Studies into the Function and Regulation of SHIP

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In Memory of

Onkel “Angelbär” Jean-Pierre

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1 Introduction

1.1 Hemopoiesis

Hemopoiesis (also known as (aka) hematopoiesis) is the process in which all the cells circulating in the blood stream are generated. Interestingly, while there are many different types of blood cells, all with different life spans and different functions (see Table 1.1), they are all generated from a common multipotent hemopoietic stem cell (HSC), which resides within the bone marrow (BM) in adult humans. These HSCs not only give rise to all terminally differentiated blood cells but to other cells as well, such as osteoclasts, which reside in and breakdown bone (Alberts 2002) and microglia, which are macrophage-like cells within the central nervous system that respond to inflammatory stimuli (Barron 2003).

Blood cells can be classified as red or white. In addition, blood contains large numbers of platelets, which are detached cell fragments from the cortical cytoplasm of large cells called megakaryocytes. Red blood cells (rbcs, aka erythrocytes) transport O_2 and CO_2 bound to hemoglobin and remain in the blood vessels (Alberts 2002). White blood cells (wbcs, aka leucocytes) have many different functions, from combating infections to phagocytosing and digesting debris. Also, unlike rbcs, most wbcs can migrate through the walls of small blood vessels into tissues (Alberts 2002). Based on their appearance in the light microscope, wbcs are grouped into three main categories: granulocytes, monocytes and lymphocytes. Granulocytes contain numerous lysosomes and secretory vesicles (or granules). According to their morphology and staining properties, granulocytes can be further subdivided into neutrophils, basophils and eosinophils. Neutrophils are also called polymorphonuclear leucocytes because of their multilobed nucleus and are the most abundant granulocytes. Their main function is to phagocytose and destroy microorganisms. Basophils secrete histamine and, in some species, serotonin to help mediate inflammatory responses. Their function is similar to mast cells (Alberts 2002, Galli 2000). Eosinophils

help to destroy parasites and modulate allergic inflammatory responses. After they leave the blood stream, monocytes mature into macrophages or dendritic cells (DCs) within tissues. Together with neutrophils, macrophages are the main phagocytes in the body. Both macrophages and neutrophils contain specialized lysosomes that fuse with newly formed phagocytic vesicles (phagosomes), exposing phagocytosed microorganisms to highly reactive molecules such as superoxide (O_2^-) and hypochlorite (HOCl) as well as to a concentrated mixture of lysosomal hydrolases (Alberts 2002).

Cell Type	Function	Life Span	Concentration (per μl)
Red Blood Cells (erythrocytes)	Transport O_2 and CO_2	120 days	5,000,000
White Blood Cells (leucocytes)			
Granulocytes			
Neutrophils (polymorphonuclear cells)	Phagocytize and destroy invading microorganisms	3 days	~ 5,000
Eosinophils	Destroy larger parasites and modulate allergic inflammatory responses	~ 12 hours	100-200
Basophils	Release histamine to start inflammatory responses	3-30 days	~ 30
Monocytes/Macrophages	Phagocytose and present Ags on the cell surface	~ 3 days	500
Lymphocytes			
B Cells	Mature to plasma cells and produce Abs	10 days (some long lived)	~ 100
T Cells	Cell mediated toxicity and regulation of other leucocytes	3 hours – 5 days	~ 2000
Natural Killer (NK) Cells	Kill virus infected cells and some tumour cells	7-14 days	100
Platelets	Initiate blood clotting	~ 10 days	300,000

Table 1.1 Different cell types present in blood. Adult humans contain about 5 liters of blood. Rbcs constitute about 45% of this volume and wbcs about 1%. The rest is comprised of the aqueous milieu, the plasma. *Adapted from* (Alberts 2002) *and* (Fernandez and Butcher 1997).

Macrophages are much larger and longer-lived than neutrophils. They recognize and remove senescent, dead and damaged cells in many tissues and they are also able to ingest large microorganisms such as protozoa. As mentioned above, monocytes also give rise to DCs, which are specialized in their ability to present foreign antigen (Ag) to T lymphocytes. Langerhans cells, for example, are DCs cells scattered in the epidermis

where they ingest foreign Ags and carry them to the lymph nodes. There are two main classes of lymphocytes. B lymphocytes make antibodies (Abs) while T lymphocytes kill virus-infected cells and regulate the activities of other wbcs. In addition, there are lymphocyte-like cells called natural killer cells which kill certain kinds of tumour cells and virus-infected cells. Often the hemopoietic cell types other than lymphocytes are referred to as myeloid cells (Alberts 2002).

All mature blood cells originate from HSCs within the BM. Only about 1 in 10,000 nucleated BM cells is a HSC. Through a series of cell divisions these HSCs lose their multipotent state and become committed to one particular differentiation pathway (Alberts 2002). Because of the stepwise nature of commitment, the hemopoietic system can be viewed as a hierarchical family tree (Fig 1.1). The first step of commitment is either to a myeloid or a lymphoid cell fate and this is followed by the stepwise amplification of progenitor cells that are committed to specific pathways of differentiation. The large number of specialized mature blood cells is generated by multiple divisions of committed progenitor cells. This extensive expansion and differentiation of committed progenitor cells allows a large proportion of HSCs to remain quiescent (out of the cell cycle) under normal, steady state conditions and thus reduces the rate of replicative senescence and damaging mutations within the HSC compartment. Hemopoiesis is regulated by many different growth factors and cytokines (see Fig 1.1) (Alberts 2002, Janeway 2001). Many of these are glycoproteins and the presence of sugar moieties on these proteins increases their stability and solubility in the aqueous environment of the interstitial fluid and plasma. Some of these growth factors and cytokines circulate in the blood and act as hormones while others act locally and are either secreted into the interstitial fluid as free or extracellular matrix bound proteins or are synthesized with hydrophobic domains that keep them bound to cell surface membranes and they act via cell-cell contact. While HSCs are strongly dependent on cell-cell contact with stromal cells for long term maintenance, their committed progenitors are less so. Some growth factors and cytokines, such as erythropoietin (EPO), which is required for rbc formation (erythropoiesis), act only on one specific pathway while others like interleukin-3 (IL-3) or stem cell factor (SCF, aka Steel Factor, c-kit ligand or mast cell growth factor) act on more than one. Typically, growth

factors and cytokines bind to specific cell surface receptors which are usually present at very low levels (Alberts 2002, Broudy 1997). By binding to these receptors, different transcription factors (TFs) become activated. Activation of TFs can lead to both activation of gene expression patterns necessary for the differentiation down a certain hemopoietic pathway and the active suppression of gene expression patterns necessary for alternate hemopoietic pathways. The combination of both effects leads to the development of one specific mature cell type (Alberts 2002). A tight regulation of hemopoiesis by these growth factors and cytokines, as well as by negative regulators of hemopoiesis, is essential to ensure proper maintenance of mature blood cell numbers under steady state conditions and also to allow for a rapid expansion of mature blood cells under crisis conditions such as during extensive bleeding, infections or low oxygen levels in the environment. Failure to maintain homeostasis under steady state conditions can lead to proliferative disorders, such as polycythemia or proliferative exhaustion of HSCs as in aplastic anemia.

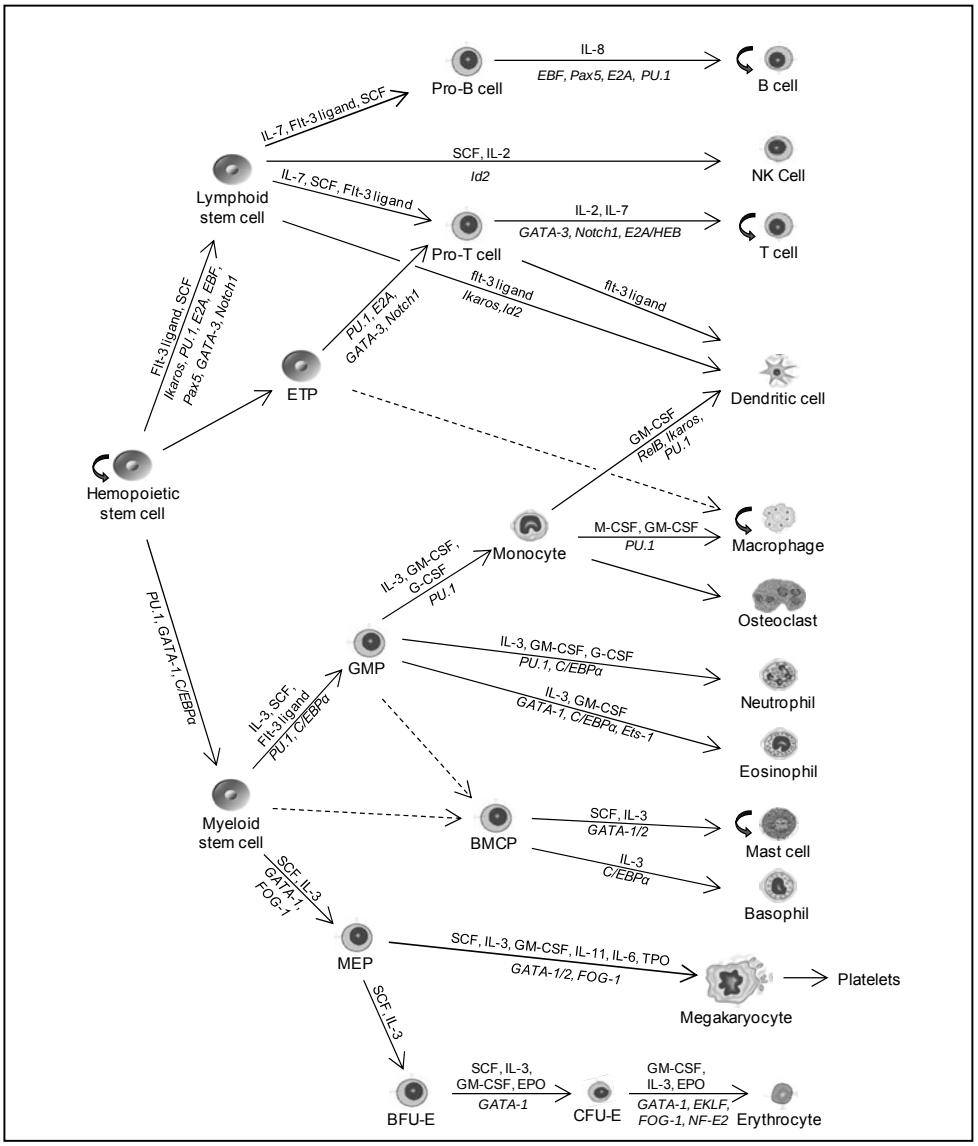


Figure 1.1 Current scheme of hemopoiesis, the growth factors and cytokines that regulate it and the TFs involved in lineage commitment. The HSC divides infrequently to generate more stem cells (self-renewal; as indicated by ↻) or progenitor cells that can generate a more restricted set of cells. Progenitors are stimulated to proliferate and differentiate by one or a combination of different growth factors (indicated above the arrow) and slowly lose their potential to divide the more mature they become. A mature cell, once formed, only has a limited life-span and usually lacks the ability to proliferate. Among mature cells, only B cells, T cells, macrophages and mast cells have been shown to possess proliferative potential (as indicated by ↻). Dotted arrows represent uncertain pathways. TFs involved in the differentiation of specific pathways are indicated in italics below the arrows. GM-CSF = granulocyte monocyte colony stimulating factor; M-CSF = monocyte colony stimulating factor; G-CSF = granulocyte colony stimulating factor; TPO = thrombopoietin; EPO = erythropoietin; CFU = colony forming unit; BFU = burst forming unit; ETP = early T lineage progenitor; GMP = granulocyte macrophage progenitor; MEP = megakaryocyte erythrocyte progenitor; BMCP; basophil mast cell progenitor. *Adapted from* (Alberts 2002, Zhu and Emerson 2002) *and* (Laiosa et al. 2006).

1.2 Mast Cells

Mast cells (MCs) were first described in 1879 by Paul Ehrlich who called them "Mastzellen" or "well fed cells". He demonstrated that these cells contained cytoplasmic basophilic granules and were present in the connective tissue of humans (Welle 1997). The important role of MCs in IgE-mediated allergic reactions was previously thought to be their main function. However, in recent years a great deal of evidence has accumulated that suggests the involvement of MCs in a number of other pathological and physiological processes including host defense against bacteria and parasites, angiogenesis and hemopoiesis (Galli 2000, Welle 1997, Okayama and Kawakami 2006).

Despite our growing understanding of hemopoiesis and its regulation by various growth factors and cytokines, MC development still remains poorly understood. However, there are some widely accepted aspects and some interesting discoveries that have been made in recent years.

MCs belong to the myeloid lineage and they are found in large numbers where the body is in contact with its environment, e.g. the skin, the respiratory system, the digestive tract and around blood vessels. This strategic distribution suggests an early role for MCs in the immune response (Welle 1997). Like all other hemopoietic cells, MCs are derived from HSCs in the BM. It is widely accepted that MCs leave the BM as morphologically unidentifiable MC progenitors and circulate in the blood and lymphatics. From there they migrate into connective and mucosal tissues where they complete their maturation into mature, morphologically identifiable MCs under the control of the surrounding microenvironment (Gurish and Boyce 2006). During inflammation there is a significant increase in MC numbers at the site of inflammation. This is thought to be achieved by a combination of enhanced recruitment of both mature MCs and MC progenitors and their subsequent proliferation at the site of inflammation (Gurish and Boyce 2006). Basophils, which are thought to play a similar function to MCs in early inflammation, have not been shown to have any proliferative potential. In fact only macrophages, T cells, B cells and MCs are known to be able to proliferate after maturation. Also, unlike MCs, basophils

typically complete their maturation in the BM before they enter the blood stream. Therefore an increase in basophil numbers at sites of inflammation is solely due to their recruitment from the blood stream (Galli 2000, Welle 1997, Gurish and Boyce 2006). The developmental relationship between MCs and basophils is poorly understood and there is an ongoing debate about whether they share a common progenitor or not. Recent findings suggest that in mice, MCs and basophils indeed share a common progenitor and that the TF C/EBPα plays a crucial role in determining whether this progenitor becomes a basophil or a MC (Gurish and Boyce 2006, Arinobu et al. 2005). There also seems to be a splenic phase in MC, but not in basophil development (Gurish and Boyce 2006).

One well known growth factor that is required for the survival, proliferation, differentiation and enhanced function of MCs is SCF. This growth factor exists in both a soluble and a membrane bound form and acts by binding to its tyrosine kinase containing cell surface receptor, c-kit (Galli 2000, Broudy 1997). The importance of proper c-kit signaling is underlined by the observation that deregulation of or mutations in either SCF or c-kit lead to MC disorders. For example, gain of function mutations occur in most, if not all, adult patients with mastocytosis (Galli 2000, Akin 2006). On the other hand severe MC deficiencies (less than 1% of normal levels) are found in mice carrying loss of function mutations in either SCF or c-kit. W/W^v mice have mutations in both c-kit alleles and SI/SI^d mice have mutations in both SCF alleles (Kitamura et al. 1978, Kitamura and Go 1979). The number of MCs in $S1/S1^d$ mice dramatically increases after treatment with SCF (Broudy 1997). Studies carried out with these mice also suggest that SCF is not important for basophil development or function. The cytokine IL-3, on the other hand, has been shown to increase basophil numbers in both mice and humans. However, IL-3 does not appear to be essential for the production of normal "baseline" levels of tissue MCs but plays an important role in increasing MC numbers, as well as basophils, upon parasitic infection since $IL\text{-}3^{-/-}$ mice lack these responses. *In vitro,* MC progenitors proliferate and mature in the presence of SCF plus IL-3 and it is thought that optimal growth and differentiation of MCs *in vivo* requires IL-3, IL-4 or IL-10 (Galli 2000, Broudy 1997).

Mature MCs are typically identified by the presence of the high affinity IgE receptor (FcεR1) and c-kit on their cell surface. In mice, FcεR1 is only expressed on the surface of

MCs and basophils. In humans, FcεR1 is also expressed on the surface of monocytes, eosinophils, platelets, and on DCs, although at significantly lower levels than on MCs and basophils. C-kit, on the other hand, is highly restricted to MCs in both mice and humans and is not expressed on basophils or other mature cells (Kinet 1999, Lantz and Huff 1995).

1.2.1 Mucosal MCs (MMCs) and Connective Tissue MCs (CTMCs)

As mentioned above, MCs complete their maturation in the tissue they reside in under the influence of the surrounding microenvironment. As a result, while they are all FcεR1 and c-kit positive, they are very heterogeneous and display differences in histochemical, biochemical and functional properties, depending on where they are situated. In mice, MCs can be classified as either connective tissue MCs (CTMCs) or mucosal MCs (MMCs), with the latter often being referred to as atypic or T-cell dependent MCs due to their absence in athymic nude mice (Welle 1997, Gurish and Boyce 2006). This division into CTMCs and MMCs is based on differences in staining properties. While CTMCs stain positive for both alcian blue and safranin, MMCs are not stained by safranin. These different dye staining properties are due to differences in the granule composition of CTMCs and MMCs. While CTMC granules contain the proteoglycan heparin, which is stained by safranin, MMC granules contain chondroitin sulfate E, which is not stained by safranin (Welle 1997, Beil et al. 1997). In addition to differences in proteoglycan content, CTMC and MMC granules also differ in their composition of both biogenic amines and proteases (summarized in Table 1.2). For example, CTMC granules contain serotonin while MMC granules do not and CTMC granules contain substantially higher levels of histamine. Also, stored in the granules are large amounts of mouse MC proteases (MMCPs) which differ in composition between CTMCs and MMCs and can comprise over 50% of the total protein content of mature MCs. They are divided into two groups; chymases (ie., MMCP-1, -2, -3, -4 and -5) and tryptases (MMCP-6 and -7) and they are serine proteases that are enzymatically active at neutral pH. In the granules they are stored in their active form and bound to proteoglycans, which prevent them from rapidly catabolizing themselves. In addition to

different MMCPs, CTMCs also contain MC carboxypeptidase (CPA) (Welle 1997). In humans, MCs cannot be classified based on their alcian blue and safranin staining properties since all human MCs contain heparin in their granules (Welle 1997, Beil et al. 1997). However, classification based on their expression of MC specific proteases has been proven to be useful and today human MCs are classified as MC_T (which contain the protease tryptase) and MC_{TC} (which contain both tryptase and chymase). MC_{TC} also contain cathepsin G protease and CPA. The distinct granular content of these two MC subtypes at least partially explains, at a molecular level, the heterogeneous nature of MCs (Welle 1997).

Mouse	**MMCs**	**CTMCs**
Biogenic amines	(low) histamine	(high) histamine & serotinin
Proteoglycans	chondroitin sulfate E	heparin
Proteases	MMCP-1, -2	MMCP-3, -4, -5, -6 & -7, MC-CPA
Human	**MC_T**	**MC_{TC}**
Biogenic amines	histamine	histamine
Proteoglycans	heparin and chondroitin sulfates A/E	heparin and chondroitin sulfates A/E
Proteases	tryptase	Tryptase, chymase, cathepsin G protease & CPA

Table 1.2 Granule content of mouse and human MCs
Adapted from (Welle 1997, Beil et al. 1997).

Today it seems clear that dividing MCs into two subgroups is an oversimplification although a very useful one since these two subtypes are found in distinct sites of the body. MMCs are found in the mucosa of the gastrointestinal tract and the lamina of the respiratory tract. CTMCs, on the other hand, are found in the skin, the peritoneum and the submucosal tissues of the gastrointestinal tract and the lung. Based on their distribution within the body and the expression of MC specific proteases in their granules, MMCs are thought to be equivalent to human MC_Ts and CTMCs to MC_{TC}s (Welle 1997, Gurish and Boyce 2006).

1.2.2 MC Functions in Normal and Disease States

Although it has long been recognized that MCs play an important role in humans, in terms of clearing parasitic worms, they have been considered a nuisance in first world countries because of their release of mediators that cause allergies, asthma and other inflammatory disorders. However, recent data suggesting an involvement of MCs in both innate and adaptive immune responses to bacteria and parasites, as well as in peripheral tolerance, have rehabilitated their image (Metz and Maurer 2007, Christy and Brown 2007).

1.2.2.1 Type I Hypersensitivity Reactions

The role of MCs in the generation of atopic or allergic diseases such as hay fever is well established. They are also involved in more serious conditions such as asthma and anaphylaxis. All these phenomena result from type I hypersensitivity reactions mediated by IgE (Corry and Kheradmand 1999). Ags that trigger the production of IgE are called allergens. Upon an initial exposure to allergens, which typically are present at very low concentrations, Ag presenting cells (APCs) such as DCs express an Ag derived peptide:MHC class II complex on their surface which stimulates allergen-specific $CD4^+$ T_H2 cells through cell-cell interactions to release IL-4 and IL-13. These cytokines stimulate B cells to produce and release Ag-specific IgE molecules. Unlike other Ab isotypes, IgE is bound by FcεR1 on the surface of MCs and basophils. A second exposure to the same Ag, which typically is multivalent, causes the crosslinking of receptor-bound IgE and the subsequent release of mediators like histamine, proteases, leukotrienes, prostaglandins, cytokines and chemokines which lead to symptoms associated with type I hypersensitivity reactions (Fig 1.2) (Janeway 2001, Corry and Kheradmand 1999). Not all individuals produce IgEs upon allergen exposure and it is still not fully understood why some develop type I hypersensitivity reactions and others do not. However, among affected individuals there is evidence that the mode of Ag presentation in the host as well as the biochemical properties of the Ag itself play important roles in triggering type I

hypersensitivity reactions. For example, presenting an Ag transmucosally and at very low doses seems to stimulate IgE production very efficiently while such an effect is usually not seen after intravenous (IV) or oral administration. Also, allergenic Ags are usually relatively small and highly soluble proteins that are carried on desiccated particles such as pollen grains and mite feces. Interestingly, many allergens are known to have enzymatic functions although there seems to be no systematic association between enzymatic activity and allergenicity (Janeway 2001, Corry and Kheradmand 1999).

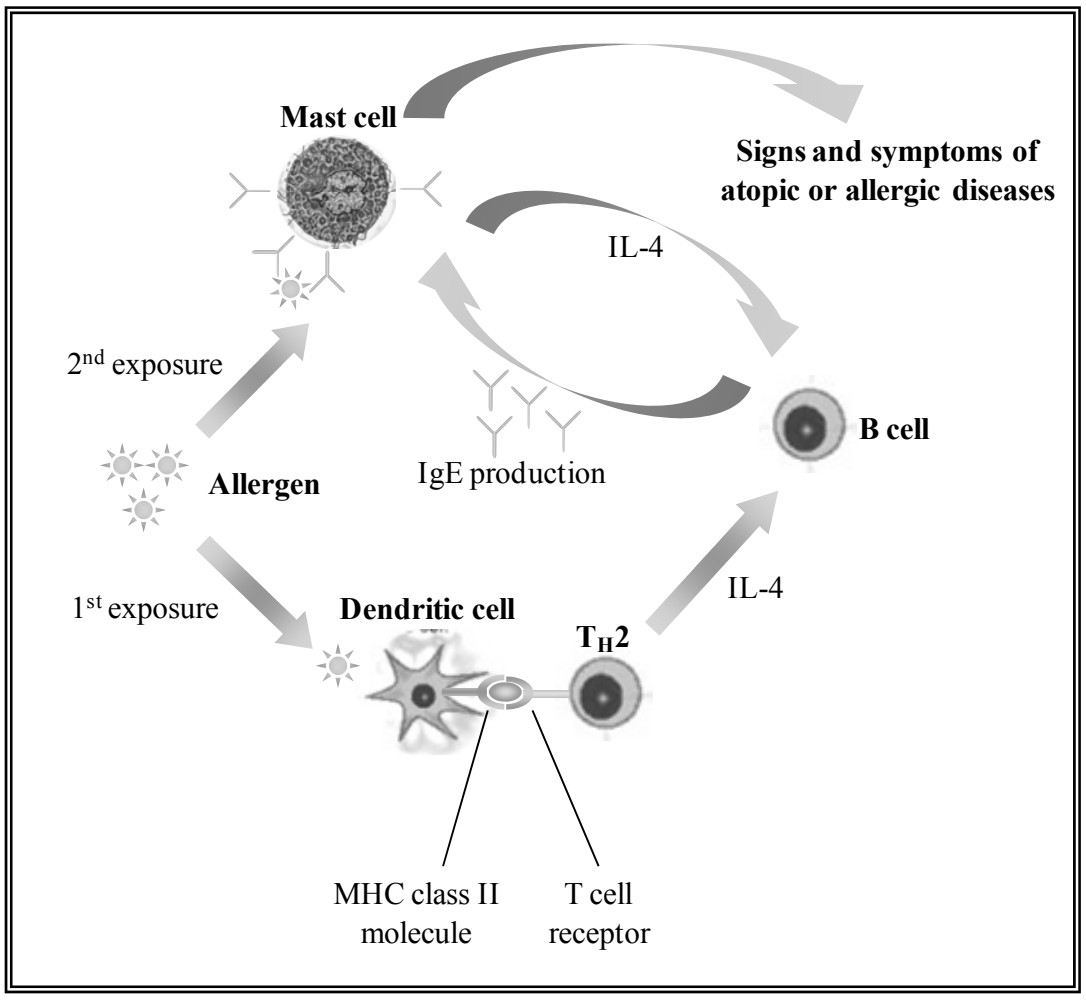


Figure 1.2 IgE-dependent MC activation. Foreign allergens or Ags are ingested and processed by APCs such as DCs. The processed Ag then is presented in the form of a peptide:MHC class II on the surface of the APC and enables the APC to activate T_H2 cells via cell-cell contact. Activated T_H2 cells produce IL-4 which stimulates B cells to produce IgE molecules which bind to the high affinity receptor FcεRI expressed on the surface of MCs. Subsequent binding of Ag induces crosslinking of receptor bound IgE molecules and activates the MC to release a wide array of inflammatory products that mediate the allergic response. *Adapted from* (Kay 2001).

