase-8 (or caspase-10 in humans)-mediated proteolysis which generates two fragments where the truncated Bid

fragment (tBid) contains the BH3 domain and has the capacity to influence the intrinsic pathway (72).

1.2.3 The Bcl-2 family

The Bcl-2 protein family contains members that safeguard cell survival (anti-apoptotic proteins) as well as proteins that promote apoptosis (pro-apoptotic proteins) and so far more than a dozen members have been identified. The Bcl-2 family members are characterized by the presence of at least one and up to four Bcl-2 homology (BH) domains. The anti-apoptotic members (including Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1 and Mcl-1) contain up to four BH domains (BH1-4) and a trans-membrane region (TM) that allows targeting to intracellular membranes. The pro-apoptotic members can be sub-divided into two groups: BH3 domain only proteins (BH3-only; such as Bcl-XS, Bcl-2 antagonist of cell death (Bad), Bim, Bcl-2–modifying factor (Bmf), BH3-interacting domain death agonist (Bid), Puma, and Noxa), which share with each other only the BH3 domain and Bax/Bak-like proteins (Bax, Bak and Bcl-2–related ovarian killer (Bok); a non-extensively studied Bax/Bak-like protein), which contain BH1, BH2 and BH3 domains and a trans-membrane region (Figure 6) (68, 73).

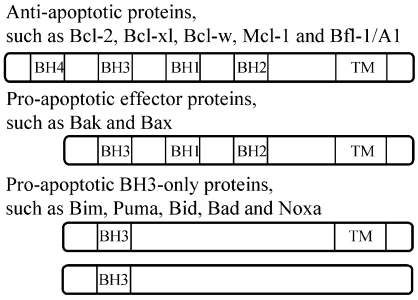

Figure 6. The Bcl-2 family consists of anti-apoptotic and pro-apoptotic members that are characterized by Bcl-2 homology (BH) domains and most of them contain a trans-membrane region (TM).

Regulation of apoptosis is dependent on the balance between the different Bcl-2 family members and through the interactions between the proteins within the three subgroups. When activated, BH3-only proteins insert their amphipatic α–helix of the BH3 domain into the hydrophobic groove of anti-apoptotic Bcl-2 family members (74). Furthermore, individual BH3-only proteins differ in their ability to bind to anti-apoptotic Bcl-2 family members and this specificity governs the function of these molecules (Figure 7) (75, 76). Bim and Puma can bind to all anti-apoptotic Bcl-2 family members while Noxa selectively binds Mcl-1 and A1, and Bad binds to Bcl-w, Bcl-2 and Bcl-XL. tBid binds all anti-apoptotic members but with low affinity to Bcl-2. The Bim, Puma and tBid proteins, being more promiscuous in their binding to anti-apoptotic members have the capacity to induce apoptosis on their own while the more selective Noxa and Bad must cooperate in order to do this. Following the interaction of BH3-only proteins and anti-apoptotic Bcl-2 proteins, which targets the cell for apoptosis, execution requires activation of Bax/Bak-like proteins (77, 78). Upon activation Bax/Bak-like proteins oligomerize and interact with the mitochondrial outer membrane, disturbing membrane integrity through their trans-membrane domain (66, 79).

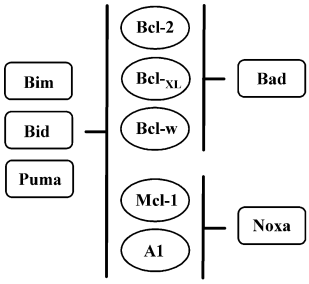

Figure 7. BH3-only proteins differ in their ability to bind to anti-apoptotic Bcl-2 family members.

Although it is well-known that Bcl-2 family members do interact, different modes of action have been proposed for the interactions between anti-apoptotic and pro-apoptotic Bcl-2 family proteins (reviewed in (2)). In the *direct activation* model, BH3-only proteins are divided into activators and sensitizers (76, 79-83). Sensitizer BH3-only proteins bind only to anti-apoptotic Bcl-2 family members, thereby releasing activator BH3-only proteins to exert their function. The activator BH3-only proteins such as Bim, Bid and possibly Puma bind directly to Bax/Bak-like proteins initiating activation leading to MOMP. There are however findings that challenge the *direct activation* model, proposing an indirect activation strategy. The *indirect activation* model demonstrates BH3-only proteins to bind only to anti-apoptotic proteins (75, 84, 85), leaving Bax/Bak-like proteins active at the mitochondrial membrane or bound to anti-apoptotic proteins in the cytosol. Upon apoptotic stimuli BH3-only proteins will bind anti-apoptotic proteins and thereby release Bax/Bak-like proteins to exert their function.

Targeting the Bcl-2 family is one of the strategies for the development of therapeutic treatments against diseases associated with dysregulation or dysfunction of apoptosis. A way of targeting the Bcl-2 family has been the development of drugs that mimic the binding of BH3-only proteins to the anti-apoptotic proteins inhibiting their function and thus promoting apoptosis. This has already proven useful in cancer therapy where the use of BH3 mimetics sensitizes tumour cells to apoptosis (86). ABT-737 is a small molecular inhibitor, mimicking the binding BH3 domain of the pro-apoptotic protein Bad. It binds with high affinity to the anti-apoptotic proteins Bcl-XL, Bcl-2 and Bcl-w but not Mcl-1 or A1 (87). It has been demonstrated to have an effect in treatment of solid tumors and lymphoid malignancies although overexpression of Mcl-1 can make cells refractory to the drug (88-92).

The anti-apoptotic Bcl-2 family member A1/Bfl-1

One Bcl-2 family member is the anti-apoptotic protein A1 (in humans Bfl-1). A1 exists as three genes and isoforms; A1a, A1b and A1d (93) whereas Bfl-1 has two isoforms due to alternative slicing; Bfl-1 and Bfl-1s (94, 95). A1/Bfl-1 confer protection from apoptosis in a number of cell types including endothelial cells, lymphocytes, neutrophils and macrophages (96-101). In lymphocytes the transcription of A1 has been demonstrated to be dependent on the NF-κB transcription factor pathway (102-

104) and a functional NF-κB binding site has been mapped within the A1 promoter. Overexpression of A1a in transgenic mice results in increased survival of T cells and activated splenocytes are offered protection from apoptosis (105, 106). In humans, Bfl-1 can be found overexpressed in some cancers, including lymphomas, breast, bladder and stomach cancer (94, 107-110). Since A1/Bfl-1 is the only known Bcl-2 family member to be induced by inflammatory cytokines it has been suggested to play a role in inflammatory processes (100-102). Mice deficient in A1a have a dampened acute inflammation after infection with pathogen and increased apoptosis of neutrophils (111). Interestingly, A1a deficient mice display mast cell numbers in the tissue comparable to wild type (wt) mice (98, 112). However, upon sensitization and subsequent provocation with allergen A1a deficient mice show a lower number of mast cell compared to wt mice (112). The expression of A1 in mast cells, following allergic activation, is not dependent on the transcription factor NF-κB but rather on NFAT (113).