There are four types of hypersensitivity reactions, types I-IV. Besides type I, types II and III are Ab mediated as well, although by IgG Abs instead of IgEs. Type II hypersensitivity reactions are initiated by IgGs raised against cell surface or matrix Ags and are responsible for drug allergies (e.g. penicillin). Type III hypersensitivity reactions involve IgGs raised against soluble Ags. Small Ag:IgG complexes can bind to Fcγ receptors expressed on MCs and other leucocytes and thus trigger inflammatory responses as seen in serum sickness. Type IV hypersensitivity reactions are T cell-mediated and contribute to conditions like chronic dermatitis and chronic asthma. They can be subdivided into three groups. In the first group an immune response is launched by T_H1 cells through the activation of macrophages while in the second group it is launched by T_H2 cell activation of eosinophils. In the third group, cytotoxic T cells (CTLs) get directly activated. Type IV hypersensitivity reactions are often referred to as delayed-type hypersensitivity reactions while the Ab mediated types I-III are called immediate-type hypersensitivity reactions (Janeway 2001).

1.2.2.2 "Alternative" MC Activation

While the "allergic activation" of MCs through IgE bound to FcεRI is the best studied mechanism of MC activation, it is now clear that there are many more signals that can lead to MC activation. Some MCs can be activated by physical stimuli or by binding IgG molecules (through their Fcγ receptors) or various cytokines, chemokines, chemical agents and endogenous and exogenous peptides. Many of the agents that activate MCs are also linked to innate immune responses (Metz and Maurer 2007). The awareness that MCs can be stimulated in many different ways and their strategic localization at places where the organism is in contact with the environment have raised an intense interest in how MCs are activated and their role in different physiological processes and pathological conditions. As a result there is now a long and still growing list of how MCs can be activated (summarized in Table 1.3) (Metz and Maurer 2007, Metz et al. 2007).

Nature of activator	Signal Recognition/Transduction
By immune receptors	
IgE	By FcεRI; IgE can also bind to IgG receptors (FcγRII and FcγRIII) and to galectin-3, which are expressed on some MC populations
IgG_1	Mouse: FcγRIII; humans: FcγRI after treatment with IFN-γ
Ig-binding superantigens	Endogenous (e.g. protein Fv in Hepatitis B and C virus infections), bacterial (e.g. *Staphylococcus aureus* Protein A), or viral (e.g. HIV gp120)
Products of complement activation	
C3a, C5a, C3b, and C4b	By their respective receptors: C3aR, C5aR, CR3 and CR4
Ligands of Toll-like receptors (TLRs)	
Peptidoglycan (PGN)	TLR2
Double-stranded (ds) viral RNA, poly I:C	TLR3
LPS	TLR4/CD14
Flagellin	TLR5 (human MCs)
Single-stranded (ss) viral RNA	TLR7
CpG-DNA	TLR9
Bacteria, parasites and their products	
E. coli FimH	CD48
Pseudomonas aeruginosa	Human MCs
Virulence factors/toxins	e.g. *Clostridium difficile* toxin A, cholera toxin
Schistosoma mansoni	Activation by cercariae
Leishmania major	Activation by living promastigotes
Viruses	
Respiratory syncytial virus, influenza virus, Dengue virus, Sendai virus	By TLR stimulation and possibly by other mechanisms
Endogenous peptides	
Nerve growth factor, substance P, CGRP, and other neuropeptides	By respective G protein-coupled receptors on MCs
ET-1	By $ET_A > ET_B$
Neurotensin	
Anti-microbial peptides	e.g. β-defensin 2 and LL-37
Venoms or venom components	
Sarafotoxin 6b	Component of *A. engaddensis* venom, by ET_A
Phospholipase A_2	Component of many different animal venoms

Table 1.3 An (incomplete) overview of different MC activators.
MCs can be activated by a variety of agents. Besides Ag-induced crosslinking of IgE bound to FcεRI, other agents include pathogen-derived molecules from bacteria, parasites or viruses, a number of venoms and endogenously derived molecules such as complement or ET-1. Unlike FcεRI crosslinking, stimulation through TLRs does not typically induce MC degranulation. *Adapted and modified from* (Metz and Maurer 2007, Metz et al. 2007).

Introduction: Mast Cells

MC activation is an early and crucial step in fighting bacterial infections. MC-deficient mice have been shown to be much more susceptible to septic peritonitis and other bacterial infections than their MC-reconstituted counterparts or wild type mice (Metz and Maurer 2007, Echtenacher et al. 1996). MC activation can result from both pathogenic components and host-derived signals and leads to degranulation and/or the synthesis and secretion of different chemokines and cytokines that help orchestrate an appropriate immune response.

Signals derived from or associated with pathogens are often recognized by Toll like receptors (TLRs). Activation of TLRs usually does not induce degranulation but the synthesis and secretion of different chemokines and cytokines that help orchestrate the accumulation and activation of different immune cells and thus contribute to optimal host defense. For example, MC-derived TNFα has been shown to be important for neutrophil recruitment (Malaviya et al. 1996).

In various other infection models, MCs (or MC-derived factors) have also been shown to be crucial for optimal T cell priming and for DC and T cell recruitment to sites of infection. However, the exact mechanisms involved are still not fully elucidated. Interestingly, some MC mediators such as histamine, TGFβ, IL-4, IL-10, and possibly others, can have anti-inflammatory properties and can lead to suppression of immune responses (Depinay et al. 2006). Thus, the appropriate activation of MCs by pathogen- and host-derived signals is important for optimal host defense.

Host-derived signals include activated complement products and endogenous peptides.

Patient levels of the endogenous vasoconstrictive peptide ET-1 correlate with severity of sepsis and thus ET-1 is thought to play a crucial role in this systemic infection (Metz and Maurer 2007). Binding of ET-1 to the endothelin-A receptor (ET_A), which is expressed on the MC surface, has been shown to induce MC degranulation and the synthesis and release of various MC mediators (Metz and Maurer 2007, Yamamura et al. 1994a, Yamamura et al. 1994b).

1.2.3 MC Mediators

After undergoing activation, MCs can release a diverse set of potent biologically active products, often called mediators. These mediators can be divided into two major categories:

(i) preformed mediators that are stored in the cells' prominent cytoplasmic granules; these include histamine (and in rodents, serotonin), certain cytokines (eg, TNFα), proteoglycans, and proteases (i.e., chymase, tryptase, and carboxypeptidase A (CPA)). These preformed mediators are usually released immediately after activation (i.e., within 1-10 min) by degranulation; and (ii) mediators that are newly synthesized following activation (eg, numerous cytokines, chemokines, lipid mediators and growth and angiogenic factors), which can mediate various pro-inflammatory, anti-inflammatory and/or immuno-regulatory effects.

It is important to note that dividing the mediators into two major categories does not reflect the complexity of their release. The decision to release only one or both types of mediators and which specific mediators are released depends on a variety of factors such as the kind, intensity, kinetics and other properties of the stimulus (Metz and Maurer 2007).

As far as granule-associated proteases are concerned, the release of CPA is important in degrading ET-1 and thereby helps to protect the organism from the toxic effects of this peptide (Metz and Maurer 2007, Maurer et al. 2004). CPA is also able to degrade some venom components such as sarafotoxin 6b of *Atractaspis engaddensis* (Israeli mole viper) or *Apis mellifera* (honeybee). Interestingly, these venoms share a high homology with ET-1 and therefore are often called endothelin (ET)-1 like venoms. Besides CPA, other proteases released from MC granules have also been shown to have specific functions in eliciting immune responses to different pathogens.

The mouse tryptase mMCP-6 is able to induce a long lasting inflammation and associated neutrophil accumulation when injected into the peritoneum of mice (Huang et al. 1998) and human tryptase β1 is important for neutrophil attraction and protects from *Klebsiella pneumoniae* infections when injected into the lungs of mice (Huang et al. 2001). Transmembrane tryptase (TMT) is implicated in increased production of the T_H2 cytokine

IL-13 by T-cells and the mediation of airway hyper-responsiveness in mice. TMTs are stored in the granules of MCs and reach the surface of the plasma membrane upon the induction of degranulation (Wong et al. 2002).
Other MC tryptases can activate the protease-activated receptor (PAR)2 which is known to be involved in inflammation of airways, joints and kidneys.
The mouse chymase mMCP4, the most likely homolog of human chymase, can activate matrix metalloprotease (MMP)-2 and MMP-9, which suggests an important role of MCs in tissue remodeling.
Parasitic infections often lead to strong IgE responses and the release of the chymase mouse MC protease (mMCP)-1. Interestingly, this chymase contributes to expulsion of certain parasites by degradation of the intestinal tight junction protein, occludin, in the epithelial barrier.
As mentioned above, this list of MC-derived mediators and their functions is still growing and thus far from complete.

1.2.4 MCs in Autoimmune Diseases

In order to properly and effectively protect an organism the immune system must be able to make 2 distinct decisions:
(i) distinguish between "self" (one's molecules) and "non-self" (pathogens);
and (ii) determine what constitutes a danger (Christy and Brown 2007).
Failure to make these decisions properly can lead to allergies (which can be seen as an overreaction to "non-self" molecules that don't constitute a threat to the organism), autoimmune diseases (when the immune system reacts against its own body) and cancer (if the immune system fails to launch a response against transformed cells and thereby allows or sometimes even supports tumour growth).
Besides their well established role in allergies it seems that MCs also contribute to certain autoimmune diseases (Metz and Maurer 2007, Christy and Brown 2007). MC-deficient mice develop neither rheumatoid arthritis (RA), a destructive inflammatory disease of the

joints nor do they develop bullous pemphigoid (BP), a disease characterized by blisters below the skin, in respective animal disease models (Nigrovic et al. 2007, Nelson et al. 2006). Similar observations have been made in experimental allergic/autoimmune encephalomyelitis (EAE), the animal model for multiple sclerosis (MS), and the classic EAE-susceptible strain SJL/J has been found to have at least 4-fold higher numbers of MCs than the less susceptible C3H strain (Secor et al. 2000, Johnson et al. 1991). Interestingly, not only are MC-deficient mice more resistant to the Ab-mediated organ specific autoimmune diseases RA, BP and EAE, they are more susceptible to the systemic Ab-mediated autoimmune disease, lupus nephritis (Lin et al. 2004). These findings not only demonstrate the devastating effects of improper MC activation, but also underline the importance of properly regulated MC activation in tissue tolerance (Christy and Brown 2007, Maurer et al. 2004).

1.3 Toll Like Receptors

Over the last decade TLRs have emerged as key receptors in the regulation of the immune system. The involvement of this family was first demonstrated in Drosophila where Toll was found to not only be critical for patterning dorso-ventral polarity in the embryo but also for immune defense of the adult fly against microbial pathogens (Lemaitre et al. 1996). A human homologue, which is able to activate the expression of inflammatory cytokines, was subsequently cloned and a point mutation in the homologous gene *Tlr4* of an LPS-unresponsive but gram negative (Gm^-) bacteria susceptible mouse strain was identified shortly after. Today, this receptor is known as TLR4. Interestingly, the pathways leading to the expression of inflammatory cytokines were found to be similar in flies and mammals. In mammals, gene expression is activated by the NF-κB pathway and in Drosophila by Dorsal and Cactus, the homologues to NF-κB and I-κB proteins, respectively (Medzhitov et al. 1997, Poltorak et al. 1998). However, while in Drosophila only one Toll protein seems to be involved in host defense against pathogens, there are at

least 10 different TLRs in humans. The discovery of TLRs and their ability to bind different conserved structures of pathogens, called pathogen associated molecular patterns (PAMPs), fundamentally changed our understanding of the immune system and today it is clear that innate immune responses are not as non-specific as once thought but rather are finely tuned to appropriately counter invading microorganisms. Furthermore, the innate and acquired arms of the immune system are now known to strongly interact with each other and TLRs play a central role in both regulating innate immune responses and in communicating between the innate and acquired immune systems (Netea et al. 2004, Takeda and Akira 2005).

As their name suggests, TLRs were originally identified based on their similarity to the Drosophila receptor Toll. After identification of the first TLR, i.e., TLR4, other TLRs were identified based on structural similarities (Rock et al. 1998, Takeda et al. 2003).
TLRs are expressed on the cell surface or in intracellular compartments such as endosomes. Specifically, TLR1, TLR2, TLR6 and TLR4 are expressed at the cell surface. TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular compartments such as endosomes and of these TLR3, TLR7 and TLR9 are known to require endosomal maturation for recognition of their ligands. TLR9 is recruited to endosomes from the endoplasmic reticulum upon non-specific uptake of CpG DNA (Takeda and Akira 2005, Heil et al. 2003, Matsumoto et al. 2003, Ahmad-Nejad et al. 2002, Latz et al. 2004). Also, TLR2 has been reported to be recruited to the phagosomal compartment of macrophages after exposure to zymosan (Underhill et al. 1999). Therefore it is thought that phagosomal/lysosomal or endosomal/lysosomal compartments might actually be the main sites for TLR recognition of microbial components.

All TLRs contain a variable number of extracellular N-terminal leucine-rich repeats (LRRs), which are involved in the recognition of pathogens, a cysteine-rich domain, a transmembrane domain and a C-terminal domain that displays substantial homology with the intracellular domain of the interleukin-1 receptor (IL-1R) family and is thus called the Toll/IL-1 receptor (TIR) domain (Takeda and Akira 2005) However, the extracellular

region of IL-1R possesses an immunoglobulin-like domain instead of LRRs and is therefore structurally distinct from TLRs.
As mentioned above, the TLR family consists of at least 10 members in humans and 13 in mice. TLRs 1 through 9 are conserved between human and mouse. TLR10 is functional in humans but not in mice due to disruption by a retroviral insertion (Hasan et al. 2005). On the other hand, TLR11 is functional in mice but not in humans due to a stop codon in the latter, which results in no human TLR11 protein (Zhang et al. 2004).

TLRs play a crucial role in detecting pathogens and launching an appropriate response designed to fight the specific pathogen recognized. In order to do so it is essential that TLRs are able to detect and discriminate amongst a variety of possible invaders. Indeed, TLRs are able to recognize and respond not only to a huge arsenal of different pathogens and their PAMPs but also to endogenous molecules that often are produced and/or released during an infection (Netea et al. 2004, Takeda and Akira 2005).
The ability of these TLRs to recognize such a wide variety of molecules is achieved by a combination of different mechanisms. First, different TLRs have different specificities for certain PAMPs. Second, upon interaction with a PAMP, some TLRs can either homodimerize or heterodimerize with a different TLR. Third, some TLRs cooperate with non-TLRs, which can be receptors or soluble factors, in order to identify and respond to pathogens.
It is thought that the combination of these mechanisms leads to the recruitment of different adaptor molecules and thus enables TLRs to induce an immune response best suited to specific invading pathogens (Netea et al. 2004, Takeda and Akira 2005).

1.3.1 TLR Ligands

Although not all the ligands for the TLRs are currently known much has been learned. For example, TLR1 heterodimerizes with TLR2 to recognize triacyl lipopeptides (see below). TLR2 has been shown to recognize a wide spectrum of microbial components, including

peptidoglycan (PGN) and lipoteichoic acid from gram positive (Gm^+) bacteria, lipoproteins/lipopeptides from various pathogens, zymosan from fungi and other compounds from various pathogens. One of the mechanisms that enables TLR2 to react to such a wide variety of ligands without reacting to harmless "self-molecules" is via cooperation with other TLRs. For example, TLR2 heterodimerizes with TLR1 to recognize triacyl lipopeptides from Gm^- bacteria and with TLR6 to recognize diacyl lipopeptides derived from mycoplasma (Takeuchi et al. 2001, Takeuchi et al. 2002). Besides the interaction with other TLRs, there is also functional cooperation between TLR2 and structurally unrelated molecules. For recognition of the fungal cell wall component, β-glucan, for example, TLR2 has been shown to cooperate with dectin-1, a lectin family receptor (Gantner et al. 2003).

TLR3 binds and becomes activated in response to dsRNA, which is produced by most viruses during their replication (Alexopoulou et al. 2001).

TLR4 is best known for its role in the recognition of lipopolysaccharide (LPS), a major component of the cell wall of Gm^- bacteria. TLR4 cooperates with CD14 and the MD2 molecule in order to bind LPS (Poltorak et al. 1998, Hoshino et al. 1999). Recognition of taxol, a diterpene from the bark of the western yew (*Taxus brevifolia*) is also mediated by TLR4 as is the recognition of some endogenous molecules such as heat shock proteins (HSP60 and HSP70), high-mobility-group box 1 (HMGB1), the extradomain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen. However, although these endogenous molecules have been shown to activate TLR4, they require high concentrations to do so (Takeda and Akira 2005, Apetoh et al. 2007, Apetoh et al. 2008).

TLR5 recognizes flagellin, a monomeric constitutent of bacterial flagella (Hayashi et al. 2001).

As mentioned above, TLR 6 heterodomerizes with TLR2 to recognize diacyl lipopeptides.

TLR7 and human TLR8 are able to recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) from certain viruses (Heil et al. 2004).

TLR9 is a receptor for unmethylated CpG DNA from bacteria and viruses. In vertebrates the frequency of CpG motifs is greatly reduced and the cytosine residues are highly methylated (Hemmi et al. 2000).

Although the ligands for TLR10 and TLR11 have not as yet been identified TLR10 has been shown to be able to heterodimerize with TLR1 or TLR2 in humans but not in mice due to a lack of functional murine TLR10 protein (Hasan et al. 2005)..

1.3.2 TLR Ligand-Induced Signaling

Binding of microbial PAMPs by TLRs facilitates TLR dimerization and subsequent activation of signaling pathways through TIR domains. Adaptor molecules containing TIR domains interact with the TIR domain of TLRs and recruit other signaling molecules, thereby activating TLR pathways. The first adaptor molecule shown to be essential for induction of inflammatory cytokines through TLRs was MyD88. Today there are two major pathways known to be activated by TLRs. One is known as the MyD88-dependent pathway and the other as the MyD88-independent/TRIF dependent pathway (Takeda and Akira 2005, Kawai et al. 1999).

TLR3 is the only TLR which relies solely on the MyD88-independent pathway and signals through the adaptor TRIF. On the other hand, TLR2, TLR5, TLR7 and TLR9 signal exclusively through the MyD88-dependent pathway and MyD88 binds directly to the intracellular region of TLR5, TLR7 and TLR9. In TLR2-mediated signaling, a complex of MyD88 and TIRAP interact with the receptor upon stimulation (see Fig 1.3). TLR4 uses a combination of the MyD88- dependent and MyD88-independent/TRIF dependent pathways. As in TLR2 signaling, the MyD88-dependent arm of TLR4 signaling is mediated by a MyD88-TIRAP complex while a complex of TRAM and TRIF mediates the MyD88-independent/TRIF dependent arm of TLR4 activation (Takeda and Akira 2005, Hazeki et al. 2007, Beutler 2004) (see Fig 1.3). TLR1, 2, 4 and 6 have been shown to be expressed in MC lines at the mRNA level. However, in these studies, TLR8 expression was not investigated (Applequist et al. 2002).

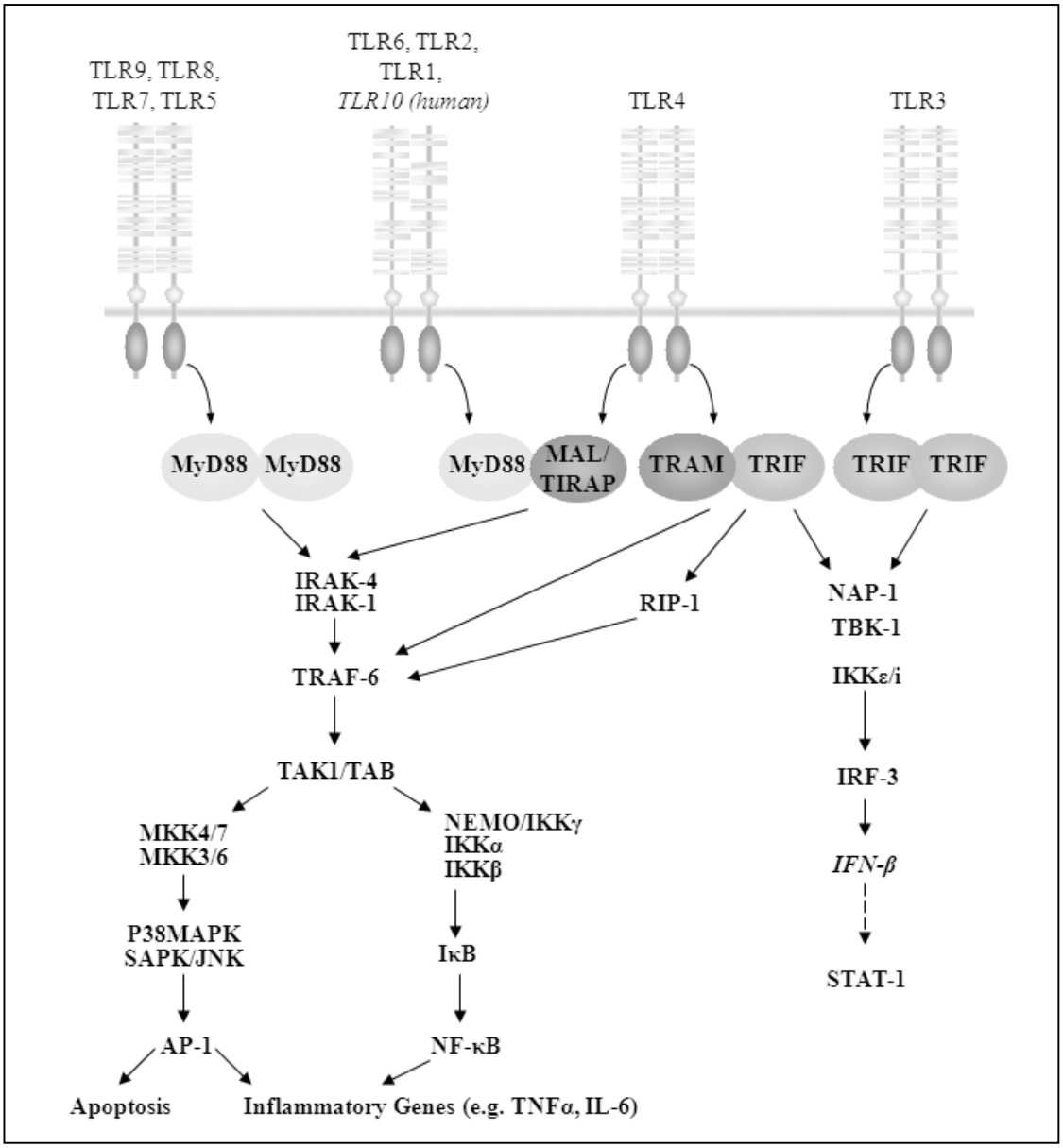


Figure 1.3 Principle of TLR signaling pathways. TLRs are located on the cell surface or in intracellular compartments such as endosomes. Upon ligand binding TLRs dimerise to induce signaling. Usually homodimers are formed but TLR2 can also heterodimerize with TLR1 and TLR2. In humans, TLR10 can form heterodimers with TLR1 and TLR6. TLRs transduce their signals through adaptor proteins. The MyD88-dependent pathway leads to the activation of NF-κB (early phase) and AP-1 which synergistically activate the production of inflammatory cytokines such as TNFα, IL-6 and others. AP-1 is also involved in the regulation of apoptosis. The MyD88-independent/TRIF dependent pathway leads to IFNβ production which activates STAT-1. TRIF is also able to activate NF-κB (late phase) through RIP-1 or direct interaction with TRAF-6. However, expression of inflammatory cytokines is not significantly induced through the TRIF pathway, compared to the MyD88-induced NF-κB pathway.

1.3.3 The MyD88-Dependent Pathway

MyD88 contains two protein-protein interaction domains, a death domain (DD) in its N-terminal region and a TIR domain in its C-terminal region, and associates with TLRs, which also contain TIR domains, via TIR:TIR interactions. Upon TLR stimulation, MyD88 recruits the IL-1R-associated kinase (IRAK)-4 through homotypic interactions of their DDs. IRAK-4 mediates phosphorylation of IRAK-1 which then associates with tumour necrosis factor (TNF) receptor-associated factor (TRAF)-6. This signaling pathway bifurcates downstream of TRAF-6 at the TAK1/TAB complex (see Fig 1.3). From there one pathway induces degradation of IκB by through phosphorylation. This phosphorylation is typically carried out by the inhibitor of κB (IκB) kinase (IKK), a complex composed of IKKα, IKKß and IKKγ (aka NEMO). Phosphorylation of IκB causes it to become ubiquitinated and degraded in the proteasome, releasing the TF, NF-κB, to enter the nucleus and turn on the expression of inflammatory cytokines. The other pathway acts through mitogen activated protein kinases kinases (MAPKs) and leads to the activation of activator protein (AP)-1, a TF which also induces expression of cytokines and/or apoptosis. The two pathways synergize to induce inflammatory gene expression by binding to AP-1 and κB sites that are present in the promoters of genes such as *Il6, Tnfα* and others. All TLRs, except for TLR3, signal, at least in part, through MyD88. A second TIR domain containing molecule, TIRAP (TIR domain-containing adaptor protein, aka Mal (MyD88-adaptor-like), has been shown to be essential for MyD88-dependent signaling through TLR2 and TLR4 (Takeda and Akira 2005, Hazeki et al. 2007, Yamamoto et al. 2002b) (see Fig 1.3).

1.3.4 The MyD88-Independent/TRIF-Dependent Pathway

MyD88-deficient macrophages cannot produce inflammatory cytokines upon TLR4 stimulation but are still capable of activating NF-κB (with delayed kinetics) and the TF interfereon (IFN) regulatory factor (IRF)-3, which leads to the production of IFNβ. This

indicates that TLR4-mediated production of inflammatory cytokines completely depends on the MyD88 pathway but also that a MyD88-independent pathway exists downstream of TLR4 (Takeda and Akira 2005, Kawai et al. 1999).

Indeed, besides MyD88 and TIRAP, two additional TIR domain-containing adaptors have been identified. The third one is called TIR domain-containing adaptor inducing IFNβ (TRIF), aka TIR domain-containing adaptor molecule (TICAM-1) (Yamamoto et al. 2002a, Oshiumi et al. 2003a) and it induces the late phase NF-κB activation and AP-1 activation through its interactions witch TRAF6 and receptor-interacting protein (RIP-1). TRIF also activates IRF-3 through NF-κB-activating kinase-associated protein-1 (NAP-1), IKKε/i and TRAF family-associated NF-κB binding kinase (TANK) binding kinase (TBK-1) complexes. IRF-3 then induces IFN-β expression which, in turn, induces signal transducer and activator of transcription (Stat)-1 activation. The fourth TIR domain-containing molecule is called TRIF-related adaptor molecule (TRAM, aka TICAM-2), and is essential for activating the TLR4-mediated MyD88-independent/TRIF dependent pathway (Oshiumi et al. 2003b) (see Fig 1.3).

1.4 SH2-Containing Inositol 5′-Phosphatase 1 (SHIP)

The Src homology 2 domain (SH2-)-containing inositol 5′-phosphatase 1 (SHIP aka SHIP1) is a master negative regulator of the immune system and hemopoietic cell survival, proliferation, differentiation and cell activation.

SHIP was identified in the mid 1990s as a 145 kDa intracellular protein that becomes both tyrosine phosphorylated and associated with the adaptor protein Shc after the stimulation of hemopoietic cells with various cytokines and growth factors (Damen et al. 1996).

In 1996 SHIP was cloned by three groups independently and in 1998 G. Krystal in collaboration with K. Humphries generated a SHIP knockout ($SHIP^{-/-}$) mouse. This mouse has become an invaluable tool for characterizing SHIP's central role within the hemopoietic system (Ware et al. 1996, Lioubin et al. 1996, Kavanaugh et al. 1996, Helgason et al. 1998).

In humans the gene that encodes SHIP, *Inpp5d*, is located on chromosome 2 (i.e., 2q36/37) and in mice on chromosome 1 (i.e., 1C5). The SHIP protein, which is only present in hemopoietic cells, consists of 3 major domains, an N-terminal SH2-domain, a central phosphoinositol 5′-phosphatase region and a proline rich C-terminus (see Fig1.4 for an overview of SHIP isoforms and binding partners) (Damen et al. 1996). The SH2-domain enables SHIP to bind to the tyrosine phosphorylated forms of proteins such as Shc, SHP-2, downstream of tyrosine kinases (Doks), Gabs, CD150, PECAM, Cas, c-Cbl, proteins with certain immunoreceptor tyrosine based inhibitory motifs (ITIMs), e.g. FcγRIIB, and proteins with certain immunoreceptor tyrosine based activation motifs (ITAMs), e.g. FcεRI and FcγRIIA. The phosphatase domain selectively hydrolyses the 5′ phosphate from phosphatidylinositol (PI)-3,4,5-trisphosphate (PIP_3), PI-4,5-P_2 and inositol-1,3,4,5-tetrakisphosphate (IP_4) (Lioubin et al. 1996, Damen et al. 2001, Huber et al. 1998a). The proline rich C-terminus binds a subset of SH3-containing proteins including Grb2, Src, Abl, PLCγI and PIASI. In addition, SHIP contains 2 NPXY motifs that, when phosphorylated, bind proteins with a phosphotyrosine binding (PTB) domain (e.g. Shc,

Dok1, Dok2) or an SH2 domain (p85α, SHIP2) (reviewed in Sly et al. 2003, Rohrschneider et al. 2000).

Besides the 145 kDa full length SHIP, aka SHIPα, there are 2 shorter SHIP proteins, the 135 kDa SHIPβ and a 110 kDa SHIPδ, which result from alternate splicing of the SHIP mRNA. Also, a 104 kDa stem cell SHIP (sSHIP or SIP-110), which is expressed in human placenta, embryonic stem cells and HSCs has been identified. However, sSHIP, which lacks the SH2-domain, is not the product of alternate splicing but instead is expressed from an alternate promoter located within intron 5 of the *INPP5D* gene (Sly et al. 2003, Rohrschneider et al. 2000, Tu et al. 2001).

SHIP negatively regulates the PI-3-kinase (PI3K) pathway, at least in part by hydrolyzing the 5′-phosphate group from the critical PI3K-generated second messenger, PIP_3. It does so after being recruited to tyrosine phosphorylated, plasma-membrane associated receptors and proteins. Hydrolysis of PIP_3 by SHIP reduces the ability of certain pleckstrin homology (PH) domain-containing proteins such as protein kinase B (PKB)/Akt or PIP_3-dependent protein kinase 1 (PDK1) to be recruited to and activated at the plasma membrane and thus downregulates the PI3K pathway (reviewed in Sly et al. 2003).

As mentioned above, with the generation of $SHIP^{-/-}$ mice, the role of SHIP within the hemopoietic system became evident. $SHIP^{-/-}$ mice are viable but have a shortened lifespan. They exhibit a pronounced splenomegaly with spleen weights and total cellularity increased five- to seven-fold compared to their wild-type (WT) counterparts. Furthermore, the lungs of $SHIP^{-/-}$ mice are enlarged and highly infiltrated by myeloid cells. $SHIP^{-/-}$ myeloid progenitors survive and proliferate better *in vitro* than their WT counterparts with suboptimal levels of CSF-1 and they also differentiate faster into mature macrophages (Helgason et al. 1998). Other abnormalities of $SHIP^{-/-}$ mice include but are not limited to, B-cell hyperactivity, perturbed natural killer (NK) cell development and deficient allograft rejection (Ono et al. 1996, Nadler et al. 1997, Wang et al. 2002, Galandrini et al. 2002, Ghansah et al. 2004).

More recently SHIP has also been found to be essential for the induction of endotoxin (aka lipopolysaccharide (LPS)) tolerance *in vivo* and *in vitro* since $SHIP^{-/-}$ mice and macrophages fail to display this phenomenon. Interestingly, the rapid and profound

upregulation of SHIP protein levels by LPS is responsible for endotoxin tolerance since treatment with antisense (AS) RNA to SHIP blocks both the increase of SHIP protein levels and the induction of endotoxin tolerance. This upregulation of SHIP by LPS is mediated by TGF-β, an anti-inflammatory cytokine that increases SHIP levels in myeloid cells(Valderrama-Carvajal et al. 2002, Sly et al. 2004).

Tumour associated macrophages (TAMs) typically display an alternatively activated (M2) or "healer" phenotype rather than a classically activated (M1) or "killer" phenotype and there is currently a lot of interest in understanding what determines whether macrophages become killers or healers. IL-4-induced healer macrophages have substantially lower SHIP levels than killer macrophages (Ho and Sly 2009). Interestingly, macrophages within $SHIP^{-/-}$ mice are also strongly M2 skewed and tumours grow substantially faster than in WT type mice. In certain hemopoietic cancers driven by oncogenic tyrosine kinases such as BCR-ABL and some mutant forms of c-kit, the half-life of the SHIP protein is dramatically shortened, which results in substantially lower SHIP levels. However, the mechanisms that lead to SHIP degradation under these conditions are still poorly understood (Rauh et al. 2005, Vanderwinden et al. 2006, Sattler et al. 1999).

In MCs, SHIP has been shown to restrain their degranulation, triggered by Ag-induced FcεRI cross-linking. SHIP does this by reducing the transient increase and duration of PIP_3 levels, which leads to restricted extracellular calcium entry as well as lower NF-κB and PDK1 activation. Cultured $SHIP^{-/-}$ MCs display a hypersensitive phenotype and become activated upon much lower IgE or Ag levels than WT MCs. Some insight has been gained into the role of TLRs and other pathogen recognition receptors (PRRs) in MC activation. However, the role of SHIP in this context still remains unknown(Metz and Maurer 2007, Huber et al. 1998a, Huber et al. 1998b, Kalesnikoff et al. 2002, Leitges et al. 2002, Stelekati et al. 2007).

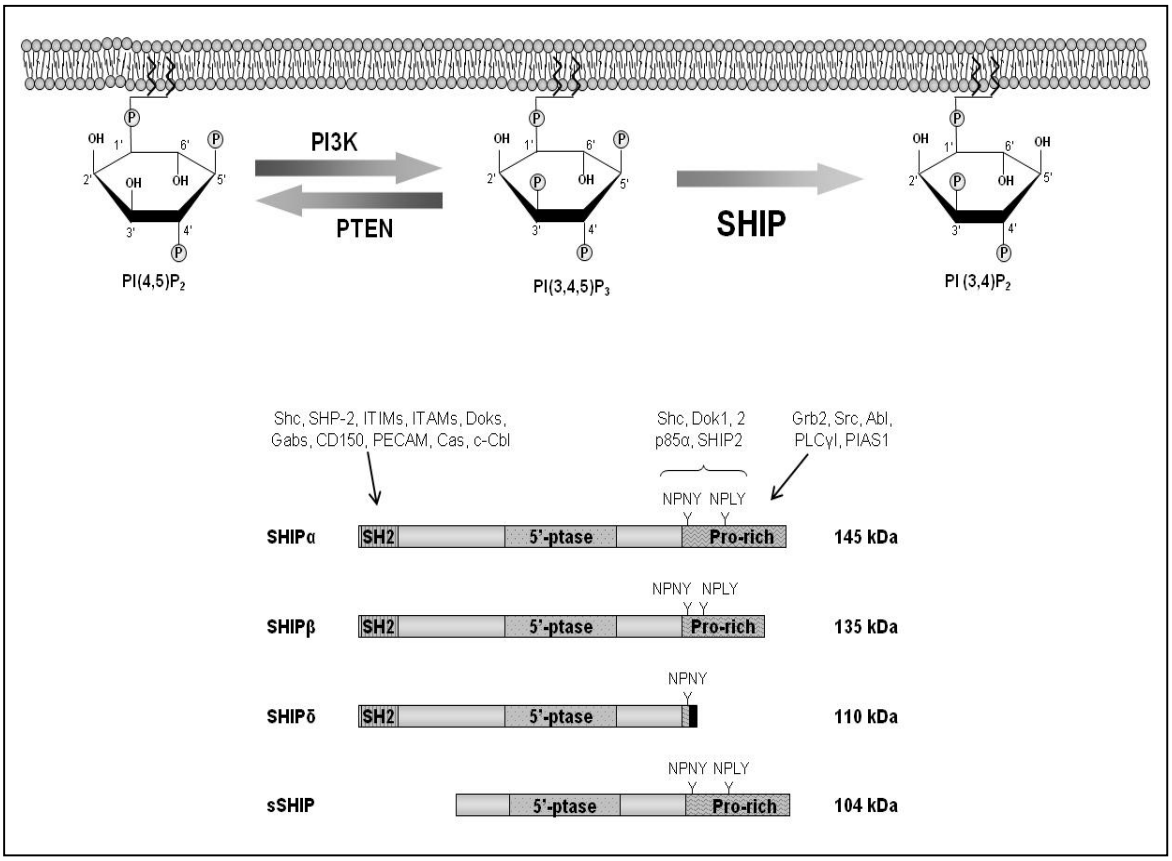


Figure 1.4 The structures of SHIP isoforms. The protein interaction motifs and binding partners of SHIP are indicated. SHIPα, SHIPβ and SHIPδ result from alternate splicing of the SHIP mRNA. In the case of SHIPβ, the alternate splicing leads to an in-frame deletion, removing part of the proline rich (Pro-rich) region between the two NPXY motifs. SHIPδ, on the other hand, lacks almost the entire Pro-rich C-terminal region and the second NPXY motif as a result of alternate splicing. In the case of sSHIP, the mRNA is transcribed from an alternative promoter located within intron 5 of the *Inpp5d* gene.

1.5 Projects and Aims

1.5.1 The Role of SHIP in the Maturation and Function of Mast Cell Subsets

Over the last few years the role of MCs as regulators of both innate and acquired immune responses has become more evident. Their expression of a variety of immune receptors such as TLRs, immunoglobulin receptors (e.g., FcεR1), functional MHC class II and others put MCs in a key position to connect the innate and acquired arms of the immune system. TLRs have been in the spotlight not only because of their ability to recognize PAMPs and thereby initiate innate immune responses but also to help dictate subsequent acquired immune responses. Studies to date investigating the function of TLRs in MCs have made use of *in vitro*-derived MCs as well as MC-deficient mice and TLR knockout (KO) mice. As a result of these studies, the central role of TLRs and some of the pathways they utilize in MCs have been elucidated. However, the majority of those studies have relied on BM-derived MCs (BMMCs), an *in vitro* model that is thought to represent mucosal type MCs only and very little is known about the role that TLRs play in connective tissue MCs (reviewed in Stelekati et al. 2007, Galli et al. 2005)). Moreover, the role of SHIP, a master negative regulator of FcεR1-mediated activation in MCs, has not as yet been investigated in TLR-mediated activation of MCs. Related to this, our laboratory has shown that SHIP plays a role in TLR signalling in macrophages (Sly et al. 2004) and so might also be important in regulating TLR-induced signalling in other cell types of the innate immune system, such as MCs. Thus, the goal of this study is to understand the role that SHIP plays in TLR-mediated activation of both mucosal and connective tissue MCs.

1.5.2 Regulation of SHIP Protein Levels

SHIP is a negative regulator of the PI3K pathway and plays a key role in restraining the immune system. Interestingly, the activity of SHIP is determined in large part by its

protein level. For example, in response to Gm^- bacterial infections, which can be mimicked by giving LPS, protein levels of SHIP increase 10 fold in macrophages and MCs and this dampens down the inflammatory response and thus saves the host from dying from systemic bacterial infections due to septic shock (Sly et al. 2004).

In contrast, when an invading micro-organism has been destroyed via classically activated (M1) macrophages and these macrophages then become skewed to an alternatively activated (M2) healer macrophage to promote tissue remodelling/healing and proliferation, SHIP levels go down dramatically in macrophages (Ho and Sly 2009).

Certain leukemias like chronic myeloid leukemia (CML) exploit this phenomenon by reducing SHIP levels to increase the PI3K pathway and thus their proliferation and survival within the host (Vanderwinden et al. 2006, Sattler et al. 1999). Thus understanding the mechanisms by which SHIP protein levels are controlled might be valuable not only in fighting cancer but also in saving patients with bacterial infections from dying of septic shock. While LPS-induced TGF-ß is known to be involved in the upregulation of SHIP protein levels, very little is known about its destruction. Related to this, the tyrosine kinase activity of BCR-ABL, the driving oncogene in CML, is essential for the degradation of SHIP since inhibition of BCR-ABL kinase activity, prevents it (Sattler et al. 1999).

Preventing cancers from abusing this mechanism to support their survival and growth might be a useful tool in fighting these diseases. Thus the aim of these studies within the thesis is to understand the mechanism(s) underlying SHIP's breakdown.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Disposable Equipment

0.4% trypan blue solution	StemCell Technologies, Vancouver
1.7 ml reaction tubes	Axygen Scientific, Union City, USA
12-well plates	Fisher Scientific, New Jersey, USA
175cm^2 tissue culture flask	Fisher Scientific, New Jersey, USA
75cm^2 tissue culture flask	Fisher Scientific, New Jersey, USA
96-well plates	Fisher Scientific, New Jersey, USA
96-well v-bottom plates	Fisher Scientific, New Jersey, USA
Acetic Acid	Fisher Scientific, New Jersey, USA
Amonium perodo-disulfate	Bio-Rad, Canada
Bromphenol blue	Sigma, Saint Louis, USA
BSA powder (fraction V)	Roche, Indianapolis, USA
$CaCl_2$	Fisher Scientific, New Jersey, USA
Cell Strainer	Fisher Scientific, New Jersey, USA
DMSO	Sigma, Saint Louis, USA
EGTA	Sigma, Saint Louis, USA
Ethanol	BC Cancer Hospital
Glycine	Sigma, Saint Louis, USA
HCl	Fisher Scientific, New Jersey, USA
HEPES	Sigma, Saint Louis, USA
Immobilon^{TM} PVDF membranes	Millipore, Bedford, USA
KCl	Sigma, Saint Louis, USA
Kodak X-Omat Blue film	Kodak

Methanol	Sigma, Saint Louis, USA
$MgCl_2$	Fisher Scientific, New Jersey, USA
Microscope slides	Fisher Scientific, New Jersey, USA
NaCl	Fisher Scientific, New Jersey, USA
NaF	Sigma, Saint Louis, USA
NaN_3	Sigma, Saint Louis, USA
NP-40	Calbiochem, UK
Pipet Tips	Molecular BioProducts, San Diego, USA
Propidium iodide	Sigma, Saint Louis, USA
Protein G agarose-beads	Pierce, Rockford, USA
SDS (20% solution)	Bio-Rad, Canada
Sodium azide (NaN_3)	Sigma, Saint Louis, USA
Sodium orthovanadate	Fisher Scientific, New Jersey, USA
Sodium pyrophosphate	Fisher Scientific, New Jersey, USA
TEMED	Bio-Rad, Canada
Triton-X-100	Sigma, Saint Louis, USA
Trizma Base	Sigma, Saint Louis, USA
Tween-20	Sigma, Saint Louis, USA
Western Lightning ECL	Perkin Elmer
α-Monothioglycerol	Sigma, Saint Louis, USA
β-mercaptoethanol	Sigma, Saint Louis, USA

2.1.2 Equipment

96-well plate EL_X808 Ultra Microplate reader	Bio-Tec Instruments, Winooski, USA
Axioplan 2 *imaging*	Carl Zeiss, Toronto, ON
Centrifuge, Allegra™ X-12R	Beckman Coulter, Mississauga, ON
Cytospin 3	Shandon
FACSCalibur™ Flow Cytometer	BD Biosciences, Mississauga, ON

FACSDiva™ Flow Cytometer & Cell Sorter	BD Biosciences, Mississauga, ON
GeneAmp® PCR System 9700 thermo cycler	Perkin Elmer Applied Biosystems
Hemocytometer	Fisher Scientific, Canada
Heraeus Biofuge Pico	DJB Labcare, UK
Protean II™ electrophoresis system	Bio-Rad, Canada
Tissue culture incubator	Forma Scientific, USA
Trans Blot Cell™ transfer system	Bio-Rad, Canada

2.1.3 Reagents, Enzymes, Cytokines and Antibodies

anti c-Abl (clone Ab-3)	Calbiochem, UK
anti Cbl (SC-170)	Santa Cruz Biotechnology
anti Cbl-b (clone G-1)	Santa Cruz Biotechnology
anti CD49b-APC (clone DX5)	eBioscience, San Diego
anti c-kit-APC (clone 2B8)	eBioscience, San Diego
anti FcεR1α-FITC (clone MAR-1)	eBioscience, San Diego
anti GAPDH (clone 6C5)	Fitzgerald, Concord
anti mouse IgG-Alexa Fluor® 647	Invitrogen, Canada
anti mouse IL-4 blocking Ab	R&D Systems®
anti mouse-HRP	Jackson *ImmunoResearch* Laboratories
anti PTEN (SC--7974)	Santa Cruz Biotechnology
anti rabbit-HRP	Jackson *ImmunoResearch* Laboratories
anti SHIP (clone P1C1, mouse IgG_1)	Santa Cruz Biotechnology
anti SHIP2	Clarkson
anti-DNP (clone SPE-7)	Sigma, Saint Louis
BD Cytofix/Cytoperm™ Kit	BD Bioscience, Mississauga, ON
BD OptEIA TMB Substrate Reagent Set	BD Bioscience, Mississauga, ON
BD OptEIA™ ELISA Set for mouse IL-10	BD Bioscience, Mississauga, ON

Materials

BD OptEIA™ ELISA Set for mouse IL-12p40	BD Bioscience, Mississauga, ON
BD OptEIA™ ELISA Set for mouse IL-4	BD Bioscience, Mississauga, ON
BD OptEIA™ ELISA Set for mouse IL-6	BD Bioscience, Mississauga, ON
BD OptEIA™ ELISA Set for mouse TNFα	BD Bioscience, Mississauga, ON
DNP_{30-40}-HSA	Sigma, Saint Louis
dNTPs (10 mM each) solution	Fermentas
Doxycycline	Calbiochem, UK
Fcγ blocking Ab (clone 2.4G2)	StemCell Technologies, Vancouver
human recombinant IL-6	StemCell Technologies, Vancouver
MG-132	Sigma, Saint Louis, USA
M-MLV RT	Invitrogen, Canada
mouse IgG_1 isotype control	BD Bioscience, Mississauga, ON
mouse recombinant EPO	StemCell Technologies, Vancouver
mouse recombinant IL-3	StemCell Technologies, Vancouver
mouse recombinant IL-4	StemCell Technologies, Vancouver
mouse recombinant M-CSF	StemCell Technologies, Vancouver
mouse recombinant SCF	StemCell Technologies, Vancouver
mTLRs RT-primers (Version # 07H09-SV)	Invivogen, Canada
oligo $(dT)_{18}$ primer	Fermentas
Phusion® High-Fidelity DNA Polymerase kit	Finnzymes
PP2	Calbiochem, UK
TRIzol® Reagent	Invitrogen, Canada
TURBO DNA-*free*™ kit	Ambion®

The phospho-SHIP (Y^{1020}) Ab used in this thesis was generated by immunizing rabbits with KLH-coupled phosphopeptides corresponding to the regions encompassing the two NPXpY peptides (EMINPNpY^{915}IGMG-Cys and EMFENPLpY^{1022}GS-Cys) in human SHIP. These sequences are identical in mouse and correspond to EMINPNpY^{917}IGMG and EMFENPLpY^{1020}GS. A C-terminal cysteine was added to facilitate coupling to

maleimide-activated mcKLH (Pierce, Rockford, USA). Antisera were affinity purified with the appropriate bead-bound immunogen.