A1 has been described to associate with several pro-apoptotic Bcl-2 members, such as Bim, Puma, and Noxa (75) and there are opposing reports regarding the ability of A1 to associate with Bax and Bak (114-116). A report has described that the interaction between A1 and Bim increases the half life of A1 and amplifies its anti-apoptotic effect, all mediated via the stabilization of the C-terminus of A1 (117). The human homologue of A1, Bfl-1, has previously been shown to interact with both Bid and tBid (118). More recently, it was demonstrated that Bfl-1 interacts with tBid and thereby suppresses the activation of Bax indirectly but also interacts directly with Bak to antagonize Bax/Bak-mediated cell death (119).

Many studies on the anti-apoptotic function of A1/Bfl-1 and its interactions with other Bcl-2 family members have been performed in systems where A1/Bfl-1 was overexpressed. Hopefully the recent derived crystal structures of both A1 (120) and Bfl-1 (121) will add to our understanding of how A1/Bfl-1 exert its anti-apoptotic function and how it might interact with other members of the Bcl-2 family.

The pro-apoptotic BH3-only proteins Bim and Puma

Other members of the Bcl-2 family are pro-apoptotic Bim (also called Bod) and Puma (also called Bbc3). Both proteins belong to the BH3-only proteins and have prominent roles since they bind with high affinity to all anti-apoptotic Bcl-2 family proteins (75). The apoptotic function of BH3-only proteins is strictly controlled by multiple mechanisms such as transcriptional and post-translational modifications and in some cases binding to cytoskeletal proteins (122).

Bim is known to be under the transcriptional control of the transcription factor FOXO3a (123, 124) and three isoforms have been characterised; Bim_{EL} and Bim_{L} and Bim_{S} (125). Bim_{EL} and Bim_{L} are found associated with the dyenin motor complex in healthy cells and upon appropriate apoptotic stimuli the protein is released to perform its apoptotic function (122). Bim has been shown to have an important role in the apoptosis of numerous cell types (in humans and rodents) including lymphocytes, myeloid cells, and neurons (126-131). Mice deficient for Bim have been shown to accumulate lymphoid and myeloid cells, display a defective T-cell development and later in life succumb to autoimmune diseases (126) demonstrating a role for Bim in homeostasis and autoimmunity.

Puma is transcriptionally upregulated by the transcription factor p53, and activated upon p53-dependent apoptotic stimuli, such as DNA damaging drugs and γ radiation (132, 133). Puma is essential for p53-mediated apoptosis in several cell types (134, 135) but has also been shown to be upregulated in response to certain p53-independent stimuli (136). Experiments with gene-targeted mice have shown that Puma is required for apoptosis of lymphoid cells and fibroblasts in response to p53-dependent stimuli (e.g. γ-radiation, etoposide) and also certain p53-independent ones, including cytokine deprivation (134, 137). Puma exist as two isoforms, Puma-α and Puma-β, and it has been demonstrated that the transcription factor FOXO3a upregulates Puma expression in response to cytokine or growth factor deprivation in lymphoid cells and mouse embryonic fibroblasts (138).

1.3 MAST CELL SURVIVAL AND APOPTOSIS

Mast cells are associated with inflammatory conditions like allergy, asthma, various skin diseases, rheumatoid arthritis and tumours. For several of these diseases mast cell numbers are increased, correlating with disease severity (5, 45, 47, 48, 139-141). Mast cells have the unique ability to survive allergy-induced activation and can be activated again which perpetuates the inflammatory reaction. In these circumstances it would be beneficial to decrease mast cell numbers and their activity. The role of Bcl-2-family members in the regulation of mast cell survival and apoptosis could be of use for specific targeting of mast cells in these mast cell-associated disorders.

1.3.1 Activation-induced survival

Mast cells have the unique ability to survive the aggregation of FcεRI (FcεRI crosslinking) and the subsequent degranulation process which is a key event in perpetuating the inflammatory response (142, 143). This increased survival of both human and murine mast cells (activation-induced survival) following FcεRI crosslinking has been demonstrated by us and others (112, 144-146) and a fundamental question in mast cell biology is how this survival is mediated.

However, opposing results have been reported regarding the effects of enhanced mast cell survival following FcεRI crosslinking (26, 147). These differences can be due to IgE type, strength and duration of the crosslinking (145, 148, 149). One study showed that weak to moderate FcεRI crosslinking led to activation-induced survival while stronger FcεRI crosslinking resulted in increased degranulation and IL-6 production but diminished survival (145). Moreover, with the use of a CD8 fusion protein the strength of FcεRI signals was manipulated demonstrating activation-induced survival even in the absence of degranulation (149). Beside the nature of the stimuli, *in vivo* findings suggest the subtype to influence the mast cell response. There is a local accumulation of IgE bearing MC_Ts upon Ag exposure in seasonal allergic rhinitis whereas MC_{TC}s do not increase (150). This considerable heterogeneity among mast cell subpopulations *in vivo*, when comparing their function and survival, suggest that the development and use of *in vitro*-derived mast cell subpopulations can be used to study the mechanism of activation-induced survival and be proved useful to mast cell research.

Upon FcεRI crosslinking mouse bone marrow derived mast cells (BMMCs) upregulate anti-apoptotic Bcl-2 family member A1 (112) and Bcl-XL and also to a lesser extent Bcl-2 on mRNA level (151, 152). These cells have been thought to share many features with MMCs (153), although BMMCs have subsequently been shown to differ in their morphology and protease phenotype compared to MMCs (154). We have also shown that mouse mast cells deficient in A1 do not exhibit activation-induced survival upon FcεRI crosslinking (112). Similarly, the human homologue of A1, *bfl-1*, is upregulated in human mast cells upon FcεRI aggregation (155) and it was also shown that human cord blood derived mast cells (CBMCs) following FcεRI crosslinking upregulate the anti-apoptotic Bcl-2 family member Mcl-1 (147, 155). These observations provide an explanation for the IgE-mediated activation-induced mast cell survival, demonstrating a major role of A1 in murine mast cells and suggest that the human homologue Bfl-1 and Mcl-1 could be of importance in human mast cells for this process.

Interestingly, FcεRI crosslinking not only regulates anti-apoptotic proteins of the Bcl-2 family but also pro-apoptotic proteins are affected. In murine mast cells, FcεRI crosslinking upregulates and induces a rapid, but transient, phosphorylation of Bim (151). Other BH3-only proteins including Bad are also phosphorylated, thereby inhibiting their pro-apoptotic functions (156). In CBMCs FcεRI crosslinking increase TNF-related apoptosis-inducing ligand (TRAIL)-induced caspase-8 and caspase-3 activation and the levels of FLICE-like inhibitory protein (FLIP), Mcl-1 and Bim are upregulated while Bid is downregulated (147). The conundrum of Bim upregulation following FcεRI crosslinking needs further investigation. It has been described that the interaction between A1 and Bim increases the half life of A1 and amplifies its anti-apoptotic effect (117) and it is possible that the transient phosphorylation of Bim inhibits its apoptotic effect (151).

Given that pro- and anti-apoptotic Bcl-2 family proteins can bind to each other and titrate each others function suggests that the regulation of the aforementioned Bcl-2 family members, both anti-apoptotic as well as pro-apoptotic, following FcεRI crosslinking is likely to determine cell fate (Figure 8).

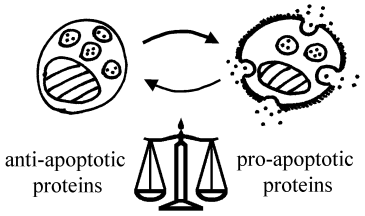

Figure 8. Regulation of apoptosis is dependent on the balance between the different Bcl-2 family members and through the interactions between them.

1.3.2 Deprivation-induced apoptosis

Mature mast cells situated in the tissue are long-lived and primarily depend on SCF for their survival. The importance of SCF is highlighted by the almost complete lack of mast cells in mice deficient in Kit or SCF (17-19) and reducing SCF production reduces mast cell numbers in the tissue (157). Furthermore, *in vitro*-derived mast cells

deprived of their requisite growth factors and thereby destined for apoptosis can be rescued by the addition of SCF (158, 159).

We have previously shown that SCF regulates mast cell survival through inactivation of FOXO3a and downregulation and phosphorylation of its target Bim (7), subsequently leading to the ubiquitination and proteasomal degradation of Bim (160-162). Overexpression of phosphorylation-deficient, constitutively active FOXO3a caused an upregulation of Bim and increased mast cell apoptosis (7). Moreover, Bim deficient mast cells are partially resistant to cytokine deprivation-induced apoptosis in culture (151). Since overexpression of Bcl-2 protected mast cells more potently than loss of Bim, it appears that other pro-apoptotic BH3-only proteins, besides Bim, might be involved in this process. The identification of such proteins remains to be done.

A way to unravel the role of Bcl-2 family members in the regulation of mast cell survival and apoptosis is the use of Bcl-2 family gene-deficient mice and cells. Many Bcl-2 family member gene deletions in mice lead to disrupted homeostasis and as a consequence cells accumulate in excess. This might cause increased tumour incidence and autoimmunity in mice reaching adulthood (126, 163). The lack of Bcl-2 family members can have implications during the development and life of mast cells both *in vitro* and *in vivo*. Mice deficient in anti-apoptotic A1 will develop a normal phenotype and display mast cell numbers in the tissue comparable to wt mice (98, 112). In contrast, anti-apoptotic Bcl-2, Bcl-XL, Mcl-1, or Bcl-w deficiency is detrimental during development in mice (164, 165). In the absence of the genes *bcl-x* or *bcl-2*, no offspring is born or born with a short life expectancy, respectively (165, 166). It is however possible to generate mast cells from murine embryonic stem cells lacking *bcl-x* or *bcl-2*, although this requires both SCF and IL-3 and produces not by far the same amount of mast cells as from wt stem cells (167).

The importance of the effector proteins Bax and Bak is demonstrated by the fact that their absence abolishes most apoptotic responses sensed by BH3-only proteins (77). Loss of both Bak and Bax results in embryonic lethality (168), whereas mice deficient in Bax or Bak will develop more or less normally (168, 169). Although both Bax and Bak have been shown to have largely overlapping functions in many cells, it has also been reported that one of them can have a dominant role over the other in certain cell types (168, 170). Increased mast cell numbers are found in the stomach mucosa of mice lacking Bax or Bak and in the back skin of mice lacking Bak (171). This may indicate a more prominent role for Bax in MMCs compared to CTMCs for cell homeostasis. Furthermore, loss of Bax conferred protection from cytokine deprivation-induced apoptosis of *in vitro*-derived BMMCs (171). Downstream of the Bax and Bak proteins, Apaf-1 and caspase-9 carry out their functions in the intrinsic pathway of apoptosis. It has been shown that lymphocytes lacking either of these proteins still have the capacity to undergo apoptosis in response to growth factor deprivation or DNA damage (172). In contrast, Apaf-1 and caspase-9 deficient mast cells fail to undergo apoptosis in response to cytokine deprivation and instead remain in a ‘senescent’ stage with no proliferative capability and lost effector functions (173).

2 AIM OF THE THESIS

The overall aim of this thesis was to study the mechanisms regulating mast cell survival and apoptosis with focus on the impact of Bcl-2 family members in this process. The specific aims of each paper were to:

Paper I. Investigate the activation-induced cell survival in two different murine *in vitro*-developed mast cell phenotypes, the mucosal-like- and connective tissue-like mast cells (MLMCs and CTLMCs) and further characterize these mast cell populations with regard to their regulation of Bcl-2 family members.

Paper II. Investigate activation-induced human mast cell survival following FcεRI crosslinking and the role of anti-apoptotic Bcl-2 family members in this process.

Paper III. Explore the role of the pro-apoptotic Bcl-2 family members Noxa, Bad, Bim, Bmf, Bid, and Puma in apoptosis of MLMCs and CTLMCs following cytokine deprivation and radiation.

Paper IV. Examine the role of the two pro-apoptotic Bcl-2 family effector proteins Bax and Bak in cytokine deprivation-induced apoptosis of MLMCs and CTLMCs.

3 METHODOLOGY

Methods used in paper I-IV are described in detail in the respective "Materials and methods" sections. Below follows an overview of each method with reference to the papers in which they were used:

Enzyme-linked immunosorbent assay [I, II]	Quantative detection of histamine, released or within cells.
Flow cytometry [I, II, III, IV]	Laser based analysis of cell viability and phenotype using fluorochrome conjugated antibodies or fluorescent molecules.
^{3}[H]thymidine incorporation assay [I]	Proliferation assay of stimulated cells by radioactive isotope incorporation and scintillation measurement.
Mast cell activation [I, II, III, IV]	Crosslinking of FcεRI on cell surface using IgE and antigen (or anti-IgE).
Mast cell generation [I, II, III, IV]	Cell cultures of mouse bone marrow cells or CD34 positive cord blood cells in the presence of requisite growth factors and cytokines to differentiate mast cells.
N-acetyl-β-D-hexosaminidase assay [I, II]	Assay measuring an enzyme stored in the mast cell granules and released with the same kinetics as histamine.
Polymerase chain reaction [I, II]	Detection of mRNA-expression of genes of interest in mast cells.
RNase protection assay (RPA) [I, IV]	Detection of RNA-expressions in mast cells.
Statistical analysis [I, II, III, IV]	Student *t* test or ANOVA (short for analysis of variance) was used.

Transfection [II, III]	Technique to introduce nucleic acids into cells using either chemical reagents or physical methods.
Western blot [III, IV]	Gel electrophoretic separation of proteins and transfer to protein-binding membranes for enzyme-conjugated antibody detection.