2.1.4 Materials, Buffers and Media for Tissue Culture

Bovine Serum Albumin (BSA) solution - 7.5%	Sigma, Saint Louis, USA
Cell Dissociation Buffer	Invitrogen, Canada
Fetal Calf Serum (FCS)	Fisher Scientific, Canada
Hanks' balanced salt solution (HBSS), modified (contains 10 mM HEPES and Sodium Bicarbonate)	StemCell Technologies, Vancouver
Iscove's Modified Dulbecco's Medium (IMDM)	StemCell Technologies, Vancouver
Phosphate Buffered Saline (PBS)	StemCell Technologies, Vancouver
Penicillin/Streptomycin	StemCell Technologies, Vancouver
Roswell Park Memorial Institute (RPMI)-1640	StemCell Technologies, Vancouver

2.1.5 TLR Ligands

E.coli LPS serotype O127:B8 was purchased from Sigma-Aldrich (St. Louis, USA). Unless otherwise stated it was used at a final concentration of 100 ng/ml to stimulate cells. Phosphorothioate-modified CpG-containing oligodeoxynucleotides were synthesized and HPLC purified by Invitrogen (Burlington, Canada) with the following sequence 5'-tccatgacgttcctgacgtt-3'. Unless otherwise stated it was used at a final concentration of 0.3 μM for cell stimulations. The dsRNA used was a synthetic analogue of polyinosine:cytosine from Sigma-Aldrich (St. Loius, USA). Unless otherwise stated it was used at a final concentration of 50 μg/ml for stimulations. PGN from *Staphylococcus aureus* was purchased from Fluka (Buchs, Switzerland). Unless otherwise stated it was used at a final concentration of 10 μg/ml for stimulations.

2.1.6 Buffers and Media

0.2 M glycine:
1.5 g glycine
H2O to 100 ml
pH 10.7

Ba/F3p210-tetOFF medium:
RPMI-1640
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS

Basophil media:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
20% FCS
0.00125% MTG
30 ng/ml IL-3

BMmac medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS
0.00125% MTG
10 ng/ml M-CSF

BMMC medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
15% FCS
30 ng/ml IL-3

BM starve medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS
0.00125% MTG

BSA starvation medium (BSA medium):
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
0.1% BSA

Complete medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
15% FCS
0.00125% MTG
10 ng/ml IL-3
10 ng/ml human IL-6
3 U/ml EPO

Connective tissue MC (CTMC) medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS
50 ng/ml SCF

HBSS plus fetal calf (HF) media:
HBSS modified
2% FCS

IL-3 medium:
RPMI-1640
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS
5 ng/ml IL-3

Mucosal MC (MMC) medium:
IMDM
100 U/mL Penicillin
100 µg/ml Streptomycin
10% FCS
10 ng/ml IL-3

Materials

p-NAG solution (in substrate buffer):
65 mg p-NAG
50 ml substrate buffer
Dissolve p-NAG and store 10 ml aliquots at -20ºC.

PSB:
50 mM HEPES
100mM NaF
10mM sodium pyrophosphate
2mM sodium orthovanadate
4mM EGTA
pH 7.4
store at 4ºC

10x SDS-PAGE running buffer:
121.1 g Trizma Base
288.3 g glycine
100 ml 20% SDS
H_2O to 2000 ml
Dilute 1:10 with H_2O to make
1x SDS-PAGE running buffer

4x SDS-Sample buffer (SB):
4 ml glycerol
4.6 ml 20% SDS
2 ml β-mercaptoethanol
Few crystals bromphenol blue
Dilute 1:4 in PSB to make 1x SDS-SB

Substrate buffer:
14.29 g Na_2HPO_4
42.05 g citric acid
H_2O to 500 ml

TBST:
150 mM NaCl
0.05% Tween 20
50 mM TRIS/HCl
pH 7.5

Tyrode's Buffer:
10 mM HEPES
130 μM NaCl
5 mM KCl
1.4 mM $CaCl_2$
1 mM $MgCl_2$
5.6 mM glucose
0.1% BSA

Western blot blocking solution:
5 g BSA
100 ml PSB
0.1 % NaN_3

10x Western blot transfer buffer:
181.5 g Trizma Base
864.75 g glycine
30 ml 20% SDS
H_2O to 6000 ml
To set up transfers dilute 1:9 with H_2O and add 1 part Methanol.

2.1.7 Mice

$SHIP^{+/+}$ (WT) and $SHIP^{-/-}$ F2 mice on a mixed C57Bl/6 x 129Sv background were used between 6-12 weeks of age. The mice were housed in the Animal Resource Centre of the BC Cancer Research Centre under specific pathogen free conditions and according to approved and ethical treatment of animal standards of the University of British Columbia. Animals were euthanized by CO_2 asphyxiation.

Femurs and tibia from mice deficient in Fyn and Lyn/Fyn were obtained from Dr. J. Rivera (National Institutes of Health, Bethesda, USA). Femurs and tibia from mice deficient in Cbl were obtained from Dr. R. Hodes (National Institutes of Health, Bethesda, USA). Femurs and tibia from mice deficient in Lyn, Hck and Lyn/Hck were obtained from Dr. T. Kawakami (La Jolla Institute for Allergy and Immunology, San Diego, USA). Femurs and tibia from mice deficient in Stat6 were obtained from Dr. R. A. Flavell (Yale University, New Haven, USA). All these knockout mice were on a C57Bl/6 background and femurs and tibia obtained from WT C57Bl/6 littermates were sent and used as controls.

2.1.8 Graphing and Statistical Analysis

Graphs were generated using GraphPad Prism 5.00 for Windows.

Statistical significance was calculated using a two-tailed unpaired student *t* test using Microsoft excel. Differences were considered significant when $p < 0.05$.

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Derivation and Stimulation of MC Subsets and Basophils

For the generation of BM derived MCs (BMMCs), BM cells were aspirated from mouse femurs and tibia using ice cold IMDM and pipetted through a 100 µm nylon cell strainer to break up cell clumps. Cells were cultured at $10x10^5$c/ml (nucleated cells, obtained by counting a 1:50 cell dilution in 3% acetic acid) in complete medium for 7 days. Non adherent cells were then counted every 7 days and cultured in BMMC medium at $3x10^5$ c/ml.

For the generation of splenic MCs, mouse spleens were cut into small pieces and pressed through a 100 µm nylon cell strainer to obtain a single cell suspension. To generate mucosal-like MCs, spleen cells were cultured at $10x10^5$ c/ml (nucleated cells, obtained by counting a 1:50 cell dilution in 3% acetic acid) in MMC medium for 7 days. Non adherent cells were then counted and cultured in MMC medium every 7 days and cultured at $3x10^5$ c/ml. To generate connective tissue-like MCs, spleen cells were cultured at $10x10^5$ c/ml (nucleated cells, obtained by counting a 1:50 cell dilution in 3% acetic acid) in CTMC medium for 7 days. Afterwards, non adherent cells were counted and cultured in CTMC medium every 7 days and cultured at $3x10^5$ c/ml.

For the generation of basophils, BM cells were aspirated from mouse femurs and tibia using ice cold IMDM and pipetted through a 100 µm nylon cell strainer to break up cell clumps. Cells were cultured at $2x10^5$c/ml (nucleated cells, obtained by counting a 1:50 cell dilution in 3% acetic acid) in basophil medium. After 7 days the medium was replaced by fresh basophil medium and after an additional 3 days, basophils were purified by staining with a CD49b-APC Ab and subjecting the cells to fluorescence activated cell sorting (FACS).

2.2.1.2 MC Survival Assay

Viable cell counts were performed by mixing 25 µl of cells with 25 µl of a 0.4% trypan blue solution. Viable cells are able to exclude the dye and therefore appear bright when counted on the hemocytometer.

To set up MC survival studies, MCs were washed with BSA medium and resuspended at $15x10^5$c/ml. 100 µl of cells were seeded in a 96-well plate and 100 µl of BSA medium or BSA medium supplemented with a monoclonal IgE Ab (10 µg/ml of monoclonal anti-DNP, clone SPE-7, herein referred to as SPE-7) was added and viable cell counts were performed daily.

2.2.1.3 MC Degranulation Assay

MCs were washed with MC starvation medium (MC starve) and cultured at $10x10^5$c/ml in MC starve for 18 h. If cells were to be stimulated with IgE + Ag, the MC starve medium was supplemented with 0.1 µg/ml SPE-7. Cells were then washed twice with Tyrode's buffer and resuspended at $62.5x10^5$c/ml. 10 µl of cells were seeded in a 96-well v-bottom plate. To trigger degranulation, 10 µl of 2x concentrated stimulus, prepared in Tyrode's buffer, was added. For non-stimulated samples, 10 µl of Tyrode's buffer was added. After 1 h incubation at 37ºC, cells were centrifuged for 5 min at 335 gs and 10 µl of cell free supernatant was transferred into a fresh 96-well (flat bottom) plate. The rest of the supernatant was carefully removed leaving only the cell pellet behind. Cells were lysed in 20 µl of 0.5 % Triton-X-100 (TX100) prepared in Tyrode's buffer and 10 µl of cell lysate was transferred into a fresh 96-well plate (flat bottom). The supernatants and cell lysates were mixed with 50 µl of p-NAG solution and incubated at 37ºC for 1 h. 150 µl of 0.2 M glycine (pH 10.7) was then added and the plate read at 405 nm in a 96-well plate EL_X808 Ultra Microplate reader. Percentage of degranulation was calculated by dividing the absorbance of the supernatants by the sum of the absorbencies of the supernatant and cell lysate.

2.2.1.4 Stimulation of MCs to quantify the production of IL-6, IL-10, IL12 and TNFα

Stimulation with TLR ligands alone.

MCs were washed once with MC starve and cultured at $10x10^5$c/ml in MC starve for 18h. MCs were then washed once with MC starve and resuspended at $10x10^5$c/ml in MC starve. For stimulations with TLR ligands, 750 µl of cells were seeded in 12 well plates. TLR ligands were made up in MC starve at twice the indicated final concentration and 750 µl added to the MCs, resulting in 1.5 ml total volumes. For non-stimulated (NS) samples 750 µl of MC starve was added. MCs were stimulated for 24 h.

Stimulation with TLR ligands plus IgE.

MCs were washed once with MC starve and cultured at $10x10^5$c/ml in MC starve for 18h. MCs were then washed once with MC starve and resuspended at $10x10^5$c/ml in MC starve. For stimulations with TLR ligands, 750 µl of cells were seeded in 12 well plates. TLR ligands were made up in MC starve plus 0.2 µg/ml SPE-7 at twice the indicated final concentration and 750 µl added to the MCs, resulting in 1.5 ml total volumes. For non-stimulated (NS) samples, 750 µl of MC starve plus 0.2 µg/ml SPE-7 were added. MCs were stimulated for 24 h.

Stimulation with TLR ligands plus IgE + Ag.

MCs were washed once with MC starve and cultured at $10x10^5$c/ml in MC starve plus 0.1 µg/ml SPE-7 for 18h. MCs were then washed twice with MC starve and resuspended at $10x10^5$ c/ml in MC starve plus 2 ng/ml DNP-HSA. For stimulations with TLR ligands, 750 µl of cells were seeded in 12 well plates. TLR ligands were made up in MC starve at twice the indicated final concentration and 750 µl added to the MCs, resulting in 1.5 ml total volumes. For non-stimulated (NS) samples, 750 µl of MC starve were added. MCs were stimulated for 24 h.

After 24 h, MCs were transferred into 1.5 ml reaction tubes, centrifuged for 5 min at 335 gs and cell free supernatants collected. Supernatants were stored at -20ºC until they were analyzed by ELISA.

2.2.1.5 Stimulation of MCs and Basophils to quantify the Production of IL-4

Freshly isolated basophils and cultured MCs were washed in MC starve and resuspended at $2x10^5$c/ml. 50 µl of cells were seeded in 96-well plates. Then 25 µl of MC starve (for non-stimulated and IL-3 stimulated samples) or 25 µl of MC starve plus 4 µg/ml SPE-7 (for IgE and IgE + Ag samples) were added and samples incubated for 2.5 h in a 37˚C tissue culture incubator. Afterwards, 25 µl of MC starve (for non-stimulated and IgE alone stimulated samples), 25 µl of MC starve plus 400 ng/ml DNP-HSA (for IgE + Ag stimulated samples) or 25 µl of MC starve plus 400 ng/ml IL-3 were added and samples incubated for 24 h in a 37˚C tissue culture incubator. After 24 h, 120 µl of PBS plus 10% heat-inactivated FCS were added to the samples. Samples were then transferred into a 96-well v-bottom plate and cells centrifuged for 5 min at 335 gs and cell free supernatants collected. Supernatants were stored at -20ºC until they were analyzed by ELISA.

2.2.1.6 Culturing and Stimulation of Ba/F3$_{p210\text{-}tetOFF}$ Cells

Ba/F3$_{p210\text{-}tetOFF}$ cells were received from Dr. A. G. Turhan (Institut Gustave Roussy, Villejuif, France) and cultured in basic Ba/F3$_{p210\text{-}tetOFF}$ medium. This medium contains no IL-3, which is required for the survival and growth of normal Ba/F3 cells. However, the BCR-ABL activity in these cells makes them IL-3 independent (Daley and Baltimore 1988). Cells were kept between $1x10^5$c/ml and $10x10^5$c/ml and diluted 10 fold with fresh medium when they reached $10x10^5$c/ml, approximately 3 times/week. Unless otherwise stated, Ba/F3$_{p210\text{-}tetOFF}$ cells were cultured in IL-3 medium for at least 24 h before stimulations. For stimulations, cells were resuspended at $1x10^5$c/ml in fresh IL-3 medium.

If Ba/F3$_{p210\text{-}tetOFF}$ cells were treated with doxycycline, control samples were treated with an equal volume of ethanol as a solvent control since the doxycycline stock (at 5 mg/ml) was dissolved in ethanol. If Ba/F3$_{p210\text{-}tetOFF}$ cells were treated with PP2 or MG-132, control samples were treated with an equal volume of dimethyl sulfoxide (DMSO) as a solvent control since both PP2 (10 mM) and MG-132 (25 mM) stocks were dissolved in DMSO. Cells were stimulated in a 37˚C tissue culture incubator. To stop the stimulations, cells were put on ice, counted and washed once with ice cold PBS. Finally, cells were lysed in 1x sodium dodecyl sulfate-sample buffer (SDS-SB) at 100x10^5c/ml and boiled for 2 min at 95ºC. Genomic DNA was sheared by passing samples 10 times through a 26 gauge needle.

2.2.1.7 Generation and Stimulation of Bone Marrow Derived Macrophages (BMmacs)

For the generation of BMmacs, BM cells were aspirated from mouse femurs and tibia using ice cold IMDM and pipetted through a 100 µm nylon cell strainer to break up cell clumps. Cells were cultured in BM starve medium in a 75cm^2 tissue culture flask for 18 h for adherence depletion. 50 ml of non-adherent cells were cultured at 5x10^5c/ml (nucleated cells, obtained by counting a 1:50 cell dilution in 3% acetic acid) in a 175cm^2 tissue culture flask in BMmac medium. The medium was replaced every 3 days. After 10-12 days BMmacs were used for stimulations. To stimulate BMmacs, the medium was aspirated from the flasks and BMmacs were lifted off the plates using Cell Dissociation Buffer. 1 ml of 5x10^5c/ml BMmacs in BM medium was plated in a 12-well dish for 18 h to allow cells to adhere. The medium was then replaced with fresh BMmac medium (control) or BMmac medium plus 10 ng/ml IL-4. If cells were treated with PP2 or MG-132, other samples were treated with an equal volume of DMSO as a solvent control.

To lyse cells, the medium was aspirated, washed once with ice-cold PBS and 100 µl of 1x SDS-SB was added. Cell lysates were transferred into fresh 1.7 ml reaction tubes. In one experiment protocol, cells were washed once with ice cold PSB and lysed in 75 µl of PSB lysis buffer (2% TX100, 0.1% NP-40 5, 2 µg/ml leupeptin, 10 µg/ml aprotinin, 500 µM

PMSF) or they were washed with PSB plus 10 µM MG-132 and lysed in 75 µl PSB lysis buffer plus 10 µM MG-132. Lysed cells were then transferred into fresh 1.7 ml reaction tubes and 25 µl of 4x SDS-SB added.
All cell lysates were boiled for 2 min at 95ºC and genomic DNA sheared by passing samples 10 times through a 26 gauge needle.

2.2.2 Proteochemical and Immunological Methods

2.2.2.1 Immunoprecipitations (IPs)

All IPs were performed on ice. First, monoclonal SHIP Ab (clone P1C1) was coupled to Protein G agarose-beads by washing 40 µl of Protein G agarose-beads 3x in PSB plus 0.1% BSA/0.1% TX100. After the final wash, the beads were resuspended in 90 µl of the same buffer and 2 µg of SHIP Ab added. The Protein G agarose-beads/SHIP Ab mix was incubated on a nutator at 4ºC for 18 h. The beads were then washed 5x in the same buffer to remove unbound Ab and resuspended in 100 µl of the same buffer.
$5x10^6$ $Ba/F3_{p210tetOFF}$ cells were washed once and resuspended in 200 µl of PSB plus 0.1% BSA. To lyse the cells 200 µl of PSB plus 0.1% BSA/2% TX100/4 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM PMSF was added and the mix incubated on a nutator at 4ºC for 1h. The nuclei were then spun down at 16000 gs for 10 min at 4ºC and the nuclei-free supernatant mixed with 100 µl of the prepared Ab-Protein G agarose-beads and incubated at 4ºC overnight. The beads were washed 5x with PSB plus 0.1% TX100, resuspended in 50 µl SDS-SB and boiled at 95ºC for 2 min. The supernatant was removed from the beads and analyzed by Western blot analysis.

2.2.2.2 SDS Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

SDS-PAGE was performed using the equipment and methods of the Protean II™ electrophoresis system. Cells were loaded as cell equivalents from total cell lysates. After separation by SDS-PAGE, proteins were transferred to Immobilon™ polyvinylidene difluoride (PVDF) membranes using a Trans Blot Cell™ transfer system (Biorad) as per the manufacturer's instructions.

For Western blot analysis, membranes were first blocked in Western blot blocking solution at 23˚C for 1h. Membranes were then incubated with appropriate primary Ab solutions, diluted in TBST plus 2% BSA and 0.1%NaN_3 at indicated concentrations, at 4ºC overnight. Membranes were then washed 5x with TBST for 10 min and incubated with 1:10000 dilution in TBST of the appropriate secondary Ab conjugated to horseradish peroxidase (HRP) for 45 min at 23˚C. Membranes were then washed 5x with TBST for 10 min and target proteins visualized using Western Lightning Enhanced Chemiluminescence (ECL) reagent (Perkin Elmer, Boston, MA) for 1min, then exposed on Kodak X-Omat Blue film.

The following table shows the dilutions that primary Abs were used at for Western blot analysis and in which species they were derived.

Antibody	Dilution	Species
anti c-Abl (clone Ab-3)	1:500	mouse
anti Cbl	1:1000	rabbit
anti Cbl-b	1:1000	mouse
anti GAPDH (clone 6C5)	1:5000	mouse
anti phospho-SHIP (phospho-Y-1020)	1:5000	rabbit
anti PTEN	1:1000	mouse
anti SHIP (clone P1C1, mouse IgG_1)	1:1000	mouse
anti SHIP2	1:2000	rabbit

Table 2.1 Dilutions for primary Abs used in Western blot analysis. The table shows the dilutions that the primary Abs were used at for Western blot analysis and the species they were derived in.

2.2.2.3 Enzyme-Linked Immunosorbent Assays (ELISAs)

Tissue culture supernatants were assayed for protein levels of some or all of the following IL-4, IL-6, IL-10, IL-12p40, TNFα, using BD OptEIA^{TM} ELISA Sets according to the manufacturer's instructions. For visualization, BD OptEIA TMB Substrate Reagent Sets were used as per the manufacturer's instructions and plates were read at 450 nm in a 96-well plate EL_X808 Ultra Microplate reader.

2.2.2.4 Fluorescence activated cell sorting (FACS)

All staining procedures for FACS were performed on ice.

To sort basophils, non adherent cells from day10 basophil cultures were resuspended at $5x10^7$c/ml in HF. 10% of the cells were removed as an unstained negative control. The remaining 90% of the cells were stained with Dx5-APC at a 1:50 dilution for 45 min at

23˚C in the dark. Afterwards, both control and stained cells were washed twice with HF and resuspended in HF plus 1 µg/ml propidium iodide (PI) at $1x10^7$c/ml. Cells were sorted using the Diva FACS machine.

To assess the maturity of MCs by FACS, MCs of each subtype were washed and resuspended at $10x10^5$c/ml in HF plus Fcγ-blocking Ab at 1:200. For each MC subtype a control sample was prepared as well. Cells were incubated for 30 min and Fcε1α-FITC and c-kit-APC Abs were added at 1:1000 and 1:200, respectively, except for the unstained controls. After an additional incubation of 45 min in the dark all cells were washed with HF and resuspended at $5x10^5$c/ml. FACS files were collected using a FACScalibur.

Intracellular FACS staining was performed using the BD Cytofix/Cytoperm™ Kit following the manufacturer's recommendations with the following specifications: Ab stainings were performed in 100 µl volumes. As a primary Ab, 0.4 µg of monoclonal anti SHIP (clone P1C1, mouse IgG_1) was used. As a secondary Ab, 1 µg of anti mouse IgG-Alexa Fluor® 647 Ab was used. For an isotype control, 0.4 µg of mouse IgG_1 isotype-control Ab was used.

2.2.2.5 Cytospin and granule staining of MCs

$5x10^4$ MCs of each subtype were resuspended in 0.5 ml of PBS and centrifuged onto a microscope slide at 500 rpm for 5 min using a Cytospin 3 centrifuge. Cells were then stained for 15 min in 0.5% alcian blue/0.3% acetic acid solution, followed by a 20 min stain in 0.1% safranin/0.1% acetic acetic acid. Cells were air dryed and pictures were taken using an Axioplan 2 *imaging* microscope.

2.2.3 Analysis of TLR expression by RT-PCR

2.2.3.1 Isolation of total RNA and 1st Strand Synthesis

Total RNA was prepared from cultured MCs with TRIzol® Reagent and genomic DNA contaminants removed using the TURBO DNA-*free*™ kit (Ambion®) according to each supplier's recommendations.

1st strand synthesis was performed using M-MLV RT. The reactions were performed as per the manufacturer's instructions with the following exception: the reactions were linearly upscaled to 25 µl. An oligo $(dT)_{18}$ primer was used.

2.2.3.2 Polymerase Chain Reaction (PCR)

PCRs were performed using the Phusion® High-Fidelity DNA Polymerase kit. Each reaction was performed in a 25 µl total volume consisting of 1 µl template, 16.5 µl PCR-H_2O, 5 µl of 5x Phusion HF buffer (containing 7.5 mM $MgCl_2$), 0.75 µl DMSO, 0.25 µl Phusion High-Fidelity DNA Polymerase (2 U/µl), 0.5 µl of a 10 µM forward primer solution, 0.5 µl of a 10 µM reverse primer solution and 0.5 µl of a dNTP (10 mM each) solution.

When using the Phusion® High-Fidelity DNA Polymerase kit, the annealing temperature is supposed to be set 3ºC higher than the Tm of the primers used. PCRs with gene specific primers for GAPDH were carried out with an annealing temperature of 63ºC and with the oligonucleotide primers 5'- TTAGCCCCCTGGCCAAGG and 5'- CTTACTCCTTGGAGGCCATG, amplifying a 541 bp fragment. PCRs for mouse TLRs (mTLR) were performed with primers purchased from Invivogen and were carried out with an annealing temperature of 58ºC. Each primer pair amplifies a DNA fragment of a specific size (see Table 2.2).

mTLR primer pair	Product Size
mTLR1	309 bp
mTLR2	400 bp
mTLR3	415 bp
mTLR4	510 bp
mTLR5	545 bp
mTLR6	575 bp
mTLR7	610 bp
mTLR8	769 bp
mTLR9	808 bp

Table 2.2 RT-PCR primers for mTLRs. RT-Primers for mTLRs were purchased from Invivogen (Version # 07H09-SV). The expected product size for each mTLR primer pair is given in base pairs (bp).

PCR amplifications were performed at 98ºC for 75 s (initial denaturing step), followed by 35 cycles at 98ºC for 15 s, 58ºC or 63ºC for 20 s, 72ºC for 30 s, followed by a final step at 72ºC for 10 min. All reactions were run in a GeneAmp® PCR System 9700 thermo cycler.

3 Results

3.1 The Role of SHIP in the Maturation and Function of Mast Cell Subsets

MCs play a critical role in the regulation of many immune responses. Their role in type I hypersensitivity reactions, which occur following cross-linking IgE bound to high affinity IgE receptors (i.e., FcεRI) by multivalent allergens, has been the focus of MC research for a long time and is thus the best understood MC function to date. However, we now know that MCs also express many other receptors that are involved in immunological responses such as TLRs, FcγRs, functional MHC class II and others. The discovery that these receptors are expressed by MCs and the availability of MC knock-in models has led to a deeper understanding of the important role MCs play in the regulation of immune responses. For example, MCs have been shown to be involved in protection from various bacterial and parasitic infections. They also play a crucial role in degrading toxic moleucles like venom components (such as sarafotoxin 6b) and ET-1 (an endogenous vasoconstrictive peptide whose levels correlate with severity of sepsis). MCs have also been shown to be involved in the development of autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). Of note, depending on the autoimmune disease, MCs appear to worsen or ameliorate the condition, highlighting the complex function(s) that MCs have.