4 RESULTS AND DISCUSSION

Mast cells are versatile effector and regulatory cells of the immune system, perhaps best known for their role in allergic reactions. Furthermore, there are several acute and chronic inflammations where mast cells are important both for the initiation as well as the perpetuation of the inflammation. Better knowledge of the mechanisms regulating mast cell survival and apoptosis could potentially help us identify molecular targets to create ways of treating mast cell disorders. In this thesis the effect Bcl-2 family members can have on mast cell survival and apoptosis have been investigated. We provide evidence that mast cell populations differ in their ability to survive allergic reactions *in vitro* and identify the Bcl-2 family member A1/Bfl-1 as a potential target for treatment of allergic diseases. Furthermore, the pro-apoptotic Bcl-2 family members Puma and Bax are shown to be essential for regulating mast cell apoptosis *in vitro* suggesting a plausible role for these proteins in the regulation of mast cell numbers *in vivo*.

4.1 FCεRI AGGREGATION PROMOTES SURVIVAL OF CONNECTIVE TISSUE-LIKE MAST CELLS BUT NOT MUCOSAL-LIKE MAST CELLS (PAPER I)

The development and phenotype of mast cells cultured in either SCF/IL-4 or SCF/IL-3/IL-9/TGF-β_1, conditions that promote development into either CTLMCs or MLMCs, respectively, were compared. We found that MLMCs show a dramatic increase in cell numbers and also displayed more vigorous proliferation, compared to CTLMCs. The kinetics of the MLMC maturation closely resembles mast cell hyperplasia seen upon nematode infections (12, 174, 175) and MLMCs expressed the β-chymase MCP-1 known to be selectively expressed in MMCs (176, 177). Morphologically, MLMC cultures exhibited fewer toluidine positive cytosolic granules compared to CTLMCs. Furthermore, *in vitro*-derived CTLMCs were found to contain more histamine and degranulate more vigorously in response to compound 48/80 compared to MLMCs. This is consistent with results obtained for CTMCs and MMCs *in vivo* (178-181). We conclude that the MLMC- and CTLMC subpopulations represent a useful *in vitro* model for committed mast cell lineages.

Having observed that culture conditions can affect FcεRI activation-induced mast cell survival (151), we compared the survival of MLMCs and CTLMCs after exposure to FcεRI crosslinking and cytokine deprivation. Strikingly, MLMCs deprived of cytokines did not respond to FcεRI crosslinking with increased survival, whereas CTLMCs did. Previous work has shown the anti-apoptotic Bcl-2 family member A1 to have an important function in activation-induced survival (112). We therefore examined the levels of A1 mRNA in MLMCs and CTLMCs, before and after FcεRI crosslinking. Only a slight up-regulation of A1 mRNA was seen in MLMCs after FcεRI cross-linking, although A1 is present in both resting and activated cells. In contrast, in CTLMCs FcεRI crosslinking potently upregulated A1 mRNA levels, and, as previously described for BMMCs (151), the levels of Bim_{EL} also increased slightly. These differences in the ability to increase *A1* expression might explain, at least in part, the differences in survival and turnover of CTMCs and MMCs. In resting cells, we detected no significant difference in mRNA expression for Bcl-2 family members

between MLMCs and CTLMCs, apart from slightly increased levels of Bim mRNA in MLMCs compared to CTLMCs.

The elevated levels of Bim present in MLMCs at resting state and a slight increase of Bim mRNA following FcεRI crosslinking in CTLMCs prompted us to examine the survival of MLMCs and CTLMCs deficient of Bim. We found that Bim deficiency enhanced survival of cytokine deprived MLMCs although FcεRI crosslinking conferred no additional survival effect. In CTLMCs, Bim deficiency increased survival similarly following cytokine deprivation and FcεRI crosslinking was still able to significantly enhance survival although this survival was somewhat diminished. Since pro- and anti-apoptotic Bcl-2 family proteins bind to each other and titrate each others function (182) this suggests that both anti-apoptotic as well as pro-apoptotic proteins, following FcεRI crosslinking is likely to determine cell fate. Interestingly, it has been reported that the interaction between A1 and Bim stabilizes the A1 protein, thereby strongly reducing A1 turnover and amplifying its anti-apoptotic effects (117). However, knowledge of the binding of A1 to pro-apoptotic Bcl-2 family proteins in mast cells is limited and Bim is not the only BH3-only protein that can bind to A1, for example Puma can also do this (75).

4.2 ANTI-APOPTOTIC BFL-1 IS THE MAJOR EFFECTOR IN ACTIVATION-INDUCED HUMAN MAST CELL SURVIVAL (PAPER II)

Having shown that FcεRI crosslinking prolongs the survival of cytokine deprived CTLMCs but not MLMCs (paper I) we next examined the survival induced by FcεRI crosslinking using different concentrations of antibody and antigen in order to investigate how this affects activation-induced survival. Our results demonstrate that with increasing IgE and antigen (TNP-BSA) concentrations the survival of CTLMCs is prolonged as opposed to MLMCs where FcεRI crosslinking diminishes the survival of cytokine deprived cells. These results demonstrate that there is a dependence of the amount of antibody and antigen administrated to the murine mast cells and that FcεRI crosslinking promotes the survival of cytokine deprived CTLMCs but not MLMCs, which highlights the role mast cell subtype and activating conditions might play in determining the outcome of FcεRI crosslinking.

We next investigated the survival capacity of human cytokine deprived CBMCs and the human mast cell line LAD-2 following FcεRI crosslinking. CBMCs as well as LAD-2 cells express tryptase and chymase (data not shown) (183-185) exhibiting a human MC_{TC} phenotype which corresponds to rodent CTMCs. The results show that FcεRI crosslinking resulted in histamine release and prolonged survival of cytokine deprived CBMCs for all antigen (anti-human IgE) concentrations used (0.2, 2 or 20 μg/ml) and also LAD-2 cells responded with an increased survival after being activated with 2 or 20 μg/ml of antigen. Although we and others previously reported FcεRI crosslinking to induce a survival program in both human and murine mast cells (145, 146, 155, 156) opposing results have been reported regarding the effects of survival induction upon FcεRI crosslinking (26, 147). We suggest that these differences can be due to activating conditions including type of IgE used, strength and duration of the crosslinking (145, 148, 149) as well as the use of different cell types and culturing conditions during *in vitro* development.