The mechanisms by which MCs start immune responses are as diverse as the functions that they possess. One important mechanism is the rapid release of preformed mediators. These mediators are stored within cytoplasmic granules within resting MCs and thus the process of release is called degranulation. Activation of MCs, however, is possible without inducing degranulation. Upon activation, MCs are also able to newly synthesize and release a large number of mediators that are important in orchestrating an immune response. This *de novo* synthesis of MC mediators can be started independent of or

together with degranulation, depending on the type, strength and duration of the activation signal.

Based on the central role MCs play in regulating immune responses and the resulting pathologies that occur when MCs are not appropriately activated, it is very important to understand how MCs are regulated. SHIP is known to be a key regulator of MC activation. The best studied role for SHIP in MC activation is its role as a master negative regulator of MC degranulation. In the absence of SHIP, MCs are much more prone to degranulate, thus starting an immune response and harming the host in the absence of an immunological threat. Since MC degranulation, a key step in the development of type I hypersensitivity reactions, has been the primary focus of MC research until recently, it is not surprising that SHIP's role within MCs is best understood in the context of degranulation. However, with the recent discovery that TLRs are expressed in MCs, there is now much interest in determining SHIP's role in regulating TLR-activated signalling in these cells. Related to this, while SHIP has been shown to be important for restraining TLR-mediated activation in macrophages, no such studies have been undertaken to date with MCs.

The goal of this study was therefore to understand the role that SHIP plays in TLR-mediated activation of MCs. Specifically, we asked if SHIP played a role in TLR-induced activation of both subtypes of MCs by deriving and testing CTMCs and MMCs (derived from WT and SHIP$^{-/-}$ mice spleens) as well as BMMCs, since they are the most common model of *in vitro* differentiated MCs.

To characterise the different WT and SHIP$^{-/-}$ MC subsets, they were assessed for their proliferation rate, maturity (via surface marker expression), subtype (by staining their granules) and TLR expression (by RT-PCR).

To functionally analyse these MCs, the different MC subsets were evaluated for their ability to survive in the presence of IgE, their ability to degranulate under various conditions and their ability to produce different cytokines upon TLR stimulation. The TLR ligands used for stimulation were LPS (100 ng/ml), dsRNA (50 μg/ml), CpG (0.3 μM) and PGN (10 μg/ml). We used these TLR ligands since, together, they covered the entire spectrum of the MyD88-dependent and MyD88-independent pathways (see section 1.3).

Results: The Role of SHIP in the Maturation and Function of Mast Cell Subsets

3.1.1 Mast Cell Model Systems: BMMCs, Spleen Derived CTLMCs, Spleen Derived MLMCs

BMMCs are the most established *in vitro* model of MCs. They are generated by culturing mouse BM cells in the presence of IL-3. This results in a large number of uniform and mature mast cells, as assessed by the cell surface expression of FcεRI and c-kit. Since BMMCs stain positive with alcian blue but not with safranin they are considered the *in vitro* counterpart of MMCs (Razin et al. 1984, Razin et al. 1982). A number of protocols have been published that allow either switching BMMCs *in vitro* to connective tissue-like MCs (CTLMCs) or the generation of CTLMCs from BM cells using IL-4 plus SCF, instead of IL-3. However, these protocols result in very low numbers of CTLMCs/mouse and hence are not very suitable for experiments requiring many cells (Levi-Schaffer et al. 1986, Ekoff et al. 2007). Other attempts to generate MCs start with peritoneal cells, i.e., peritoneal cell-derived MCs (PCMCs). This approach generates a larger number of MCs than can be obtained with IL-4 plus SCF cultured BM cells or by converting BMMCs into CTLMCs, but overall these numbers are still relatively low and the MCs obtained are phenotypically comparable to CTLMCs only (Malbec et al. 2007). Fetal skin-derived cultured MCs (FSMCs) can be obtained in large numbers by culturing the skin of mouse embryos in the presence of SCF plus IL-3. The MCs obtained are considered CTLMCs based on the presence of heparin in their granules (Yamada et al. 2003). However, this method also only generates CTLMCs and not both MLMCs and CTLMCs.

Being able to generate both CTLMCs and MLMCs in large numbers using the same starting cell population would be preferred since any differences observed can more likely be attributed to the different subtype rather than being due to intrinsic differences that exist between different populations of starting cells such as BM versus peritoneal cells.

Since the spleen contains MC progenitors, differentiating MCs *in vitro* from spleen cells has been considered a good way to obtain both CTLMS and MLMCs. Indeed, if cultured in the presence of IL-3, spleen cells will differentiate into MLMCs and if cultured in the presence of SCF, they will differentiate into CTLMCs (Kataoka et al. 2005). Throughout this thesis splenic CTLMCs are called “WT CTMCs” if derived from WT and “SHIP$^{-/-}$

CTMCs" if derived from SHIP$^{-/-}$ mice. Similarly, splenic MLMCs are called "WT MMCs" if derived from WT and "SHIP$^{-/-}$ MMCs" if derived from SHIP$^{-/-}$ mice.

3.1.2 SHIP is a negative Regulator of MC Differentiation

To investigate the influence of SHIP on the maturation and proliferation of MCs, we derived different MC subsets as described in Materials and Methods. Cell counts were performed on a weekly basis to monitor proliferation. To test MCs for their maturity they were stained for the MC surface markers c-kit and FcεR1α, starting 3 weeks after initiation of cultures. Cells that expressed both c-kit and FcεR1α were considered mature MCs. Cells were stained with safranin and alcian blue to determine their subtype.

We found that SHIP$^{-/-}$ MCs matured faster than WT MCs (Figure 3.1.2-A). While this increased rate of differentiation was very apparent with CTMCs and BMMCs, it was also detectable, albeit to a lesser degree, with MMCs. Also of note, CTMCs matured faster than MMCs or BMMCs, i.e., after 3 weeks of culture, over 80% of WT and 95% of SHIP$^{-/-}$ spleen-derived cells were mature CTMCs, while only 65% of WT and 80% of SHIP$^{-/-}$ spleen-derived cells were MMCs. Similarly, only 35% of BM-derived WT and 80% SHIP$^{-/-}$ cells were BMMCs. In both MMC and BMMC cultures, we found a strong increase in mature MCs between weeks 3 and 4. By week 5 over 90% of the cells in all cultures were mature MCs.

When looking at the proliferation of MCs, we found a mixed pattern (Figure 3.1.2-B). While SHIP$^{-/-}$ CTMC cultures proliferated faster than WT CTMC cultures, we found no significant difference in WT and SHIP$^{-/-}$ MMC cultures. In BMMC cultures, WT cells actually proliferated faster than SHIP$^{-/-}$ cells. The safranin and alcian blue stains confirmed the MC subtypes**,** i.e., CTMC granules were stained red by safranin, confirming the presence of heparin. Since CTMCs are thought to be an *in vitro* model for connective tissue MCs (Kataoka et al. 2005) this was expected. In both of the mucosal MC models, i.e., MMCs and BMMCs, granules were stained blue by alcian blue, confirming the presence of chondroitin sulphate. SHIP$^{-/-}$ MCs stained like their WT counterparts,

indicating that the absence of SHIP did not affect the granule content of these MC subtypes (Figure 3.1.2-C).

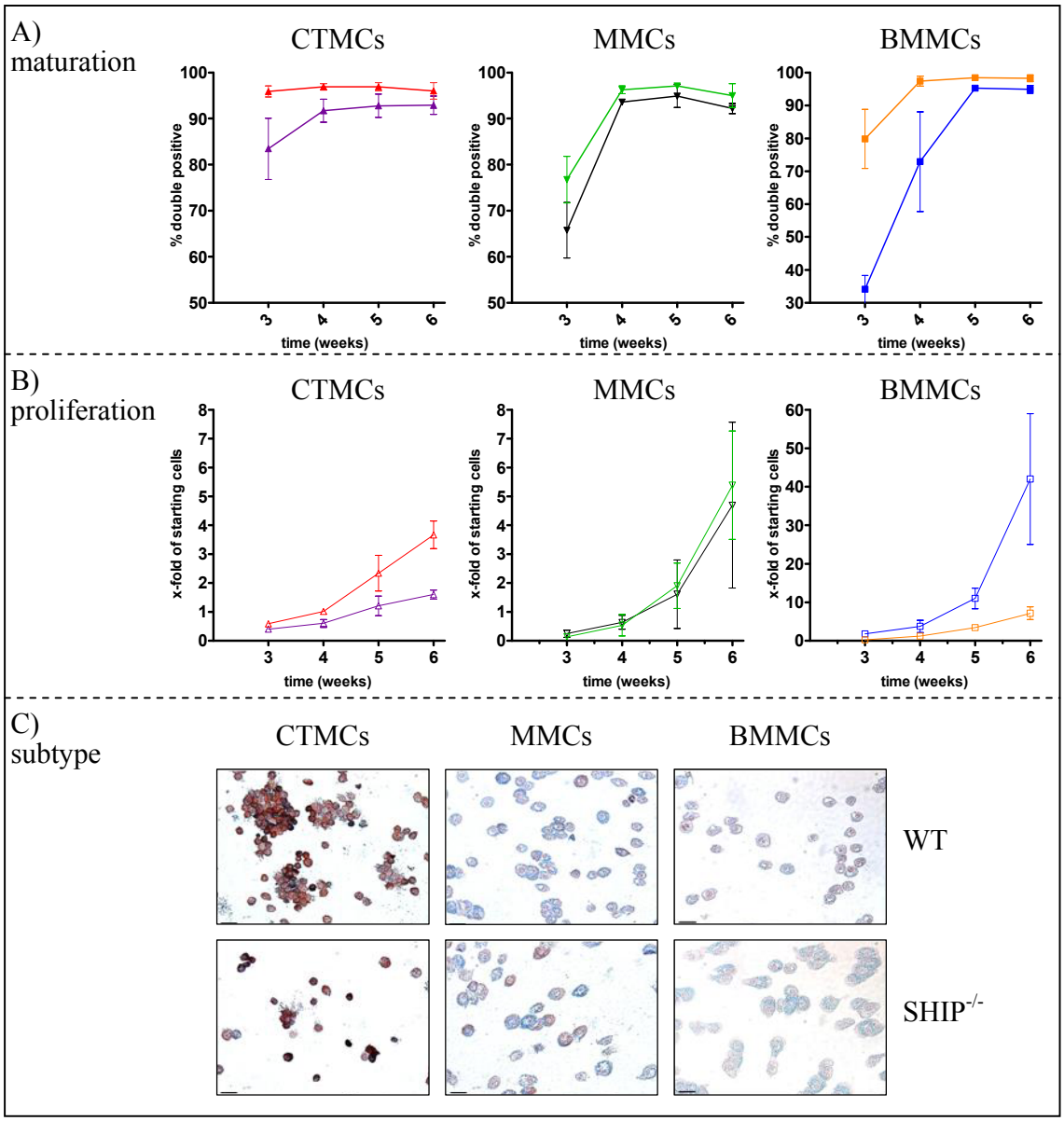


Figure 3.1.2 Influence of SHIP on *in vitro* MC proliferations and differentiation. Different MC subtypes were differentiated from either spleen or BM and proliferation and maturity tested from week 3-6. CTMCs were derived in IMDM + 10% FCS + 50 ng/ml SCF. MMCs were derived in IMDM + 10% FCS + 5 ng/ml IL-3 and BMMCs were derived in IMDM + 15% FCS + 30 ng/ml IL-3 (see Material and Methods for a detailed protocol). **(A)** The maturity of MCs was assessed by staining for the surface expression of c-kit and FcεR1α. Cells that were doubly positive were considered mature. **(B)** Cell numbers were counted on a weekly basis. Shown is the absolute cell number divided by the starting number of cells at week 0. (A & B) WT CTMCs are represented as purple triangles, $SHIP^{-/-}$ CTs as red triangles, WT MMCs as black triangles, $SHIP^{-/-}$ MMCs as green triangles, WT BMMCs as blue squares and $SHIP^{-/-}$ BMMCs as orange squares. **(C)** Cells were stained with safranin, which stains heparin (present in CTMC granules) red and with alcian blue, which stains chondroitin sulphate (present in MMC granules) blue. Black bars represent 50 µm.

3.1.3 SHIP influences TLR Expression Patterns

Due to their strategic positioning, MCs are among the first cells to recognize invading pathogens and do so, in large part, via their expression of TLRs. To date, expression studies of TLRs on mast cells has been restricted to mast cell lines, fetal skin derived mast cells (FSMCs) or BMMCs (reviewed in Stelekati et al. 2007). Also, the influence of SHIP on TLR expression has not yet been investigated. Hence, we wanted to test which TLRs are expressed in the different MC subtypes and determine if TLRs are expressed at different levels in WT and SHIP$^{-/-}$ MCs. Due to the lack of good Abs for some of the TLRs, it was not possible to test the expression of all TLRs with Ab-based techniques, such as Western blots or flow cytometry. Thus, we decided to check the expression of TLRs by RT-PCR. To do this, we isolated total RNA from mature *in vitro* derived MCs and RT-PCR analyzed the expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8 and TLR9 mRNAs.

Depending on the MC subtype we found different expression patterns (see Figure 3.1.3). For example, both WT and SHIP$^{-/-}$ CTMCs expressed TLRs 1, 2, 4, 6, 8 and 9 with TLRs 1, 2, 8 and 9 being more highly expressed in SHIP$^{-/-}$ MCs. Also, SHIP$^{-/-}$, but not WT, MCs expressed TLRs 3 and 7, but only at low levels. In MMCs and BMMCs we found expression of TLR1, TLR2, TLR4 and TLR6. However, in SHIP$^{-/-}$ cells we detected higher levels of these TLR mRNAs than in WT cells. In BMMCs, TLR1 and TLR6 were more highly expressed in SHIP$^{-/-}$ cells compared to WT cells. Since both MMCs and BMMCs are models of mucosal MCs, it is not surprising that both expressed the same TLRs.

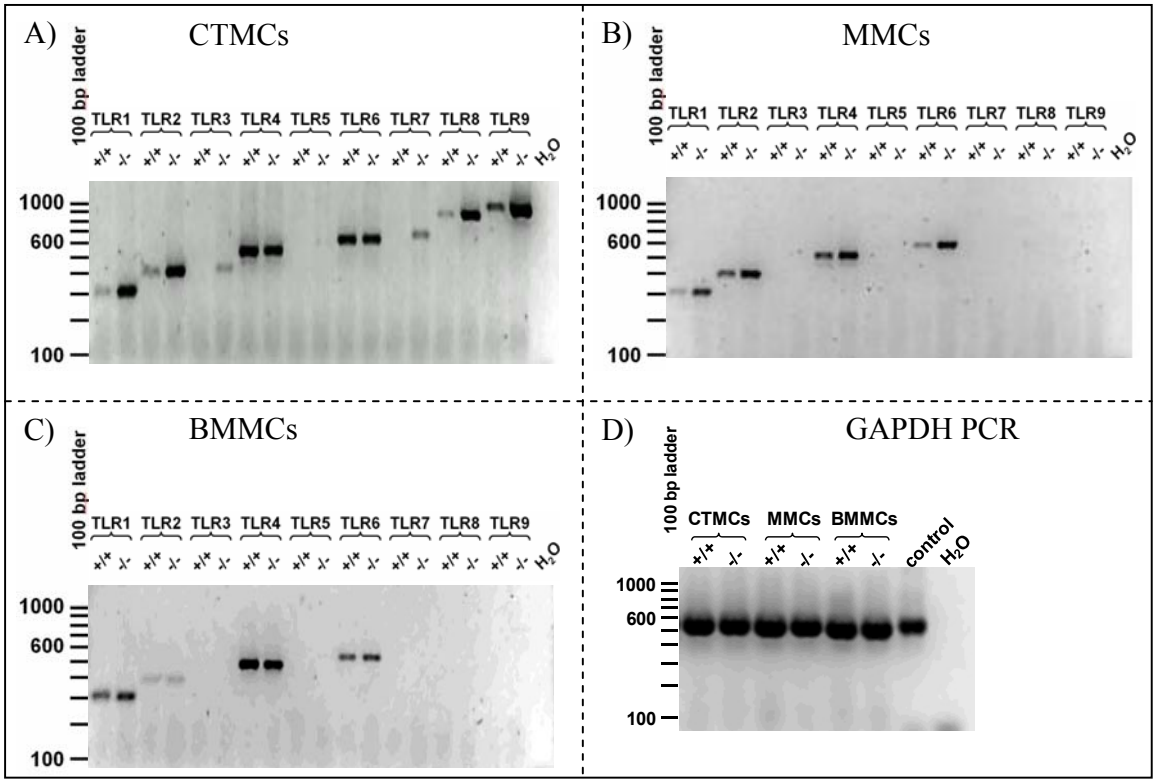


Figure 3.1.3 Analysis of TLR expression by RT-PCR. Total RNA was isolated from mature *in vitro* differentiated MCs. After reverse transcription using M-MLV RT and an oliogo(dT)$_{18}$ primer, equal amounts of cDNA were used in each reaction to test for TLR expression. **(A-C)** Lanes labeled with "+/+" indicate WT and lanes indicated with "-/-" indicate SHIP$^{-/-}$. (A) CTMC RT-PCRs were performed using 200 ng of cDNA. 160 ng of cDNA were used for MMCs **(B)** and BMMCs **(C)**. **(D)** As a loading control 100 ng of cDNA were used as template in a PCR reaction with primers specific for GAPDH. Genomic DNA (10 ng) was used as a positive control.

3.1.4 SHIP enhances IgE-induced BMMC Survival

IgE + Ag-induced crosslinking of FcεR1α is the best studied activation pathway for MCs. However, IgE alone (without Ag present) has been shown to enhance BMMC survival, with some IgEs being much more potent than others (Kalesnikoff et al. 2001, Sly et al. 2008). We therefore compared the ability of different MC subsets to survive in the presence of IgE alone when deprived of their normal growth factors such as IL-3 and/or SCF (Figure 3.1.4). After four days there were almost no surviving WT or SHIP$^{-/-}$ CTMCs in the absence of IgE (Figure 3.1.4-A). However, in the presence of IgE, both WT and SHIP$^{-/-}$ CTMCs showed a significant increase in survival, but there was no difference in viability between WT and SHIP$^{-/-}$ CTMCs. Interestingly, MMCs (Figure 3.1.4-B) survived better without IgE than CTMCs and the addition of IgE also resulted in increased survival. But again, there was no significant difference in survival between WT and SHIP$^{-/-}$ MMCs, with or without IgE. Lastly, WT and SHIP$^{-/-}$ BMMCs survived equally well, and the best of the 3 MC subtypes, in the absence of IgE (Figure 3.1.4-C). Interestingly, while IgE increased the survival of both WT and SHIP$^{-/-}$ BMMCs over the 4 day timecourse, it was far more effective at promoting the survival of WT BMMCs. This is in marked contrast to a previous study with WT and SHIP$^{-/-}$ BMMCs (Liu et al. 1999). However, in those studies BMMC survival assays were performed in the presence of 2% FCS while our survival assays were performed in serum free conditions.

Overall, MMCs survived better, with or without IgE, than CTMCs but there was no significant difference if SHIP was present or not with either of these two MC subsets. In the presence of IgE, both MMCs and CTMCs showed an increased survival. However, with IgE-treated BMMCs, we saw a dramatic improvement in cell survival/proliferation when SHIP was present. This may be related to our finding that IgE alone can trigger degranulation of SHIP$^{-/-}$ BMMCs (Huber et al. 1998b).

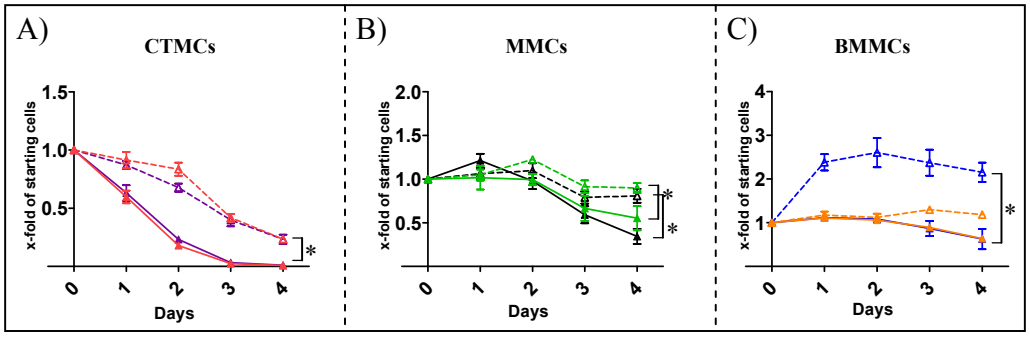


Figure 3.1.4 IgE-induced survival of MCs. Different MC subsets derived from Spleen and BM were washed out of their growth factors and resuspended in 0.1% BSA media (closed triangles, solid lines) or plus 5 µg/ml SPE-7 IgE (open triangles, dashed lines) and viable cells counted on the days indicated. **(A)** CTMCs (WT in purple, SHIP$^{-/-}$ in red). **(B)** MMCs (WT in black, SHIP$^{-/-}$ in green). **(C)** BMMCs (WT in blue, SHIP$^{-/-}$ in orange). Values are the mean ± SEM from two independent experiments in triplicate. Significant differences ($p<0.05$) are indicated (*).

3.1.5 TLR Activation does not trigger MMC, CTMC or BMMC Degranulation and SHIP Deficiency does not alter this

MCs can quickly release preformed mediators by degranulation, and this degranulation plays a crucial role in the development of Type I hypersensitivity reactions. In this context, MC degranulation is triggered by IgE + Ag-induced crosslinking of FcεR1α. SHIP has been shown to play a critical role in the negative regulation of this process (Huber et al. 1998b).

We wanted to determine if CTMCs and MMCs showed the same IgE + Ag-induced degranulation properties as BMMCs and if TLR ligands affected degranulation. Moreover, we wanted to see if SHIP played a role in these degranulations.

To test if and how strong MCs degranulate in response to IgE + Ag, we preloaded MCs with IgE (0.1 µg/ml) and then stimulated them with increasing doses of Ag (Figure 3.1.5-A). We found that SHIP$^{-/-}$ MCs degranulated significantly more than WT MCs at all Ag concentrations tested, underlining SHIP's function as a master negative regulator of MC degranulation. Interestingly, while WT CTMCs and MMCs degranulated to approximately the same extent, SHIP$^{-/-}$ MMCs degranulated more strongly than CTMCs. Since MMCs are

mucosal like MCs, we expected their degranulation properties to be similar to those of BMMCs. However, we found that WT BMMCs degranulated more vigorously than wild type MMCs and, intriguingly, $SHIP^{-/-}$ MMCs degranulated more than $SHIP^{-/-}$ BMMCs. When MCs were stimulated with only TLR ligands (Figure 3.1.5-B), no degranulation was observed in any MC subset.

Synergistic interactions between TLRs and IgE + Ag have been reported for cytokine production(Masuda et al. 2002, Qiao et al. 2006) but not for degranulation. However, since those studies used BMMCs, we decided to investigate if there was any synergy in MCs derived from spleen. To test this, MCs were preloaded with IgE (0.1 μg/ml) and then stimulated with Ag (2 ng/ml) in the presence or absence of TLR ligands (Figure 3.1.5-C). We repeated this experiment with the $SHIP^{-/-}$ BMMCs and MMCs at a lower Ag concentration (0.2 ng/ml) since these cells degranulated to maximal levels with IgE + 2 ng/ml Ag (Figure 3.1.5-D). The addition of TLR ligands did not change the degranulation observed with IgE + Ag alone. This indicated that there were no synergistic effects between TLRs and IgE + Ag with respect to degranulation.

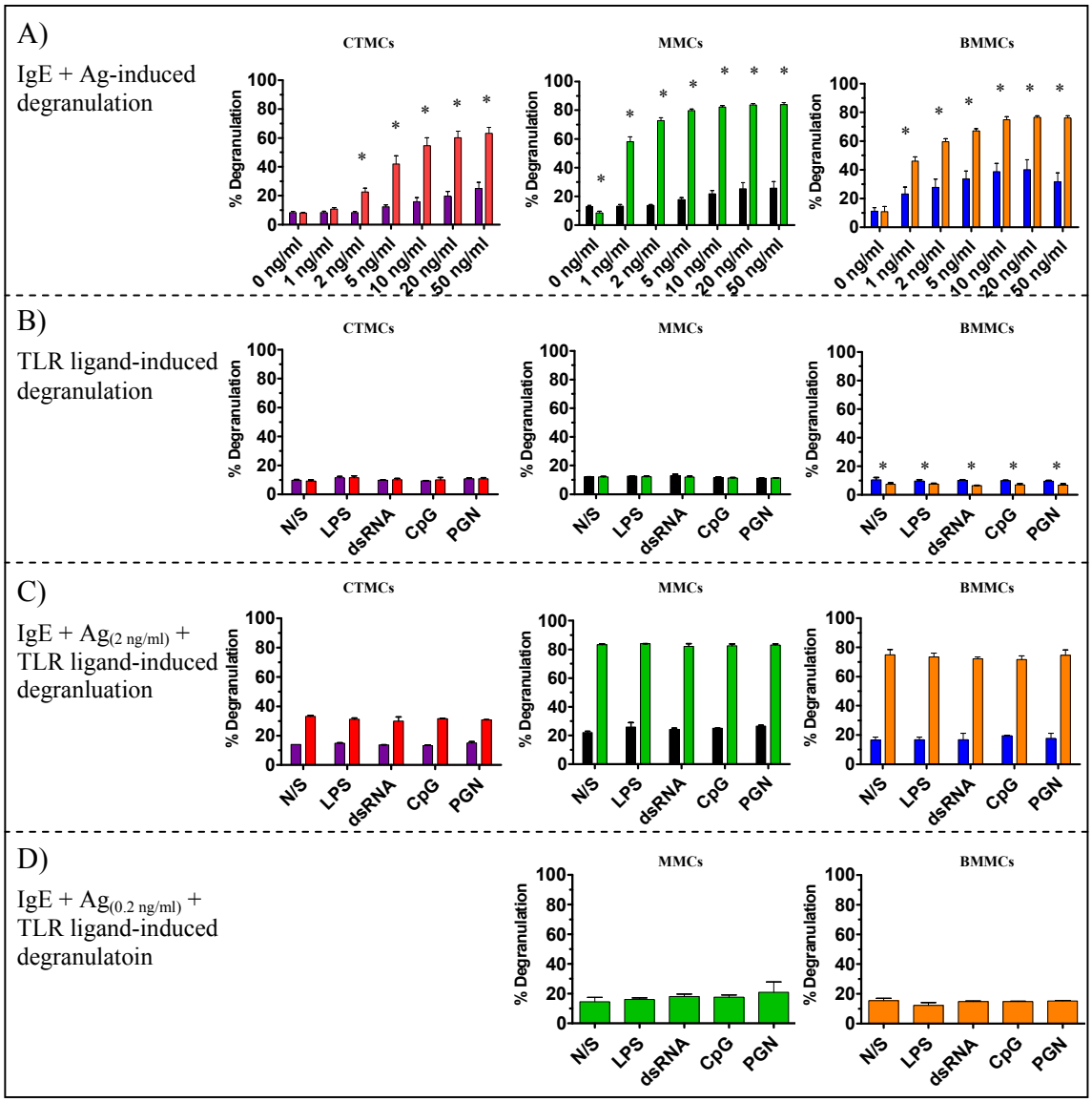


Figure 3.1.5 Degranulation properties of different MC subsets. Different MC subsets from WT and SHIP$^{-/-}$ mice were tested for their degranulation properties. These subsets included WT CTMCs (purple bars), SHIP$^{-/-}$ CTMCs (red bars), WT MMCs (black bars), SHIP$^{-/-}$ MMCs (green bars) as well as WT (blue bars) and SHIP$^{-/-}$ BMMCs (orange bars). For experiments involving IgE + Ag stimulation **(A,C and D)**, MCs were incubated with 0.1 µg/ml IgE for 18 hrs at 37˚C and then (A) Increasing concentrations of Ag were added (final concentrations indicated below x-axis) for 1 h. **(B)** MCs were treated with buffer alone (not stimulated, N/S), LPS (100 ng/ml), dsRNA (50 µg/ml), CpG (0.3 µM) or PGN (10 µg/ml) for 1 h. (C and D) MCs were preloaded with IgE as above and then treated with either buffer (not TLR stimulated, N/S) or with TLR ligands (same concentrations as in B) + 2 ng/ml Ag **(C)** or 0.2 ng/ml Ag **(D)** for 1 h. Significant results ($p<0.05$) between WT and SHIP$^{-/-}$ samples are indicated (*). For **(C)**, all samples were preloaded with IgE (0.1µg/ml) and stimulated with Ag (2 ng/ml) and the significance for these conditions has been shown in **(A)**. Since the addition of TLR ligands did not lead to any additional changes, significance in these graphs was not indicated. Results are shown as mean ± SEM from at least 2 independent experiments carried out in duplicate **(A-C)** or 1 experiment in triplicate **(D)**.

3.1.6 SHIP is a central Regulator of Cytokine Production in MCs

In addition to IgE + Ag-induced degranulation, which releases preformed mediators such as histamine and cytokines, it is now clear that MCs also play a central role in initiating and regulating innate and adaptive immune responses. They do so, at least in part, by releasing newly formed cytokines and chemokines upon TLR stimulation (reviewed in (Metz et al. 2007) and (Stelekati et al. 2007)). These secreted proteins are able to attract and stimulate or suppress different immune cells.

Therefore, we tested the ability of different subsets of MCs to produce the cytokines IL-6, IL-10, IL-12 and TNFα upon TLR stimulation alone or in combination with IgE or IgE + Ag (Figure 3.1.6/1-3). Each of these cytokines has an established role in the development of inflammation and has been shown to be produced by MCs(Stelekati et al. 2007, Nakano et al. 2007).

First, the ability of MCs to secrete cytokines in response to TLR ligands alone was tested by starving MCs over night and then treating with or without (NS) LPS (to activate TLR4), dsRNA (TLR3), CpG (TLR9) or PGN (TLR2). As can be seen in Figure 3.1.6/1, WT CTMCs were by far the most robust producers of all 4 cytokines in response to stimulation with these TLR ligands alone. In marked contrast, the $SHIP^{-/-}$ CTMCs produced little or no cytokines in response to these TLR agonists, indicating that SHIP was a potent positive regulator of TLR-induced cytokine production in CTMCs. Both WT and $SHIP^{-/-}$ MMCs, on the other hand, produced little to no cytokines in response to TLR stimulation. Lastly, BMMCs, like MMCs, were poor responders (compare ordinate scales) but WT BMMCs were slightly better than their $SHIP^{-/-}$ counterparts at producing IL-6 and TNFα. IL-10 and IL-12 levels were not detectable from MMCs or BMMCs.

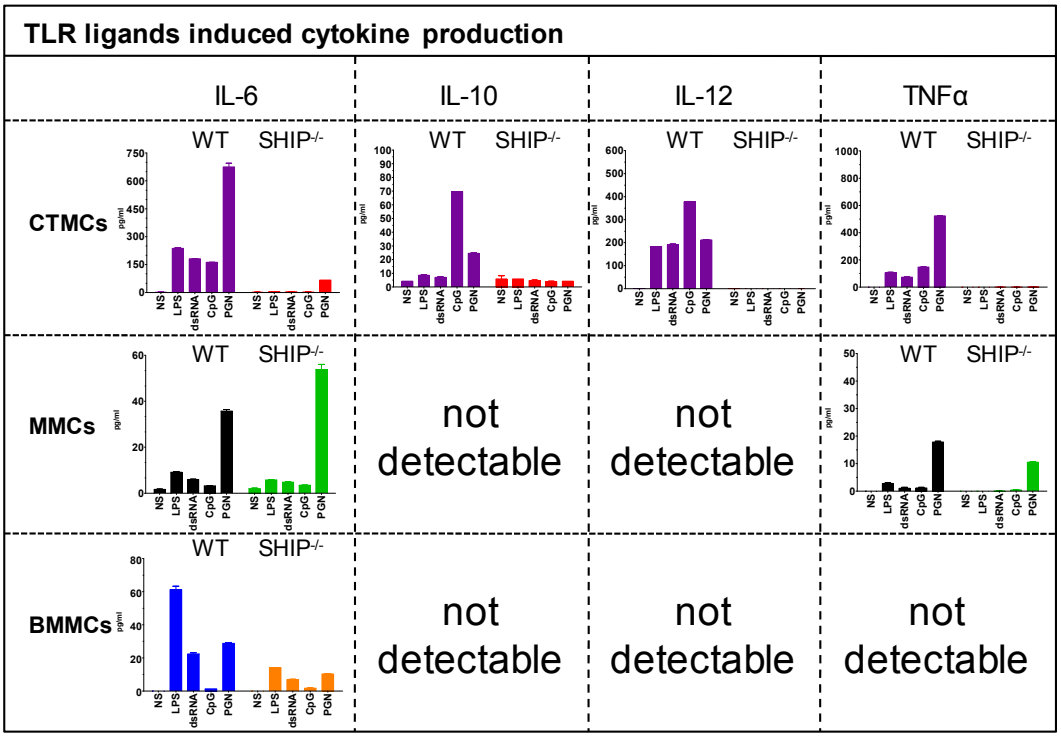


Figure 3.1.6/1 MC stimulation with TLR ligands alone. CTMCs (WT in purple, SHIP$^{-/-}$ in red), MMCs (WT in black, SHIP$^{-/-}$ in green) and BMMCs (WT in blue, SHIP$^{-/-}$ in orange) were stimulated for 24h with TLR ligands. After 24 h, cell free supernatants were collected and the indicated cytokines measured by ELISA. Values are the mean ± SD from one representative experiment, measured in duplicate. Similar results were obtained in 2 independent experiments.

When MCs were stimulated for 24h with TLR ligands in the presence of 0.1µg/ml SPE-7 IgE, there was no change in the level or pattern of cytokine production from either WT or SHIP$^{-/-}$ CTMCs (Figure 3.1.6/2). Interestingly, however, both SHIP$^{-/-}$ MMCs and BMMCs now produced higher levels of IL-6 and TNFα than their WT counterparts. As with TLR ligands alone, IL-10 and IL-12 were not detectable from MMCs or BMMCs. These results suggest that SHIP is a negative regulator of IgE-induced cytokine secretion from MMCs and BMMCs. This is consistent with our previously reported studies showing that IgE alone stimulates more IL-6 from SHIP$^{-/-}$ than from WT BMMCs (Kalesnikoff et al. 2002).

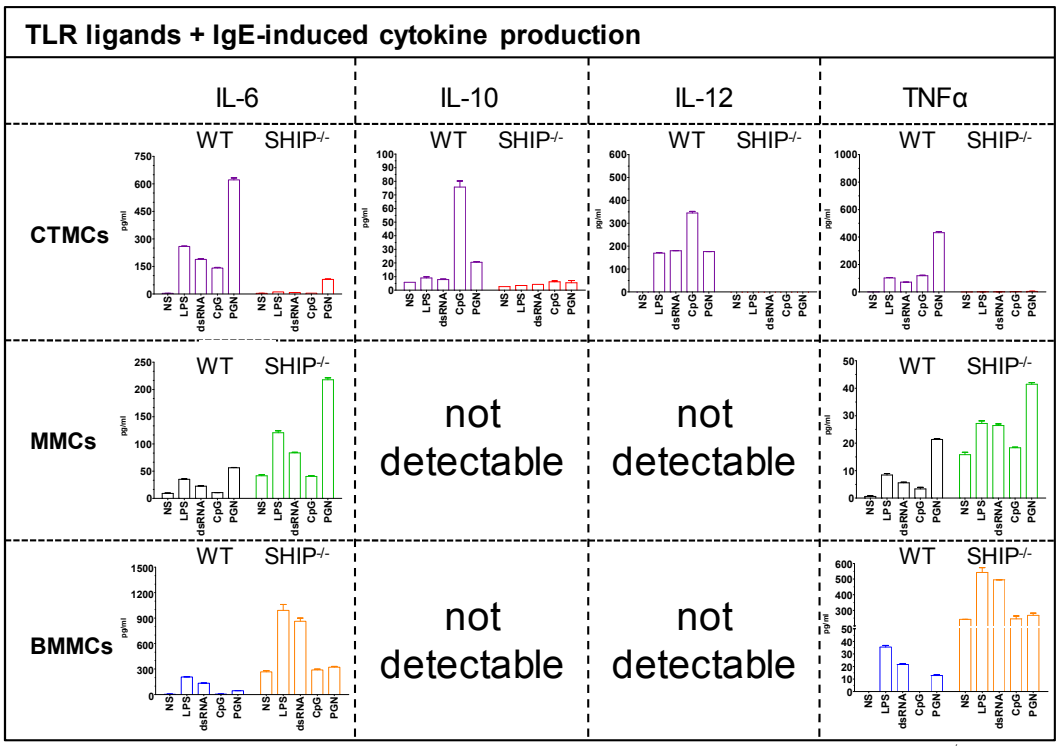


Figure 3.1.6/2 MC stimulation with TLR ligands plus IgE. CTMCs (WT in purple, SHIP$^{-/-}$ in red), MMCs (WT in black, SHIP$^{-/-}$ in green) and BMMCs (WT in blue, SHIP$^{-/-}$ in orange) were stimulated for 24h with TLR ligands + 0.1 µg/ml SPE-7 IgE. After 24 h, cell free supernatants were collected and the indicated cytokines measured by ELISA. Values are the mean ± SD from one representative experiment, measured in duplicate. Similar results were obtained in 2 independent experiments.

When the different MC subsets were stimulated with IgE + Ag in combination with TLR ligands, the WT but not SHIP$^{-/-}$ CTMCs showed some differences in their response, compared to stimulations with TLR ligands alone. While IgE with or without Ag had no effect on these cells (i.e., NS levels in these figures signify IgE without TLR ligand or IgE + Ag without TLR ligand), IgE + Ag with CpG led to much higher levels of secreted IL-12 compared to stimulation with CpG alone or IgE + CpG. In a similar manner, TNFα levels were also higher, especially when WT CTMCs were stimulated with PGN (Figure 3.1.6/3). Importantly, there was a dramatic increase in the levels of IL-6 and TNFα produced by SHIP$^{-/-}$ MMCs, even without TLR ligand stimulation (NS), but the levels increased with the addition of TLR ligands. No such effect was observed with WT MMCs, demonstrating the repressive role that SHIP plays in IL-6 and TNFα production from this MC subset. Interestingly, the absence of SHIP did not increase IL-10 or IL-12 from these cells,

revealing that the production of these cytokines is not regulated in the same way. In keeping with the similarity between MMCs and BMMCs, SHIP$^{-/-}$ BMMCs showed increased production of IL-6 and TNFα. SHIP$^{-/-}$ MMCs and BMMCs, produced high levels of IL-6 and TNFα, even in the absence of TLR agonists (Figure 3.1.6/3; N/S column). The addition of TLR ligands increased the amount of IL-6 and TNFα. As with Spl MMCs, IL-10 and IL-12 were not detectably secreted from either WT or SHIP$^{-/-}$ BMMCs.

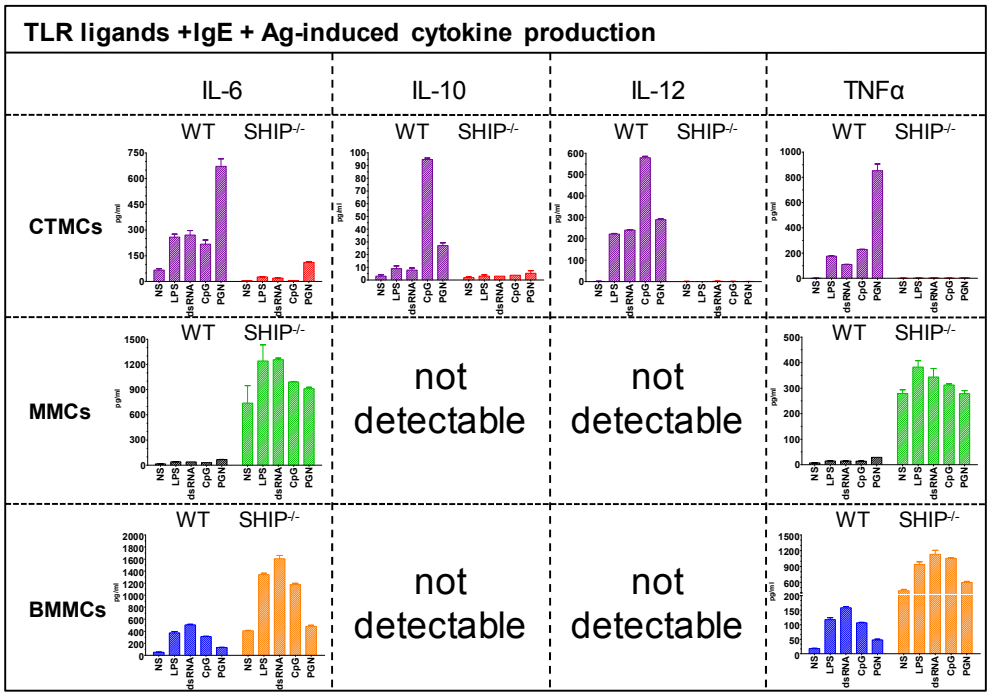


Figure 3.1.6/3 MC stimulation with TLR ligands and IgE + Ag. CTMCs (WT in purple, SHIP$^{-/-}$ in red), MMCs (WT in black, SHIP$^{-/-}$ in green) and BMMCs (WT in blue, SHIP$^{-/-}$ in orange) were stimulated for 24h with TLR ligands and 0.1 µg/ml SPE-7 IgE + 1 ng/ml Ag. After 24 h, cell free supernatants were collected and the indicated cytokines measured by ELISA. Values are the mean ± SD from one representative experiment, measured in duplicate. Similar results were obtained in 2 independent experiments.

3.1.7 MCs and Basophils have Distinct Roles in the Regulation of Immune Responses

As mentioned earlier, studies in our laboratory have shown that macrophages in SHIP deficient mice become skewed at an early age toward an alternatively activated (M2) phenotype and this promotes tumour growth (Rauh et al. 2005). More recent studies in our lab suggest that basophils, via their production of high levels of IL-4, play a critical role in this M2 skewing and that SHIP negatively regulates this IL-4 production from these basophils (Kuroda, E.; *et.al.* submitted). Since both MCs and basophils are important mediators of type I hypersensitivity reactions, which involve T_H2 skewing by IL-4, we compared the ability of *in vitro* derived MCs and basophils to produce IL-4 in response to either IgE (with or without Ag) or IL-3 (see figure 3.1.7). We found that neither IL-3 nor IgE could stimulate the secretion of detectable levels of IL-4 from WT or $SHIP^{-/-}$ CTMCs, MMCs or BMMCs but that IgE + Ag did trigger a low but significant level of IL-4 from $SHIP^{-/-}$ MMCs and BMMCs. On the other hand, both WT and $SHIP^{-/-}$ basophils, produced much higher levels of IL-4 in response to IL-3 and especially in response to IgE alone or IgE + Ag. As well, SHIP acted as a negative regulator of IL-4 production in these cells. Since we were able to detect low levels of IL-4 from $SHIP^{-/-}$ MMCs when stimulated with IgE + Ag we decided to test if further stimulation with LPS led to increased IL-4 production. This, however, was not the case (data not shown).

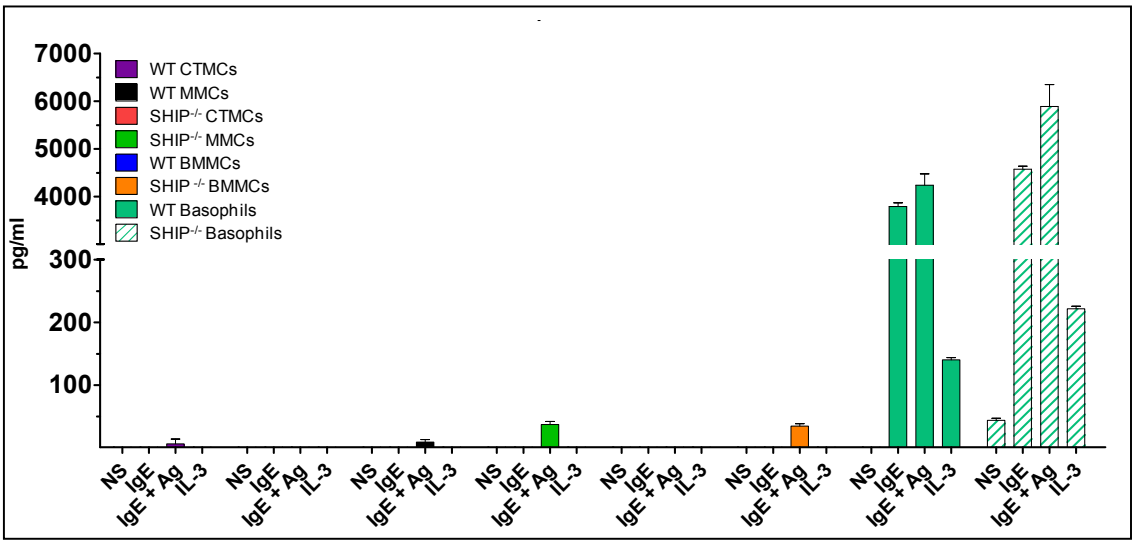


Figure 3.1.7 IL-4 production in MCs and basophils. WT and SHIP$^{-/-}$ CTMCs, MMCs, BMMCs and basophils were derived as described in Material and Methods. To test IL-4 production, 10,000 cells were stimulated in 100 µl final volume with either media alone (not stimulated, NS), IL-3 (100 ng/ml), IgE (SPE-7, 1 µg/ml) alone or + Ag (100 ng/ml) and after 24 h, cell free supernatants were collected and IL-4 levels measured by ELISA. Values are the mean ± SD from one representative experiment, measured in duplicate. Similar results were obtained in 2 independent experiments.

3.2 Regulation of SHIP Protein Levels

SHIP activity appears to be regulated, in large part, via modulation of its protein levels. For example, during a Gm^- bacterial infection, which can be mimicked by treating *in vitro* derived macrophages with LPS, SHIP protein levels are increased 10-fold via the production of autocrine-acting TGFβ, thus protecting the host from septic shock (Sly et al. 2004). On the other hand, downregulation of SHIP protein levels seems to be an important event in switching macrophages from a "killer" M1 phenotype to a "healer" M2 phenotype and IL-4 is thought to play an important role in this switch (Ho and Sly 2009, Rauh et al. 2005). Interestingly, certain cancers, such as BCR-ABL driven CML, also downregulate SHIP during their progression, which increases the activity of the pro-survival/proliferation PI3K pathway and thus likely gives these cells a selective advantage over normal hemopoietic progenitors (Sattler et al. 1999). However, very little is known about the mechanisms involved. Thus, we decided to study the mechanisms involved in the downregulation of SHIP in two different models, $BaF3_{p210\text{-}tetOFF}$ cells, a model for BCR-ABL-induced SHIP downregulation, and IL-4-induced SHIP downregulation in bone-marrow derived macrophages (BMmacs), as a model for normal primary cell SHIP downregulation.

3.2.1 SHIP Levels In $Ba/F3_{p210\text{-}tetOFF}$ Cells

3.2.1.1 $BaF3_{p210\text{-}tetOFF}$ Cells as a Model System

BCR-ABL is the product of the t(9,22)(q34;q11) Philadelphia chromosome (Ph) translocation and is the oncogene responsible for chronic myelogenous leukemia (CML) (Ben-Neriah et al. 1986). Besides other effects, its tyrosine kinase activity leads to decreased protein levels of SHIP protein (Sattler et al. 1999). Considering the central role that SHIP plays in hemopoiesis and the importance of its protein level in determining its effect on the PI3K pathway (Valderrama-Carvajal et al. 2002, Sly et al. 2004, Sly et al.

2009), we thought an inducible system for BCR-ABL might provide a useful tool to study the mechanisms by which BCR-ABL reduces SHIP levels. Relevant to this, Dougray *et al.* had recently infected Ba/F3 cells, an IL-3 dependent mouse pro B-cell line, with a retroviral construct carrying the BCR-ABL p210 cDNA, the Tet repressor-VP16 fusion protein (tTa) and Tet-OP (tetop) sequences, under the control of a minimal CMV promoter (CMV_{min}) (see figure 3.2.1.1 A). This cell line, termed $Ba/F3_{p210\text{-}tetOFF}$, expresses BCR-ABL and tTa from the same transcript. Because of the weak promoter activity of CMV_{min}, protein levels of BCR-ABL and tTa are very low initially but eventually increase to high levels due to the positive feedback loop of tTa, which leads to increased CMV_{min} activity. However, this is a slow process and takes 2 weeks before BCR-ABL and tTa reach maximal levels. Upon addition of doxycycline, a member of the tetracycline family, tTa can no longer bind to the tetop sequence resulting in reduced activity of the CMV_{min} activity and with it a decrease in BCR-ABL and tTa protein levels (Dugray et al. 2001).