The anti-apoptotic Bcl-2 family member A1 is essential for activation-induced survival in a murine model (112) and the human homologue of A1, *bfl-1*, was found to be upregulated upon FcεRI crosslinking in CBMCs. With the use of the small BH3 mimetic compound ABT-737 alone or in combination with the cyclin-dependent kinase (CDK) inhibitor roscovitine we further investigated the potential role of anti-apoptotic proteins of the Bcl-2 family apart from Bfl-1 in activation-induced survival of human mast cells (Figure 9). Cell viability of human mast cells decreased upon treatment with non-cytotoxic concentrations of ABT-737 and roscovitine. Using ABT-737 alone or in combination with roscovitine as death stimuli in human mast cells we next investigated if FcεRI crosslinking still had the capacity to induce survival in human mast cells. FcεRI crosslinking resulted in prolonged survival of ABT-737-treated cells. This result is consistent with previous reports where mainly Bfl-1 and Mcl-1 were observed to be upregulated following FcεRI crosslinking in human mast cells (147, 155). This points them out as likely candidates to play a major part in activation-induced survival. Interestingly, FcεRI crosslinking also resulted in prolonged survival of ABT-737- and roscovitine-treated cells. These results show that the ability to induce survival is sustained in an experimental setting using ABT-737 alone or in combination with roscovitine as a death stimuli and this would implicate a minor role of Bcl-XL, Bcl-2, Bcl-w and Mcl-1 in activation-induced survival in human mast cells.

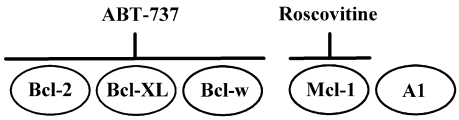

Figure 9. ABT-737 binds with high affinity to the anti-apoptotic proteins Bcl-XL, Bcl-2 and Bcl-w but not Mcl-1 or A1 whereas the cyclin-dependent kinase (CDK) inhibitor roscovitine has been reported to downregulate Mcl-1.

Although *bfl-1* has been found to be upregulated in human mast cells upon FcεRI crosslinking and FcεRI crosslinking results in increased mast cell survival the importance of Bfl-1 in this process has not been fully established. Using quantative PCR we confirmed that Bfl-1 is regulated on mRNA level following FcεRI crosslinking and we next investigated if downregulation of *bfl-1* would affect activation-induced survival of CBMC after FcεRI crosslinking. The use of *bfl-1* siRNA led to diminished upregulation of *bfl-1* following activation and this was accompanied by a decrease in activation-induced survival as compared to cells transfected with non-targeting siRNA. These results establish the importance of Bfl-1 for the activation-induced survival process in human mast cells.

4.3 THE BH3-ONLY PROTEIN PUMA PLAYS AN ESSENTIAL ROLE IN CYTOKINE DEPRIVATION INDUCED APOPTOSIS OF MAST CELLS (PAPER III)

The BH3-only proteins Noxa, Bad, Bid, Bmf and Puma have previously all been implicated in growth factor withdrawal-induced apoptosis (73). To study their involvement in cytokine deprivation-induced apoptosis in MLMCs and CTLMCs, mast cells were cultured from mice lacking these BH3-only proteins or, as controls, from wt mice or mice expressing a *bcl-2* transgene. We also generated MLMCs and CTLMCs from p53 and FOXO3a deficient mice to establish the influence these transcription factors have on cytokine deprivation-induced apoptosis.

It has previously been shown that Bim deficient mast cells are partially resistant to cytokine deprivation-induced apoptosis (151). In addition to Bim, our study identifies the BH3-only protein Puma to be essential for cytokine deprivation-induced apoptosis in mast cells. Mast cells lacking only one allele of *puma* showed significantly increased viability compared to wt cells, the degree of protection being similar to that obtained with complete loss of Bim. Moreover, Puma is upregulated in response to cytokine deprivation and loss of Puma promotes long-term, clonogenic survival of mast cells deprived of their requisite cytokines.

Puma was first identified as a BH3-only protein transcriptionally upregulated by p53 and activated in response to p53-dependent apoptotic stimuli (132, 133). Protection from apoptosis through loss of Puma was not restricted to cytokine deprivation, but was also seen when Puma deficient mast cells were treated with etoposide. In addition, p53 deficient mast cells exhibited increased viability and no upregulation of Puma protein levels compared to corresponding wt mast cell populations upon etoposide treatment. This identifies etoposide-induced apoptosis in mast cells as a process that is dependent on p53-mediated induction of Puma.

Puma is also upregulated in response to certain p53-independent stimuli (136, 137) and has been shown to play a critical role in cytokine deprivation-induced apoptosis of lymphoid cells and myeloid progenitors (134, 135, 186). Cytokine deprivation in mast cells was found to be a p53-independent stimulus, since cells lacking p53 died in response to cytokine deprivation with similar kinetics as wt mast cells. The upregulation of Puma in cytokine deprived p53 deficient mast cells indicates that another transcription factor is critical for this process. The transcription factor FOXO3a has been shown to regulate Bim (123) and was also described to control Puma expression in response to growth factor deprivation in lymphoid cells and mouse embryonic fibroblasts (138). FOXO3a deficient mast cells showed increased survival compared to wt mast cells following cytokine deprivation and BMMCs transfected with an inducible FOXO3a protein, identifies Puma as a FOXO3a downstream target in mast cells. However, loss of FOXO3a did not confer as much protection from apoptosis as loss of Puma or Bim and FOXO3a deficient mast cells were still able to upregulate Puma and Bim in response to cytokine deprivation. Thus, we believe that additional factors must be involved. Redundancy between FOXO3a and other FOXO members (187) has been suggested but other (unrelated) transcription factors may also play a role in this process.

We and others have previously demonstrated mast cell viability to increase upon FcεRI crosslinking (112, 145, 146) and this was associated with increased levels of anti-apoptotic as well as pro-apoptotic Bcl-2 family members (112, 151, 155). However, Puma was found not to be upregulated following FcεRI crosslinking and Puma deficiency did not further increase the survival of mast cells in response to FcεRI crosslinking suggesting that Puma does not play a critical role in this anti-apoptotic pathway.

In conclusion, our results demonstrate an important role for Puma in p53-dependent as well as p53-independent mast cell apoptosis where removal of only one allele of this BH3-only gene protects mast cells from apoptosis more sufficiently than complete removal of any of the other BH3-only proteins investigated.

4.4 PRO-APOPTOTIC BAX IS THE MAJOR AND BAK AN AUXILIARY EFFECTOR IN CYTOKINE DEPRIVATION-INDUCED MAST CELL APOPTOSIS (PAPER IV)

Although both Bax and Bak have been shown to have largely overlapping functions in several cell types (168, 170), it has also been reported that one of these proteins can have the dominant role. To investigate whether both Bax and Bak are necessary for mast cell apoptosis we assessed cytokine deprivation-induced apoptosis in CTLMCs and MLMCs deficient in *bax*, *bak* or both and wt.

The two effector proteins Bax and Bak were found to be expressed on both mRNA and protein level in wt CTLMCs. RNase protection assay (RPA) on mRNA revealed that *bax* was more abundant than *bak*. Loss of either *bax* or *bak* did not cause any compensatory alteration in the expression of the anti-apoptotic genes *bcl-w, A1, bcl-*x_L or *bcl-2*. On protein level, both Bax and Bak were detected in wt mast cells, although the levels of Bak appeared to be lower than the levels of Bax.