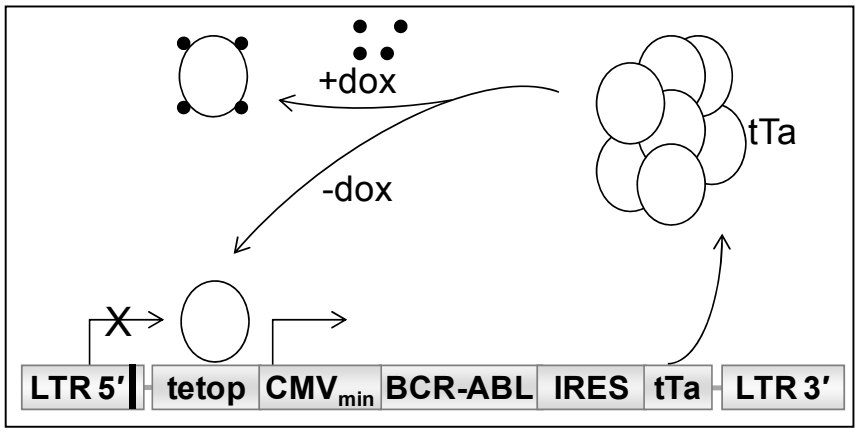


Figure 3.2.1.1 Construct for the suppression of the BCR-ABL transgene. $Ba/F3_{p210\text{-}tetOFF}$ cells were derived from Ba/F3 cells as described in(Dugray et al. 2001). In the absence of doxycycline (dox) both BCR-ABL and the tTa (Tet repressor-VP16 fusion protein) get expressed. Increasing levels of tTa lead to higher activity of the minimal CMV (CMV_{min}) promoter which in turn leads to increasing BCR-ABL and tTa levels. Upon addition of doxycycline, tTa cannot bind to the CMV_{min} promoter and BCR-ABL as well as tTa levels decrease. *Adapted and modified from* (Dugray et al. 2001).

3.2.1.2 BCR-ABL Reduces SHIP but not SHIP2 or PTEN Protein Levels

To confirm previous reports that BCR-ABL reduces SHIP protein levels (Sattler et al. 1999), we first tested the effect of BCR-ABL on SHIP expression in the Ba/F3$_{p210\text{-}tetOFF}$ system. We also examined the effect of BCR-ABL levels on SHIP2 and phosphatase/tensin homolog deleted on chromosome 10 (PTEN) since these 2 lipid phosphatases are also involved in negatively regulating the PI3K pathway. Specifically, Ba/F3$_{p210\text{-}tetOFF}$ cells, cultured in cytokine-free medium (since the expression of BCR-ABL enables these cells to grow in the absence of IL-3 (Daley and Baltimore 1988), were transferred either into medium containing IL-3 or into medium containing IL-3 plus doxycycline (to turn off BCR-ABL expression) and the expression of SHIP, SHIP2 and PTEN was monitored for up to 7 days by Western blot analysis. As can be seen in figure 3.2.1.2-A, the addition of doxycycline dramatically reduced BCR-ABL levels within 24 h and they remained at this low level for the duration of the study. SHIP levels, in response to this decrease in BCR-ABL, increased dramatically within 24h and plateaued at this level for the 7 days of the study. Interestingly, however, SHIP2 levels did not change upon the addition of IL-3 or doxycycline. PTEN levels, on the other hand, were higher in the presence of IL-3 alone compared to cytokine-free medium. However, the further addition of doxycycline did not increase PTEN expression. These results were consistent with BCR-ABL affecting SHIP but not SHIP2 or PTEN levels. Since IL-3 increased the expression of PTEN we carried out all further experiments in the presence of IL-3 to ensure that SHIP2 and PTEN levels remained constant throughout.

Cells from day 0 to day 3 (marked with "*" in figure 3.2.1.2-A) were also subjected to intracellular staining with anti-SHIP Ab (see figure 3.2.1.2-B) and confirmed that (a) IL-3 alone does not change SHIP levels in the presence of BCR-ABL and (b) doxycycline (which lowers BCR-ABL levels) increases SHIP levels to the same extent in all cells and reaches maximal levels within 24h.

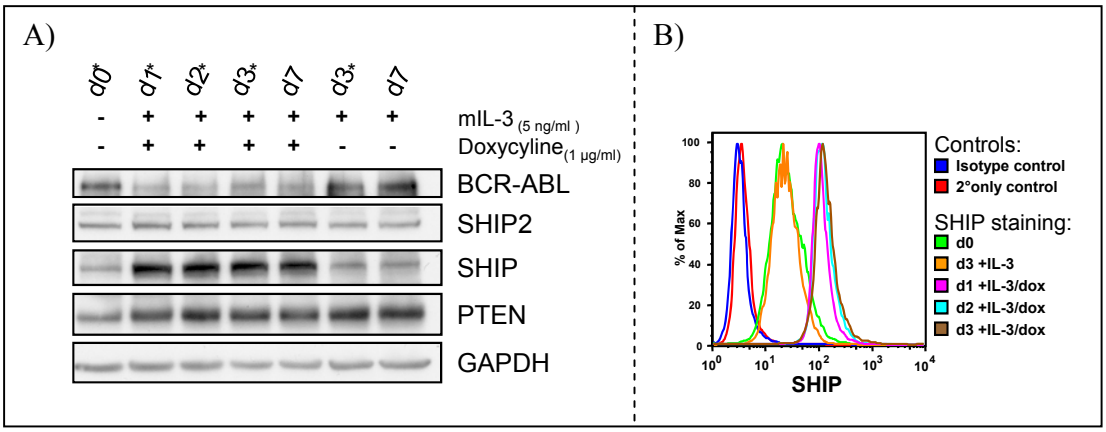
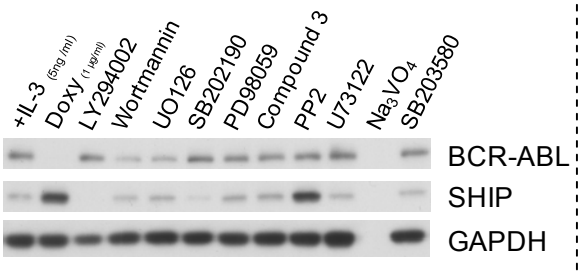


Figure 3.2.1.2 Effect of BCR-ABL on SHIP, SHIP2 and PTEN. Ba/F3$_{p210\text{-}tetOFF}$ cells were incubated with IL-3 or IL-3 plus doxycycline for up to 7 days. **(A)** Western blot analysis of total cell lysates was carried out, using anti-c-ABL, anti-SHIP2, anti-SHIP, anti-PTEN and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as a loading control). The blot shown is representative of 2 independent experiments.
(B) On days marked with an asterisk in **(A)**, intracellular staining for SHIP was performed and this showed that SHIP levels changed equally in all cells in response to doxycycline.

3.2.1.3 The BCR-ABL-Induced Drop in SHIP Levels is Inhibited by The Src Kinase Inhibitor PP2

As shown above, BCR-ABL levels drop upon the addition of doxycycline in Ba/F3$_{p210\text{-}tetOFF}$ cells and, as a result, SHIP levels increase. However, since the CMV$_{min}$ promoter activity is very weak in this cell system, it takes a long time for BCR-ABL levels to rise again after washing out doxycycline from the cells and therefore, we could not carry out the reciprocal experiments. This prevented us from testing pathway specific inhibitors to determine if we could prevent the increasing levels of BCR-ABL from reducing SHIP levels as it would require exposure of the cells to these toxic inhibitors for 1-2 weeks. Thus, to gain some insight into which pathways BCR-ABL uses to reduce SHIP levels we treated Ba/F3$_{p210\text{-}tetOFF}$ cells growing constitutively in the absence of doxycycline (so BCR-ABL expression is turned on) with different pathway specific inhibitors (see figure 3.2.1.3). Of the inhibitors tested only PP2, an inhibitor of Src kinases, reduced the reduction of SHIP levels that occurs in the presence of BCR-ABL.

A)



B)

Name	Target	Concentration
Ly	PI3K	10 µM
WM	PI3K	100 nM
UO126	MEK 1/2	10 µM
SB202190	p38	10 µM
PD98059	MEK 1	50 µM
Compound 3	PKC	30 nM
PP2	Src	10 µM
U73122	PLC3	1 µM
Na Orthovanadate	Tyr Phosphatases	1 mM
SB203580	p38 MAPK	10 µM

Figure 3.2.1.3 PP2 maintains SHIP levels in the presence of BCR-ABL. (A) Ba/F3$_{p210\text{-}tetOFF}$ cells were incubated with IL-3 alone (+IL-3), IL-3 plus doxycycline (Doxy) or IL-3 plus the indicated inhibitors for 24 h and total cell lysates subjected to Western blot analysis. The blot shown is representative of 2 independent experiments. **(B)** List of inhibitors, their targets and the final concentration used (based on previous studies showing that these concentrations blocked the targeted pathways in Ba/F3 cells (unpublished).

3.2.1.4 Total and Phosphorylated Protein Levels of SHIP Have an Inverse Relationship

Our finding that PP2 prevented the BCR-ABL-induced reduction of SHIP levels was interesting given BCR-ABL is known to lead to the constitutive tyrosine phosphorylation of SHIP (Sattler et al. 1999) and that SHIP tyrosine phosphorylation is thought to be carried out in response to many extracellular stimuli by members of the Src kinase family (Giuriato et al. 2000, Hibbs et al. 2002, Baran et al. 2003, Hernandez-Hansen et al. 2004). To explore the possible relationship between the tyrosine phosphorylation of SHIP and SHIP protein levels we first compared the effects of PP2 and doxycycline on total and phosphorylated levels of SHIP protein at 24 h (see figure 3.2.1.4-A). We found that in the presence of BCR-ABL, SHIP levels were low, as expected, but highly phosphorylated on its two NPXY motifs. However, 24 h after the addition of doxycycline, when BCR-ABL levels are very low, we detected high levels of total SHIP protein with a very low level of phosphorylation. After a 24 h treatment with PP2, which did not significantly affect BCR-

ABL levels, we observed an intermediate result, i.e., total SHIP levels were higher than under normal culture conditions but lower than after treatment with doxycycline. Phosphorylated SHIP levels, on the other hand, were lower than under normal culture conditions but higher than in doxycycline-treated cells.

To determine how fast SHIP levels reach their maximal levels after the addition of doxycycline to Ba/F3$_{p210\text{-}tetOFF}$ cells, we monitored total and tyrosine phosphorylated levels of SHIP, as well as BCR-ABL protein levels, by Western blot analysis over a 24 h period. As shown in figure 3.2.1.4-B, BCR-ABL levels started to drop steeply after 7 h of doxycycline treatment and were barely detectable at 24 h. With decreasing BCR-ABL levels, total SHIP levels started to increase, reaching their maximum at 19 h. Tyrosine phosphorylated SHIP levels, on the other hand, decreased rapidly and showed a nice correlation with BCR-ABL levels.

To gain some insight into how quickly SHIP levels drop in the presence of BCR-ABL, we preincubated Ba/F3$_{p210\text{-}tetOFF}$ cells growing in the absence of doxycycline (i.e., high BCR-ABL levels) with PP2 (to maintain SHIP levels in the presence of BCR-ABL) and, after 24 h we washed out the PP2 and monitored total and phosphorylated SHIP levels by Western blot analysis (see figure 3.2.1.4-C). As expected, BCR-ABL was expressed over the entire course of the experiment. After 24 h with PP2 we found low levels of phosphorylated SHIP and high total SHIP levels compared to control samples without PP2 treatment. After washing out the PP2 we observed a large increase in SHIP phosphorylation within 30 min and after 3h total and phosphorylated SHIP levels were back to control levels.

Taken together, our data show that total and tyrosine phosphorylated levels of SHIP have an inverse relationship in Ba/F3$_{p210\text{-}tetOFF}$ cells. When SHIP's tyrosine phosphorylation level increases, its total protein level decreases and vice versa. Importantly, after washing out PP2 we see SHIP levels fall within 3 h to control levels. Given that previous studies in our lab have shown that SHIP is a relatively long-lived protein, with a half life of 18 h (Damen et al. 1998) it is unlikely that the drop in SHIP levels we observe within 3 h can be attributed to marked reductions in SHIP mRNA synthesis or translation and thus propose that our data are consistent with BCR-ABL triggering the tyrosine phosphorylation and subsequent degradation of SHIP. Further, both, doxycycline and PP2 prevent the

degradation of SHIP by inhibiting its tyrosine phosphorylation, although by different mechanisms. Doxycycline prevents the expression of BCR-ABL while PP2 inhibits the pathways BCR-ABL utilizes to phosphorylate SHIP. Since PP2 has been shown to be able to inhibit both Src kinases and BCR-ABL (Tatton et al. 2003), we cannot say at this time whether SHIP is getting tyrosine phosphorylated directly by BCR-ABL or via Src kinases or both.

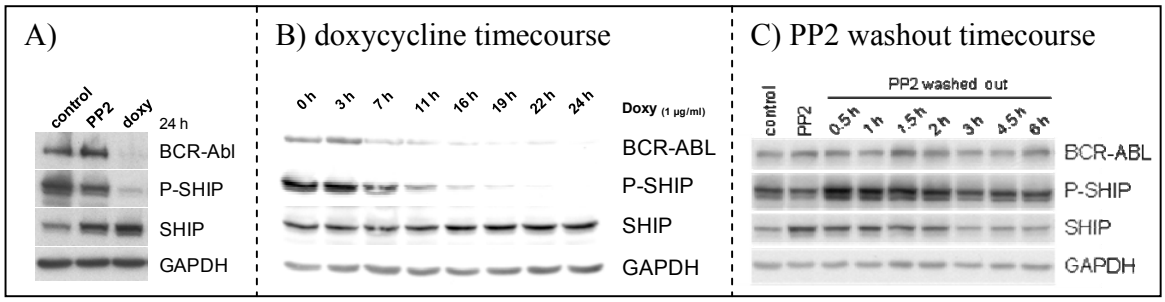


Figure 3.2.1.4 Total and tyrosine phosphorylated protein levels of SHIP share an inverse relationship. Western blot analysis of Ba/F3$_{p210\text{-}tetOFF}$ cells for BCR-ABL, SHIP, P-SHIP (phospho Y-1020) and GAPDH. **(A)** Ba/F3$_{p210\text{-}tetOFF}$ cells were treated for 24 h with PP2 (10 µM) or doxycycline (1 µg/ml). **(B)** Ba/F3$_{p210\text{-}tetOFF}$ cells were incubated with doxyycline (1 µg/ml) and total cell lysates taken at the indicated time points. **(C)** Ba/F3$_{p210\text{-}tetOFF}$ cells were incubated with PP2 (10 µM) for 24 h. Cells were then washed twice to remove PP2 and cell lysates taken at the indicated time points. All blots shown are representative of 2 independent experiments.

3.2.1.5 SHIP Forms a Complex with the Ubiquitin Ligases Cbl and Cbl-b and is Degraded via the Proteasome

In a different study our laboratory is currently trying to identify binding partners of phosphorylated SHIP. To do this, SHIP$^{-/-}$ BMMCs expressing SHIP with an N-terminal hemagglutinin (HA) tag (HA-SHIP) were stimulated with SCF, a potent physiological inducer of SHIP phosphorylation (Damen et al. 2001). After stimulation with SCF, HA-SHIP was immunoprecipitated using an anti-HA antibody and the eluate was subjected to mass spectroscopy revealing CIN85 as a binding partner of phosphorylated SHIP (Lam et al. unpublished data). The interaction between SHIP and CIN85 has been shown previously in HEK293T cells overexpressing both proteins (Kowanetz et al. 2004). CIN85 is a scaffolding protein which associates with the ubiquitin ligases Cbl and Cbl-b. This

protein complex is involved in the downregulation of several receptor tyrosine kinases (RTKs) by facilitating their ubiquitination as well as triggering their internalization (Soubeyran et al. 2002, Szymkiewicz et al. 2002, Take et al. 2000).

Since we found that SHIP gets degraded via the proteasome in BMmacs (see below) and also interacts with CIN85 we wanted to determine if SHIP is degraded via the proteasome in Ba/F3$_{p210\text{-}tetOFF}$ cells as well and whether Cbl and Cbl-b also interact with SHIP. To test the involvement of the proteasome in SHIP's degradation, we incubated Ba/F3$_{p210\text{-}tetOFF}$ cells with the proteasome inhibitor MG-132 for 24 h and analyzed total and phosphorylated SHIP levels by Western blot analysis. As seen in figure 3.2.1.5/1, blocking of the proteasome leads to an increase of both phosphorylated and total SHIP protein levels.

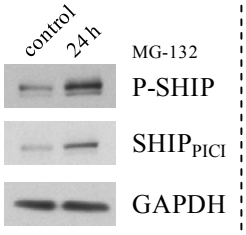


Figure 3.2.1.5/1 SIHP is degraded through the proteasome in Ba/F3$_{p210\text{-}tetOFF}$ cells. Ba/F3$_{p210\text{-}tetOFF}$ cells were treated with MG-132 (100 nM) for 24 h and phosphorylated and total SHIP levels were measured by Western blot analysis. The blot shown is representative of 2 independent experiments.

To test if SHIP interacts with Cbl or Cbl-b, we treated Ba/F3$_{p210\text{-}tetOFF}$ cells with PP2 for 24 h to achieve high levels of SHIP protein. After 24 h we washed out the PP2, in order to increase BCR-ABL mediated SHIP phosphorylation, and added the proteasome inhibitor MG-132 for 3h. We then immunoprecipitated SHIP and checked if Cbl and Cbl-b co-precipitated together with SHIP. Indeed, as seen in figure 3.2.1.5/2, we were able to detect both Cbl and Cbl-b by Western Blot analysis in the SHIP immunoprecipitate.

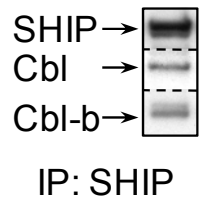


Figure 3.2.1.5/2 Cbl and Cbl-b co-precipitate with SHIP. Ba/F3$_{p210\text{-}tetOFF}$ cells were treated with PP2 (10 µM) for 24 h to increase SHIP levels. The PP2 was then washed out, to allow for BCR-ABL-induced SHIP phosphorylation, and the proteasome inhibitor MG-132 (3 µM) was added for 3h to prevent SHIP degradation through the proteasome. After 3h, cells were lysed and SHIP was immunoprecipitated. The eluate was subjected to Western Blot analysis to see if Cbl and Cbl-b coprecipitate.

3.2.2 IL-4-Induced Reduction in SHIP Levels in BMmacs

3.2.2.1 IL-4-Induced Reduction in SHIP Protein Levels in BMmacs as a Model System

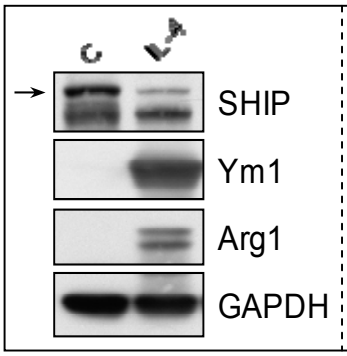


Figure 3.2.2.1 IL-4-induced reduction of SHIP protein levels in BMmacs. Western blot analysis of BMmacs treated with IL-4 (10 ng/ml) for 2 days. The arrow indicates intact full length SHIP protein. GAPDH was used as a loading control. *Adapted and modified from* (Ho and Sly 2009).

To study the regulation of SHIP protein levels in primary cells we used another model system, M-CSF-derived BM macrophages (BMmacs; derivation described in Material and Methods). Once mature, we treated BMmacs with IL-4 (10 ng/ml) for 2 days and SHIP levels were monitored by Western blot analysis. We found that IL-4 skewed BMmacs to an alternatively activated, M2 phenotype (i.e., they expressed the M2 markers Ym1 and arginase 1 (Arg1) and that during this skewing process, SHIP levels are reduced (see figure 3.2.2.1) (Ho and Sly 2009).

3.2.2.2 IL-4 Directly Induces SHIP Breakdown

To study the regulation of SHIP protein levels in primary cells and in response to a physiologically normal stimulation we used another model system, IL-4-treated M-CSF-derived BMmacs. This project came about as a result of studies carried out in our lab where we were investigating the skewing of BMmacs to an M2 phenotype with IL-4. We discovered that a 2 day treatment with IL-4 not only skewed the cells to an M2 phenotype but substantially reduced their SHIP protein levels. To explore this phenomenon further, we first examined the kinetics of IL-4-induced reduction of SHIP levels. To do this, we incubated BMmacs either in BMmac medium supplemented with IL-4 (10 ng/ml) alone for

3 days or with IL-4 plus an anti-IL-4 blocking Ab (αIL-4; 1 μg/ml) and determined for the expression of SHIP by Western blot analysis. As can be seen in figure 3.2.2.2-A, the presence of IL-4 started to reduce SHIP levels significantly on day 2 and full length SHIP was completely undetectable by day 3. The addition of αIL-4 for 3 days clearly prevented this loss of SHIP. To determine if IL-4 was directly responsible for reducing SHIP levels or if it was triggering the production of a secreted factor that acted in an autocrine manner to reduce SHIP levels, we added the supernatant (SN) from BMmacs treated with IL-4 for 3 days to fresh BMmac cultures in the presence or absence of αIL-4. As can be seen in the last 2 lanes of figure 3.2.2.2-B, the αIL-4 prevented SHIP breakdown indicating that IL-4 itself rather than IL-4-induced secreted factors induce the degradation of SHIP.

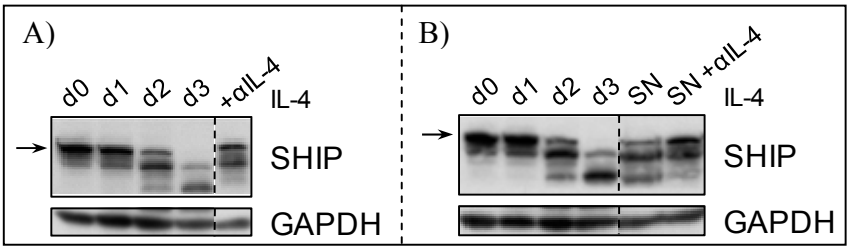


Figure 3.2.2.2 IL-4-induced reduction in SHIP levels in BMmacs. Western blot analysis of SHIP protein expression. The arrows indicate full length SHIP. **(A)** BMmacs were incubated with IL-4 (10 ng/ml) only for up to 3 days or with IL-4 + anti-IL-4 blocking Ab (+αIL-4 at 1 μg/ ml for 3 days). **(B)** Supernatant (SN) from IL-4 treated cultures (d3) was diluted 1 in 2.5 (so IL-4 is at 4 ng/ml) and used to incubate BMmacs directly or plus αIL-4 for 2 days. Blots shown are representative of two independent experiments. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

3.2.2.3 PP2 Prevents the IL-4-Induced Loss of SHIP via the Proteasome

Based on our results with Ba/F3$_{p210\text{-}tetOFF}$ cells, we asked if Src kinases might also be involved in inducing the drop in SHIP protein levels following IL-4 treatment of BMmacs. To test this, we incubated BMmacs with IL-4 (10 ng/ml) for up to 2 days with or without PP2 (10 μM) and analysed SHIP levels by Western blot analysis. As shown in figure 3.2.2.3-A, we found that the presence of PP2 resulted in more full length SHIP protein after a 2 day exposure to IL-4. Since we had shown that the IL-4-induced loss of SHIP was most profound after 3 days with IL-4 (Fig 3.2.2.2), and the most significant drop in total

SHIP levels occurred between day 2 and 3 we assessed the involvement of the proteasome in the degradation of SHIP by adding the proteasome inhibitor MG-132 (10 nM) during the last 24 h of IL-4 treatment. This was necessary because MG-132 is quite toxic and we could not expose the BMmacs to MG-132 for 3 days without causing significant cell death. We found that MG-132 inhibited the degradation of full length SHIP that occurs between days 2 and 3 (without any detectable loss in cell viability), consistent with IL-4-induced SHIP degradation taking place within the proteasome (see figure 3.2.2.3-B).

Finally, given that we had shown in earlier studies that SHIP is rapidly degraded from the C-terminus if cells are lysed with non-ionic detergents (eg, NP40 or Triton-X-100 (TX100)) (Damen et al. 2001) rather than with SDS sample buffer, we asked if SHIP is already primed for rapid degradation in the proteosome and all that is needed is a trigger, such as Src kinases, to induce this process. To test this we treated two sets of BMmacs for 2 days with IL-4 with or without PP2. One set of cells was then washed with PBS and lysed with Triton-X-100 (2%) plus NP40 (0.1%) in the presence of protease inhibitors to prevent non-specific protein degradation. The other set was washed and lysed in the same way except that both the PBS and the lysis buffer were supplemented with MG-132 (10 μM) to inhibit proteasomal degradation. As seen in figure 3.2.2.3-C, we found that SHIP levels were greatly reduced in the absence of MG-132 in all samples, independent of IL-4 or PP2 treatment.