Loss of Bax conferred partial resistance to cytokine deprivation, whereas loss of Bak did not significantly affect cytokine deprivation-induced apoptosis of mast cells. Only combined loss of both Bax and Bak conferred complete resistance to cytokine deprivation, this effect resembling the effect seen in mast cells overexpressing *bcl-2*, or those lacking the BH3-only pro-apoptotic proteins Bim and Puma.

Furthermore we investigated if FcεRI crosslinking not only alters the expression of anti-apoptotic proteins and pro-apoptotic BH3-only proteins (112), but also the effector proteins Bax and Bak. We could observe a minor upregulation of Bak but no prominent effect on Bax, suggesting that FcεRI crosslinking primarily controls mast cell survival by regulating the levels and function of survival proteins and BH3-only proteins.

In conclusion, Bax was found to have a more prominent role than Bak in mast cell apoptosis induced by cytokine deprivation although both effector proteins are expressed in the cells. This data are in accordance with a previous report in which IL-3 derived BMMCs from Bax deficient mice were shown to have an increased survival upon cytokine deprivation (171). Previous *in vivo* data has indicated a more prominent role for Bax in MMCs compared to CTMCs for cell homeostasis (171). However, our *in vitro* data reveal no difference in the importance of Bax for the induction of apoptosis in CTLMCs and MLMCs, which suggests other additional mechanisms *in vivo*.

5 CONCLUSIONS

Paper I. In this study we cultured mast cells in two different ways, generating MLMCs and CTLMCs. Our *in vitro*-derived MLMCs and CTLMCs were found to display the characteristic differences in proliferation and turnover rate of MMCs and connective CTMC *in vivo*. Characteristic differences seen for MMCs and CTMCs, such as reactivity to compound 48/80, histamine content and chymase expression were also seen in our MLMCs and CTLMCs. These observations suggest that MLMCs and CTLMCs represent a useful *in vitro* model for committed mast cell lineages. These cells were used to analyze activation-induced cell survival and we show that CTLMCs but not MLMCs exhibit FcεRI stimulation-induced upregulation of anti-apoptotic *A1* and enhanced cell survival.

Paper II. Here we describe FcεRI crosslinking to promote activation-induced survival in human mast cells and that this is associated with an upregulation of anti-apoptotic *bfl-1*. *bfl-1* is a mediator of activation-induced mast cell survival as demonstrated by *bfl-1* siRNA inhibiting this effect. Our results, utilizing the inhibitors ABT-737 and roscovitine, show that activation-induced mast cell survival following FcεRI crosslinking is sustained indicating a minor role for the targeted anti-apoptotic Bcl-2 family members Bcl-XL, Bcl-2, Bcl-w and Mcl-1.

Paper III. Herein, we describe for the first time that Puma is critical for the induction of mast cell apoptosis following cytokine deprivation and treatment with the DNA-damaging agent etoposide in MLMCs and CTLMCs. Using mast cells deficient for p53, we found that cytokine deprivation-induced apoptosis, in contrast to that elicited by etoposide, is p53-independent. Our data suggest the involvement of FOXO3a in the regulation of cytokine deprivation-induced apoptosis and the expression of Puma. Mast cells deficient for FOXO3a were markedly resistant to cytokine deprivation compared to wt cells and overexpression of phosphorylation-deficient, constitutively active FOXO3a resulted in an upregulation of Puma.

Paper IV. In this study we examined the role of the two pro-apoptotic effector proteins Bax and Bak in cytokine deprivation-induced apoptosis in MLMCs and CTLMCs. Both Bak and Bax protein were expressed at readily detectable levels but we found a major role for Bax in mediating mast cell apoptosis. Upon cytokine withdrawal, Bak deficient mast cells died at a similar rate as wt mast cells, whereas Bax deficient mast cells and mast cells deficient for both Bak and Bax were partially or completely resistant to apoptosis, respectively. The total resistance seen in mast cells deficient for both Bak and Bax was comparable to mast cells deficient in both pro-apoptotic Bim and Puma or mast cells overexpressing anti-apoptotic Bcl-2.

In summary, the work in this thesis delineates the relative importance of different Bcl-2 family members for mast cell survival or apoptosis following various stimuli. The results highlights the anti-apoptotic A1/Bfl-1 as a major effector in activation-induced mast cell survival and the importance of the pro-apoptotic proteins Puma, Bim and Bax following cytokine deprivation-induced apoptosis. This thesis contributes to our understanding of specific targets for regulation of the number of mast cells and their activity in the tissue.

6 FUTURE PERSPECTIVES

Mast cells have a central role in the initiation and perpetuation of inflammatory responses, as seen in allergy and asthma. To reduce mast cell numbers, thereby reducing the severity of inflammation, has been suggested as an alternative therapeutical treatment in mast cell-associated diseases. A way to do this would be to sensitize mast cells to apoptosis. In order to achieve this we need to understand how mast cell survival and apoptosis is regulated in these settings. Pro- and anti-apoptotic proteins belonging to the Bcl-2 family is known to regulate apoptosis and our knowledge of how these regulate mast cell survival and apoptosis is continuously increasing. Still, the structural and functional difference between the members and their relative impact on mast cell survival and apoptosis need to be clarified. It is known that anti-apoptotic Bcl-2 family proteins bind to and neutralize pro-apoptotic members. This suggests that, once the key Bcl-2 family members that affect the severity of inflammation have been identified, the use of small molecular inhibitors could block their binding and thereby sensitize mast cells for apoptosis. The results of this thesis delineate the relative importance of different Bcl-2 family members for mast cell survival or apoptosis following various stimuli. The results highlights the anti-apoptotic A1/Bfl-1 as a major effector in activation-induced mast cell survival and the importance of the pro-apoptotic proteins Puma, Bim and Bax following cytokine deprivation-induced apoptosis.

The surrounding microenvironment in the tissue affects mast cell phenotype (13) and the mast cells are usually divided into two main subtypes; MMCs (MC_T in humans) and CTMCs (MC_{TC} in humans). However, depending on their local milieu mast cells will adapt an effector profile appropriate for that environment and it is anticipated that more than two subpopulations of mast cells can be found in both mouse and human. Therefore, it is of importance when studying mast cells both *in vivo* and *in vitro* to carefully examine the mast cells and to determine their type since subpopulations might respond differently to stimuli. This was clearly demonstrated by our MLMC- and CTLMC subpopulations (paper I) thus making them useful as an *in vitro* model for committed mast cell lineages. Furthermore, we showed that CTLMCs but not MLMCs exhibit FcεRI stimulation-induced upregulation of the anti-apoptotic gene *A1* and enhanced cell survival. Since we previously have linked this activation-induced cell survival to the upregulation of *A1* in the mouse system, these differences in the ability to increase *A1* expression might explain, at least in part, the differences in survival of MLMCs and CTLMCs. The reason for this differential expression of *A1* remains to be determined. A possible explanation could be epigenetic factors influencing the expression pattern. This imprinting of the gene, caused by environmental factors, would not change the underlying DNA sequence but rather lead to changes in gene expression.

This differential expression of *A1* is of great interest since our previous studies have shown *A1* levels to be quickly modulated, assuring survival of the mast cells following an allergic activation. Beside mast cells, other cell types such as B-cells are also dependent on A1 upregulation to reassure cell survival (188). The transcription of *A1* is regulated by the transcription factor NF-κB in most cells whereas the expression of *A1* in mast cells, following allergic activation, is dependent on the transcription factor

NFAT (113). Thus, what appears to be a differential regulation of *A1*, depending on mast cell subtype (paper I), and the transcriptional regulation of *A1* distinguish mast cells from other cell types. This indicates A1 to be of particular interest for further studies and as a potential target for new therapeutics in mast cell-associated disorders. To further prove the usefulness of our MLMC- and CTLMC subpopulations, as an *in vitro* model for differential expression of *A1* in mast cell lineages *in vivo*, it would be of interest to test MMCs and CTMCs *ex vivo* for their capacity to survive an allergic reaction and their regulation of *A1*.

In addition to the mouse system, we also demonstrate Bfl-1, the human homologue of A1, to be a mediator of activation-induced mast cell survival in the human system. Our results also indicate a minor role for the anti-apoptotic Bcl-2 family members Bcl-XL, Bcl-2, Bcl-w and Mcl-1 (paper II). The CBMCs found to upregulate *bfl-1* and display enhanced survival were also characterized as belonging to the MC_{TC} subtype which resembles mouse CTMCs. Given the differential expression pattern of *A1* depending on mouse mast cell subtype *in vitro* it would be interesting to evaluate MC_{TC} and MC_T subtypes for their response to an allergic activation. Would a MC_T subpopulation of human mast cells (corresponding to the mouse MMCs) display any differences in their response to an allergic activation in terms of activation-induced survival and regulation of *bfl-1* compared to CBMCs (corresponding to the mouse CTMCs)? A differential regulation of Bfl-1 in human mast cell subtypes *in vivo* would have implications, distinguishing mast cell-associated disorders that would benefit from the use of Bfl-1 as a target for new therapeutics. Our results highlights the anti-apoptotic A1/Bfl-1 as a major effector in activation-induced mast cell survival and a way to sensitize mast cells for apoptosis would be to inhibit A1/Bfl-1 by blocking the interactions of A1/Bfl-1 with its pro-apoptotic binding partner.

Unfortunately, not much is known about which pro-apoptotic Bcl-2 family members A1/Bfl-1 might interact with. A report has described that the interaction between A1 and Bim increases the half life of A1 and amplifies its anti-apoptotic effect, all mediated via the stabilization of the C-terminus of A1 (117). Bfl-1 has been shown to interact with tBid and thereby suppresses the activation of Bax indirectly but also interacts directly with Bak. Deletion of the C-terminus decreased Bfl-1 interaction with Bak and tBid and hence impaired its ability to prevent cell death (119). Interestingly, in our mouse system FcεRI crosslinking upregulates and induces a rapid, but transient, phosphorylation of Bim (151). Similarly, FcεRI crosslinking of CBMCs leads to Bim upregulation while Bid is downregulated (147). Since pro- and anti-apoptotic Bcl-2 family proteins bind to each other and titrate each others function this suggests that both anti-apoptotic as well as pro-apoptotic proteins, following FcεRI crosslinking is likely to determine cell fate. In our first paper (paper I) we were mainly interested in the differential expression of Bim when comparing the two subtypes of mast cells in a resting state, MLMCs and CTLMCs, although we touched upon the possibility that A1 and Bim might interact following FcεRI crosslinking. Revisiting paper I, the data on viability following FcεRI crosslinking from Bim deficient and wt CTLMCs do indicate that Bim deficient CTLMCs display a diminished cell survival although the ability to induce activation-induced survival is sustained. I believe it to be of interest to verify this observation, since we in paper III show another potential binding partner of A1, Puma, to be dispensable of CTLMC activation-induced survival in a similar experimental setting. Puma, in contrast to Bim, is not upregulated in mast cells following FcεRI crosslinking. These reports and our own findings suggest Bim and/or Bid to be of particular interest when trying to identify a potential binding partner of

A1/Bfl-1 in mast cells, although other Bcl-2 family members shown to bind A1/Bfl-1(75) should also be investigated.

Curiously, A1/Bfl-1seem to differ from other anti-apoptotic Bcl-2 proteins in the sense that its C-terminal end is unlikely to act as a trans-membrane domain (189) and the two previous reports suggest that the C-terminus of A1/Bfl-1 might be involved in the stability and perhaps interaction of this protein with pro-apoptotic proteins of the Bcl-2 family. This could be a unique feature of A1/Bfl-1 since pro- and anti-apoptotic protein interactions normally involve the BH3 domain of BH3-only proteins and the hydrophobic groove of the anti-apoptotic proteins (74). Thus, the recently derived crystal structures of both A1 (120) and Bfl-1 (121) are scientific contributions likely to add to our understanding of how A1/Bfl-1 exerts its anti-apoptotic function and how it might interact with other members of the Bcl-2 family. This knowledge will aid when designing a small molecular inhibitor to abrogate the interactions of anti-apoptotic A1/Bfl-1 with its yet unidentified binding partner.

Although pro-apoptotic Puma was found to be dispensable for activation-induced survival in CTLMCs it is critical for the induction of mast cell apoptosis following cytokine deprivation. Our results (paper III and IV) identify the BH3-only proteins Bim and Puma and the pro-apoptotic effector protein Bax to be the most important Bcl-2 family members involved in this process. Mast cells lacking only one allele of *puma* showed significantly increased viability compared to wt cells, the degree of protection being similar to that obtained with complete loss of Bim. Most likely Puma and Bim have overlapping functions in regulating mast cell survival following cytokine deprivation since both proteins bind with high affinity to all anti-apoptotic Bcl-2 family proteins (76). Furthermore, an inducible FOXO3a protein identifies Puma and Bim as FOXO3a downstream targets in mast cells. However, FOXO3a deficient mast cells were still able to upregulate Puma and Bim in response to cytokine-deprivation. Thus, redundancy between FOXO3a and other FOXO members (187) has been suggested but other (unrelated) transcription factors may also play a role in this process. We did not find evidence to support that Puma deficiency compromises the normal development and regulation of mouse mast cell numbers *in vivo.* However, during nematode infections in the gut, mast cells differentiate, are activated and hyperplasia occurs before numbers decline as the infection resolves. This resolution of the hyperplasia involves both apoptosis and recirculation back to the spleen for elimination. In this setting, it remains to be elucidated if Puma and/or Bim deficiency lead to an increased accumulation of mast cells and a delay in clearance.

A way of targeting the Bcl-2 family has been the development of drugs that mimic the binding of BH3-only proteins to the anti-apoptotic proteins inhibiting their function and thus promoting apoptosis. This has already proven useful in cancer therapy where the use of BH3 mimetics sensitizes tumour cells to apoptosis (86). In paper III we used ABT-737 a small molecular inhibitor that mimicks the binding BH3 domain of the pro-apoptotic protein Bad. It binds with high affinity to the anti-apoptotic proteins Bcl-XL, Bcl-2 and Bcl-w but not Mcl-1 or A1 (87). In this setting it was used with the purpose of elucidating the relative importance of anti-apoptotic Bcl-XL, Bcl-2 and Bcl-w compared to Mcl-1 or A1 during activation-induced survival. However, ABT-737 and other BH3 mimetics could possibly also be used as therapy to impair mast cells in inflammatory diseases such as asthma and allergy.

Although systemic abolishment of mast cells is often not desirable, targeting of mast cells within a specific tissue could have beneficial therapeutic effects. New strategies

targeting mast cell survival should decrease the severity of inflammation in mast cell-associated disorders. The anti-apoptotic Bcl-2 family member Mcl-1 has been suggested as a novel, interesting target in mastocytosis (190), a disease characterized by abnormal accumulation of mast cells. Thus, small molecular inhibitors of crucial Bcl-2 family members involved in mast cell survival and/or apoptosis offer an attractive strategy. As a first step, the effects of such compounds should be investigated *in vitro*. In particular, the toxicity of the compounds and the effects on cell viability should be addressed. Moreover, *in vivo* mouse models of mast cell-associated disease and human *ex vivo* material can be used assessing the effects further. Ideally, the compounds would reduce mast cell numbers without affecting other cell types substantially.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Mastceller är en viktig del av vårt immunförsvar. De är framför allt kända för att framkalla de symptom vi ser vid en allergisk reaktion. Idag vet man att mastceller även har stor betydelse vid regleringen av inflammationer samt i kroppens försvar mot olika bakterier, virus och parasiter.

Vid allergi och andra typer av inflammatoriska reaktioner brukar man se en ansamling av mastceller och dessa stimuleras till att frigöra ämnen som orsakar inflammation. När ett allergiframkallande ämne, t.ex. pollen, kommer in i kroppen på en allergisk person binder det till IgE-antikroppar på mastcellens yta. När detta sker aktiveras mastcellen (allergisk aktivering) vilket leder till att olika ämnen, t ex histamin, släpps ut av mastcellen (degranulering). Aktivering och degranulering av mastceller är orsaken till uppkomsten av allergiska symtom så som snuva, klåda och svullnad. Idag behandlas allergiker och astmatiker framför allt med läkemedel som lindrar symptomen. Ett alternativ kan vara att kontrollera antalet mastceller i vävnaden och därmed också inflammationens svårighetsgrad.

I kroppen finns två olika typer av mastceller; bindvävsmastceller, CTMC (connective tissue mast cell) och slemhinnemastceller, MMC (mucosal mast cell). Bindvävsmastceller återfinns bland annat kring blodkärl i de flesta vävnader och slemhinnemastceller i slemhinnor, lungor och tarmen. Mastcellsantalet på ett visst ställe i kroppen regleras bland annat av cellerna själva. Cellerna har en inneboende reglering som bestämmer om de ska fortsätta leva eller begå ”självmord” (programmerad celldöd, apoptos) som svar på stimuli. Stimuli kan vara tillgång respektive avsaknad av tillväxtfaktorer, men även aktiveringen av cellerna vid en allergisk reaktion kan fungera som stimuli. Cellerna svarar på stimuli genom att reglera vissa proteiner i cellen som fungerar som överlevnads- eller dödsproteiner. Beroende på balansen mellan dessa proteiner räddas antingen cellen från att dö eller genomgår programmerad celldöd. En familj av överlevnads- och dödsproteiner är Bcl-2 familjen. I studierna som beskrivs i denna avhandling har vi studerat vilka proteiner tillhörande Bcl-2 familjen som styr mastcellernas programmerade celldöd vid tillväxtfaktorsvält samt vid en allergisk reaktion.

I delarbete I och II har jag beskrivit hur det vid en allergisk aktivering av mastceller induceras ett överlevnadsprogram hos bindvävsmastceller från både mus och människa. I detta program uppregleras en gen som kallas A1 hos möss och Bfl-1 hos människa. A1/Bfl-1 tillhör Bcl-2 familjen och detta överlevnadsprotein är nödvändigt för att cellerna ska överleva. Trots att även slemhinnemastceller från mus aktiverades på samma sätt befrämjades varken överlevnad eller uppreglering av A1 i denna mastcellstyp. Dessa resultat tyder på att det är viktigt att karaktärisera vilken typ av mastceller man studerar och jag har utvecklat ett protokoll för att odla fram bindvävsmastceller respektive slemhinnemastceller från benmärgsceller i mus. Dessa mastcelltyper i mus påminner i mångt och mycket om motsvarande mastcelltyper hos

människa och kan användas för att studera skillnader respektive likheter mellan bindvävs- och slemhinnemastceller i kroppen.

För att mastcellerna ska kunna överleva ute i kroppen är de beroende av tillväxtfaktorer. I delarbete III och IV har jag identifierat två dödsproteiner som reglerar programmerad celldöd i mastceller vid tillväxtfaktorsvält. Detta har vi gjort genom att studera mastceller som odlats fram från möss som saknar ett eller flera dödsproteiner tillhörande Bcl-2 familjen. Resultaten visar att mastceller (både bindvävs- och slemhinnemastceller) som saknar genen för antingen dödsproteinet Bim eller Puma överlever tillväxtfaktorsvält bättre jämfört med normala mastceller. Detta visar att dessa proteiner är viktiga vid regleringen av programmerad celldöd i mastceller. Jag visar även att vid brist på tillväxtfaktorer i mastceller regleras Puma (mängden Puma som ska bildas) av transkriptionsfaktorn FOXO3a, något som även visats för Bim tidigare. För att programmerad celldöd ska kunna äga rum i mastceller vid tillväxtfaktorsvält måste ytterligare ett eller möjligen två dödsproteiner från Bcl-2 familjen aktiveras: Bax och/eller Bak. Vi visar att Bax är viktigare än Bak för programmerad celldöd vid tillväxtfaktorsvält i mastceller eftersom celler (både bindvävs- och slemhinnemastceller) som saknar genen för dödsproteinet Bax men inte Bak överlever bristen på tillväxtfaktorer bättre jämfört med normala mastceller.

Den här avhandlingen har bidragit till att ge ökade kunskaper om vilka proteiner tillhörande Bcl-2 familjen som styr mastcellernas programmerade celldöd vid avsaknad av tillväxtfaktorer samt vid en allergisk reaktion. Genom att blockera produktionen av A1/Bfl-1 vid en allergisk aktivering eller öka produktionen av Puma, Bim eller Bax skulle man på så sätt kunna oskadliggöra mastceller. Denna kunskap kan utnyttjas i framtida forskning och användas i försöken att hitta behandlingar mot skadliga inflammationer orsakade eller delvis orsakade av mastceller.

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