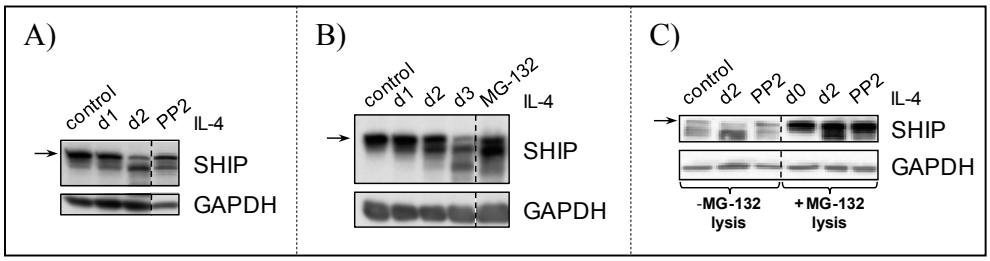


Figure 3.2.2.3 PP2 prevents IL-4-induced SHIP degradation within the proteasome. (A) BMmacs were incubated with IL-4 (10 ng/ml) for up to 2 days or with IL-4 + PP2 (10 μM) for 2 days and total cell lysates subjected to Western analysis. **(B)** BMmacs were incubated with IL-4 for up to 3 days. For one sample 10 nM MG-132 was added for the last 24h. **(C)** BMmacs were incubated with IL-4 or IL-4 + PP2 for 2 days. The cells were then either washed 1x with PBS and lysed with PSB +2%Triton-X-100 +0.1% NP40 in the presence of protease inhibitors (-MG-132 lysis) or washed and lysed the same way but in the presence of 10 μM MG-132 (+MG-132 lysis). The arrows indicate full length SHIP. The blots shown are representative of two independent experiments. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

3.2.2.4 Src Kinases have Different Effects on the Phosphorylation of SHIP and may Substitute for each other to Induce SHIP's Breakdown

The finding that PP2 prevented SHIP breakdown indicated the likely involvement of Src kinases. Indeed, Src kinases have been shown to interact with SHIP (Giuriato et al. 2000, Hibbs et al. 2002, Baran et al. 2003, Hernandez-Hansen et al. 2004). The Src kinase Lyn in particular has been shown to be important for proper SHIP signalling upon stimulation with various cytokines in BMMCs and BMmacs and it also has been speculated that another Src kinase, Fyn, can partially substitute for Lyn in Lyn-deficient cells (Giuriato et al. 2000, Hibbs et al. 2002, Baran et al. 2003, Hernandez-Hansen et al. 2004). A recent study also showed that mice that are deficient in the Src kinases Lyn and Hck, are phenotypically similar to $SHIP^{-/-}$ mice (Xiao et al. 2008). Thus, we decided to test the effect of IL-4 treatment on the phosphorylation and degradation of SHIP in BMmacs derived from mice lacking either Lyn ($Lyn^{-/-}$), Fyn ($Fyn^{-/-}$), Hck ($Hck^{-/-}$), Fyn and Lyn ($Fyn^{-/-}/Lyn^{-/-}$) or Lyn and Hck ($Lyn^{-/-}/Hck^{-/-}$) (see figure 3.2.2.4/1). We found that the lack of a single Src kinase had different effects on the phosphorylation of SHIP. $Lyn^{-/-}$ BMmacs, for example, showed lower SHIP phosphorylation than WT cells while $Hck^{-/-}$ cells had increased levels. $Lyn^{-/-}/Hck^{-/-}$ cells, similar to $Lyn^{-/-}$ cells also had lower levels of phosphorylated SHIP. Interestingly, SHIP levels in unstimulated cells seemed higher in the absence of Lyn and/or Hck. However, after two days of IL-4 treatment SHIP was still degraded to the same extent as it was in WT cells (see figure 3.2.2.4/1-A). Similar results were obtained for $Fyn^{-/-}$ and $Fyn^{-/-}/Lyn^{-/-}$ cells. $Fyn^{-/-}$ cells showed no change in the phosphorylation of SHIP, but $Fyn^{-/-}/Lyn^{-/-}$ had reduced levels and in both cases we still observed SHIP breakdown after two days of IL-4 treatment (see figure 3.2.2.4/1-B).

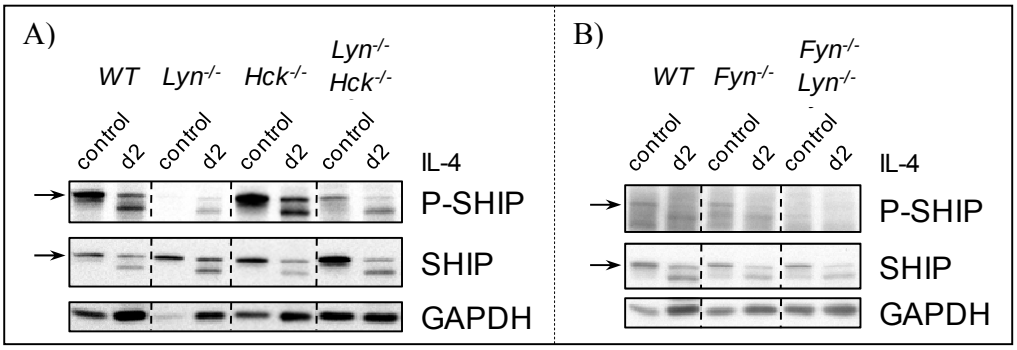


Figure 3.2.2.4/1 Effects of different Src kinase deficiencies on IL-4-induced SHIP breakdown. BMmacs from either **(A)** WT, $Lyn^{-/-}$, $Hck^{-/-}$ and $Lyn^{-/-}/Hck^{-/-}$ mice or **(B)** WT, $Fyn^{-/-}$ and $Fyn^{-/-}/Lyn^{-/-}$ mice were incubated with IL-4 (10 ng/ml) for 2 days and phosphorylated and total levels of SHIP were measured by Western blot analysis. The blots shown are representative of two independent experiments. The arrows indicate full length SHIP. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

Since we still observed some SHIP phosphorylation and breakdown we wondered whether SHIP might get phosphorylated by kinases other than Src kinases or if a Src kinase that we have not tested might be able to substitute for the missing Src kinases. To test this we derived BMmacs from the same knockout mice as above and treated them with IL-4 or IL-4 plus PP2 for 2 days (see figure 3.2.2.4/2). We found that treatment with PP2 completely abolished SHIP phosphorylation and prevented degradation in all cases, indicating that another Src kinase was able to substitute for the deficient Src kinases in the cells tested or that PP2 was inhibiting a critical non-Src family tyrosine kinase.

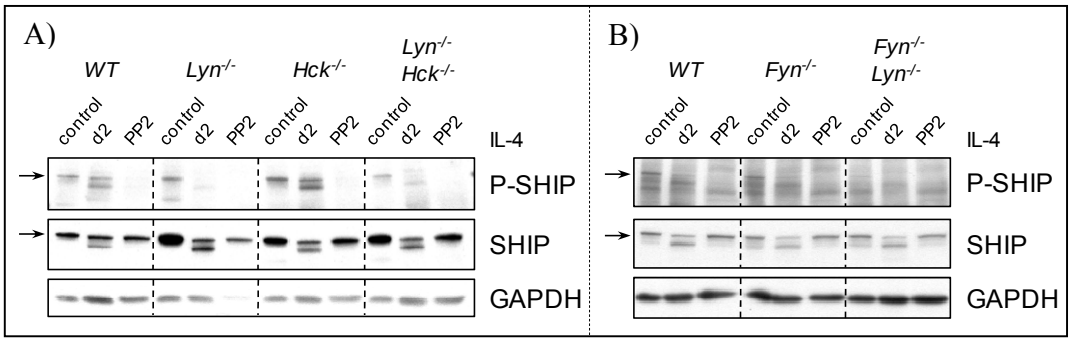


Figure 3.2.2.4/2 Inhibition of Src kinases prevents IL-4-induced SHIP phosphorylation and breakdown. BMmacs from either **(A)** WT, $Lyn^{-/-}$, $Hck^{-/-}$ and $Lyn^{-/-}/Hck^{-/-}$ mice or **(B)** WT, $Fyn^{-/-}$ and $Fyn^{-/-}/Lyn^{-/-}$ mice were incubated with IL-4 (10 ng/ml) or IL-4 + PP2 (10 µM) for 2 days and phosphorylated and total levels of SHIP measured by Western blot analysis. The blots shown are representative of two independent experiments. The arrows indicate full length SHIP. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

3.2.2.5 Lack of the Ubiquitin Ligase Cbl does not Prevent IL-4-Induced SHIP Degradation

Given our results showing that SHIP was likely being degraded through a proteasome-dependent mechanism since it could be blocked with the proteasome inhibitor MG-132 and, from our studies with $Ba/F3_{p210\text{-}tetOFF}$ cells, that SHIP could form complexes with the ubiquitin ligases Cbl and Cbl-b (shown by co-immunoprecipitation with SHIP) we asked whether Cbl deficiency could prevent IL-4-induced SHIP degradation in BMmacs. Interestingly, using BMmacs derived from Cbl KO ($Cbl^{-/-}$) mice we found that this was not the case (Fig 3.2.2.5).

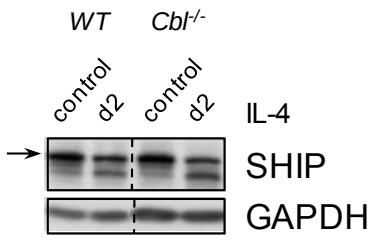


Figure 3.2.2.5 Cbl deficiency does not prevent IL-4-induced SHIP breakdown. BMmacs from either WT or $Cbl^{-/-}$ mice were incubated with IL-4 (10 ng/ml) for 2 days and total levels of SHIP were measured by Western blot analysis. The blot shown is representative of 2 independent experiments. The arrow indicates full length SHIP. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

3.2.2.6 IL-4 Induces SHIP Breakdown Through Stat6

IL-4 induces many of its biological effects through the activation of Stat6 (Urban et al. 1998, O'Shea et al. 2002). Thus, we wanted to determine whether the IL-4-induced degradation of SHIP was also mediated through Stat6 triggered pathways. To test this, we treated BMmacs derived from WT and Stat6 KO ($Stat6^{-/-}$) mice with IL-4 for 2 or 3 days and analyzed the expression of full length SHIP by Western blot analysis. As shown in Fig 3.2.2.6, we found that Stat6 was essential for the IL-4-induced degradation of SHIP.

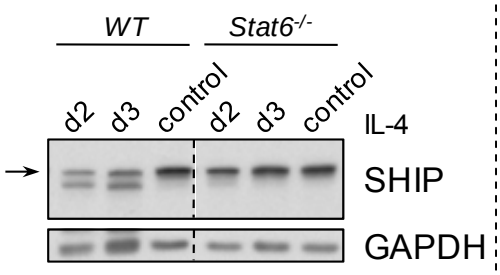


Figure 3.2.2.6 Stat6 is essential for IL-4 induced SHIP breakdown. BMmacs derived from WT or Stat6 KO ($Stat6^{-/-}$) mice were incubated with IL-4 (10 ng/ml) for 2 or 3 days and total cell lysates subjected to Western blot analysis for SHIP and GAPDH (loading control) levels. The blot shown is representative of 2 independent experiments. The arrow indicates full length SHIP. The dashed lines on the blots indicate that while the lanes to the right of the dashed lines were from the same blot and the same exposure, they were not adjacent and so the blot was cut to juxtapose the lanes.

4 Discussion

4.1 The Role of SHIP in the Maturation and Function of Mast Cell Subsets

The goal of this study was to understand the role that SHIP plays in TLR-induced activation of MCs. While SHIP is well known for its role in IgE-stimulated MC activation, very little is known about its role in TLR-triggered MC activation. However, since MCs play a central role in the early recognition of invading pathogens, understanding their regulation is of great importance. To assess the effect SHIP has on the development of MCs, we derived different WT and SHIP$^{-/-}$ MC subsets from spleen cells as model of CTMCs and MMCs and used BMMCs as another model for MMCs. We compared their growth rate, maturation rate (as measured by cell surface expression of both c-kit and FcεR1α) and expression of subtype specific granules. Safranin/alcian blue staining revealed that both WT and SHIP$^{-/-}$ CTMC granules contained heparin, a marker of CTMCs in mice, and that both WT and SHIP$^{-/-}$ MMC, as well as BMMC, granules, contained chondroitin sulphate, a marker of MMCs in mice (figure 3.1.2-C). This indicated that granule formation in these MC subsets, at least based on heparin and chondroitin sulphate, was not affected by the absence of SHIP. However, SHIP did have an impact on how fast MCs matured. All three subsets matured faster in the absence of SHIP (figure 3.1.2-A), suggesting that SHIP may act as a negative regulator of MC differentiation in vivo. Indeed, there are several studies reporting that SHIP deficiency leads to perturbations in hemopoietic cell maturation. Specifically, lineage-depleted BM cells from SHIP$^{-/-}$ mice have been shown to mature into macrophages in the presence of M-CSF much more quickly than from WT BM (Rauh et al. 2003) and that SHIP$^{-/-}$ mice have decreased numbers of lymphoid cells but increased numbers of myeloid cells (Helgason et al. 1998, Nakamura et al. 2004). We also found that SHIP affected the proliferation rate of some MC subsets (figure 3.1.2-B). Specifically, while WT CTMCs had reduced proliferation

compared to SHIP$^{-/-}$ CTMCs, WT BMMCs proliferated more than their SHIP$^{-/-}$ counterparts. WT and SHIP$^{-/-}$ MMCs, on the other hand, proliferated at the same rate. While these results suggest that SHIP, depending on the MC subset, acts as a positive or negative regulator of proliferation, we measured cell proliferation by simply performing viable cell counts, and since SHIP has a profound effect on MC differentiation, activation and survival, it is possible that the differences we observed in proliferation are confounded by its effects on these properties (Huber et al. 1998b, Liu et al. 1999, Rauh et al. 2003).

MCs can sense the presence of various pathogens by virtue of their different TLRs. We measured the mRNA expression levels of TLR1, 2, 3, 4, 5, 6, 7, 8 and 9 by RT-PCR (figure 3.1.3) and found that WT and SHIP$^{-/-}$ CTMCs expressed TLRs 1, 2, 4, 6, 8 and 9. SHIP$^{-/-}$ CTMCs also expressed TLR3 and 7, although at very low levels. We found that the expression pattern of TLRs was more limited in the mucosal-like MCs, with both WT MMCs and BMMCs, expressing only TLRs 1, 2, 4 and 6.

Although the TLR expression pattern we observed with WT MCs was similar to that reported in the literature (reviewed in Stelekati et al. 2007), there were some differences. For example, TLR3 and TLR7 have been reported to be expressed, as assessed by RT-PCR, in FSMCs but we did not observe any expression of these TLRs in our WT CTMCs. While both FSMCs and our CTMCs are *in vitro* models for connective tissue MCs, FSMCs are derived from fetal skin (ie, fetal skin-derived cultured MCs (FSMCs) while our CTMCs were derived from adult spleen cells, and this could explain the difference in our results (Stelekati et al. 2007, Matsushima et al. 2004). Also, Matsushima, *et al.* derived FSMCs in the presence of IL-3 and SCF while the media we used to derive our CTMCs only contained SCF. In the same report, Matsushima, *et al.* showed the expression of TLR3 and TLR7 in BMMCs, although at levels that were barely above background so it is not clear whether or not these 2 TLRs really are expressed. As well, unlike our results, TLR8 has been reported to be expressed by BMMCs (Supajatura et al. 2001). However, in these studies the BMMCs were derived with 10% pokeweed mitogen-stimulated spleen-conditioned medium, and this ill-defined mix of growth factors might yield BMMCs with different properties than when derived, as we did, with recombinant IL-3. As well, in their studies, the authors derived their BMMCs from C3H/HeN and C3H/HeJ mice while we

derived them from C57BL/6 mice and this may be responsible for the difference as well (Stelekati et al. 2007, Supajatura et al. 2001).

Comparing TLR expression levels in the presence and absence of SHIP revealed that SHIP$^{-/-}$ CTMCs expressed higher levels of all TLRs, with the exception of TLR4 and 6, which were expressed at equal levels in SHIP$^{+/+}$ and $^{-/-}$ cells. Interestingly, however, even though WT CTMCs did not express detectable levels of TLR3 and far lower levels of TLR 2 and 9 (Fig 3.1.3), these cells responded much more robustly to their ligands (dsRNA, PGN, and CpG, respectively), in terms of cytokine production, than their SHIP$^{-/-}$ counterparts (Fig 3.1.6/1-3). The caveat of RT-PCR studies is, of course, that they may not reflect protein levels. Although we tried to measure TLR protein levels, where antibodies were commercially available, the levels were too low to detect (i.e., far lower than in DCs, which were used as positive controls).

Binding of IgE to the FcεRI, with or without inducing crosslinking, has been shown to enhance survival of BMMCs in the absence of growth factors (Kalesnikoff et al. 2001). To study the role of SHIP in IgE-induced survival we seeded WT and SHIP$^{-/-}$ CTMCs, MMCs and BMMCs in medium containing 0.1% BSA, with or without IgE (5 μg/ml SPE-7 IgE) and counted viable cells on a daily basis (figure 3.1.4). Generally, MMCs survived better than CTMCs, but in all subsets tested we didn't find any differences in MC survival between WT and SHIP$^{-/-}$ MCs in the absence of IgE. This suggested that SHIP had no effect on MC survival in resting MCs or MCs that were deprived of their growth factors. However, we found mixed results for MC survival in the presence of IgE. While again there was no difference in survival between WT and SHIP$^{-/-}$ MCs in the splenic derived MCs, WT BMMCs actually proliferated in the presence of IgE while SHIP$^{-/-}$ BMMCs simply survived without a significant change in viable cell numbers. This big difference in MC survival between MMCs and BMMCs is surprising since both are mucosal MC models. However, it could suggest that MMCs are quite heterogeneous in vivo and that their properties depend in large part on the tissue environment in which they develop. Overall, our results suggest that SHIP plays a mixed role in IgE-induced MC survival.

It is well established that SHIP is a key negative regulator of IgE-induced MC degranulation (Huber et al. 1998b). Our results confirmed this finding for BMMCs and

also expanded this important role for SHIP to other MC subtypes (figure 3.1.5-A). TLR ligands, however, did not induce MC degranulation in WT or SHIP$^{-/-}$ MCs (figure 3.1.5-B-D). This and the fact that SHIP$^{-/-}$ BMMCs degranulate upon stimulation with growth or survival factors that normally don't induce degranulation, such as SCF or IgE (Huber et al. 1998a), suggests that the pathways leading to degranulation are not activated by TLRs. This contrasts with one report saying that PGN, a ligand for the TLR2:TLR6 heterodimer, induces degranulation in BMMCs (Supajatura et al. 2002). Interestingly, this report is from the same group that reported TLR 8 expression in BMMCs, which we could not confirm as well. It is possible that our different results are attributable to the unknown factors in the media they use for BMMC differentiation. Their observations, however, show that MCs in principle can be sensitized for PGN-induced degranulation, which highlights the ability of MCs to sense and adjust to changes in their immediate environment.

One crucial mechanism by which MCs launch and regulate an immune response is by the synthesis and secretion of cytokines. We compared different MC subsets for their ability to synthesize and secrete IL-6, IL-10, IL-12 and TNFα and found significant differences between MC subsets (figure 3.1.6/1-3). Upon stimulation with TLR ligands alone we found that WT CTMCs produced higher levels of all 4 cytokines than either of the two mucosal subtypes, which barely responded at all (figure 3.1.6/1). Amongst WT CTMCs, PGN was the strongest stimulator of IL-6 and TNFα while CpG was the most potent stimulator of IL-10 and IL-12. Although the responses were weak in general, WT BMMCs did produce higher levels of IL-6 in response to LPS, dsRNA and PGN than their SHIP$^{-/-}$ counterparts. Also, the level of cytokines secreted from SHIP$^{-/-}$ CTMCs were much lower compared to WT CTMCs. Thus, SHIP is a positive regulator of TLR stimulation in MCs.

When MCs were stimulated with TLR ligands in the presence of IgE (figure 3.1.6/2) there was no change in cytokine production in CTMCs. However, in mucosal subtypes we saw an increase of IL-6 and TNFα production. This effect was stronger in SHIP$^{-/-}$ cells than in WT cells. For IL-10 and IL-12 production, however, we did not observe any differences between TLR stimulation alone or in the presence of IgE.

When we stimulated TLRs in the presence of IgE + Ag, we observed synergistic effects in IL-6 and TNFα production in SHIP$^{-/-}$ mucosal subtypes (figure 3.1.6/3). Both, SHIP$^{-/-}$

MMCs and SHIP$^{-/-}$ BMMCs produced much higher levels compared to stimulations with TLR ligands alone. This effect was also present in WT BMMCs but not in WT MMCs. In CTMCs we only observed such an effect with WT cells in CpG-induced IL-12, production and in TLR ligand-induced TNFα production (most prominently with PGN). Qiao, *et al.* also observed synergistic effects between TLR and FcεR1-mediated signalling in the production of IL-6 and TNFα in BMMCs (Qiao et al. 2006). However, their study did not include IL-10 and IL-12 for which we did not observe a synergistic effect in BMMCs. Their study also did not include CTMCs or other MMCs. This indicates that parallel stimulation of TLRs and FcεR1 in MCs results in an enhanced activation of specific pathways rather than making MCs generally "hyper responsive". The high levels of IL-6 and TNFα produced by SHIP$^{-/-}$ MMCs and BMMCs in the presence of IgE alone or IgE + Ag reflects the well established role of SHIP as a master negative regulator of FcεR1-mediated mucosal MC activation. However, the observation that SHIP$^{-/-}$ CTMCs responded very poorly under all conditions tested shows that SHIP, depending on the MC subtype, can also be a positive regulator of MC activation. Our studies also suggest that basophils rather than MCs are a major source of IL-4 (figure 3.1.7), which plays an important role in skewing an immune response towards a T_H2 phenotype. If this skewing is not tightly regulated and is too strong, the immune system is unable to properly respond to threats. In SHIP$^{-/-}$ mice the extremely high levels of IL-4 produced by basophils skews macrophages towards an M2 phenotype, which supports the growth of tumour cells instead of killing them (Rauh et al. 2005, Kuroda et al. submitted).

Therefore our studies suggest distinct roles for the different MC subsets and for basophils in regulating immune responses, with SHIP playing a unique role in each cell type (see Figure 4.1).

4.1.1 Model and Future Perspectives

The inability of SHIP$^{-/-}$ CTMCs to produce significant levels of the pro-inflammatory cytokines IL-6, IL-12 and TNFα might suggest that loss of SHIP makes CTMCs unable to

induce an inflammatory response. Related to this, SHIP$^{-/-}$ mice do not develop graft versus host disease (GVHD) (Paraiso et al. 2007) and IL-12 has been shown to play an important role in acute GVHD (Williamson et al. 1996). As well, IL-6 and TNFα have been reported to act synergistically in rejecting allografts (Shen and Goldstein 2009). This underlines the important function SHIP plays in tissue homeostasis. It would be interesting to see the effect of specifically knocking down SHIP within the CTMC compartment on the development of GVHD.

In MMCs, SHIP deficiency has the opposite effect. While SHIP$^{-/-}$ MMCs do not produce IL-12, they produce, in the presence of IgE alone, high levels of IL-6 and TNFα and these levels dramatically increase in the presence of Ag and/or TLR ligands. This is accompanied by high IL-4 levels from SHIP$^{-/-}$ basophils in response to IL-3 or IgE and this, in turn, induces Ig class switching of B cells to make more IgE and thus more IL-4 from basophils (see model in Fig 4.1). Thus, in SHIP$^{-/-}$ mice, there is a vicious, self perpetuating cycle of increased IL-4, IL-6 and TNFα, leading to high systemic levels of these cytokines. The high levels of IL-4 likely induce the development of M2 macrophages which are unable to kill pathogens or tumour cells but, instead, actually promote tumour growth (Rauh et al. 2005). The high levels of IL-6 and TNFα produced by MMCs in mucosal tissues keep on attracting and activating other immune cells, thus driving the development of chronic inflammation, as seen in the lungs of SHIP$^{-/-}$ mice (Helgason et al. 1998).

Based on our studies, the next logical step will be to elucidate the role of MMCs and CTMCs in the generation of the SHIP$^{-/-}$ phenotype. Tools that might be useful in carrying this out include MC knock-in models, i.e., injecting SHIP$^{-/-}$ MMCs, CTMCs and/or basophils and BMMCs into MC-deficient mice to see if this recapitulates the SHIP$^{-/-}$ mouse phenotype. Another strategy might be blocking the production of IL-4, IL-6 and TNFα in SHIP$^{-/-}$ mice using monoclonal Abs to see if this reduces the characteristic macrophage M2 skewing and chronic inflammation observed in these mice. The combination of both strategies should provide valuable insights into the role of MCs and basophils in regulating immune responses.

Discussion: The Role of SHIP in the Maturation and Function of Mast Cell Subsets

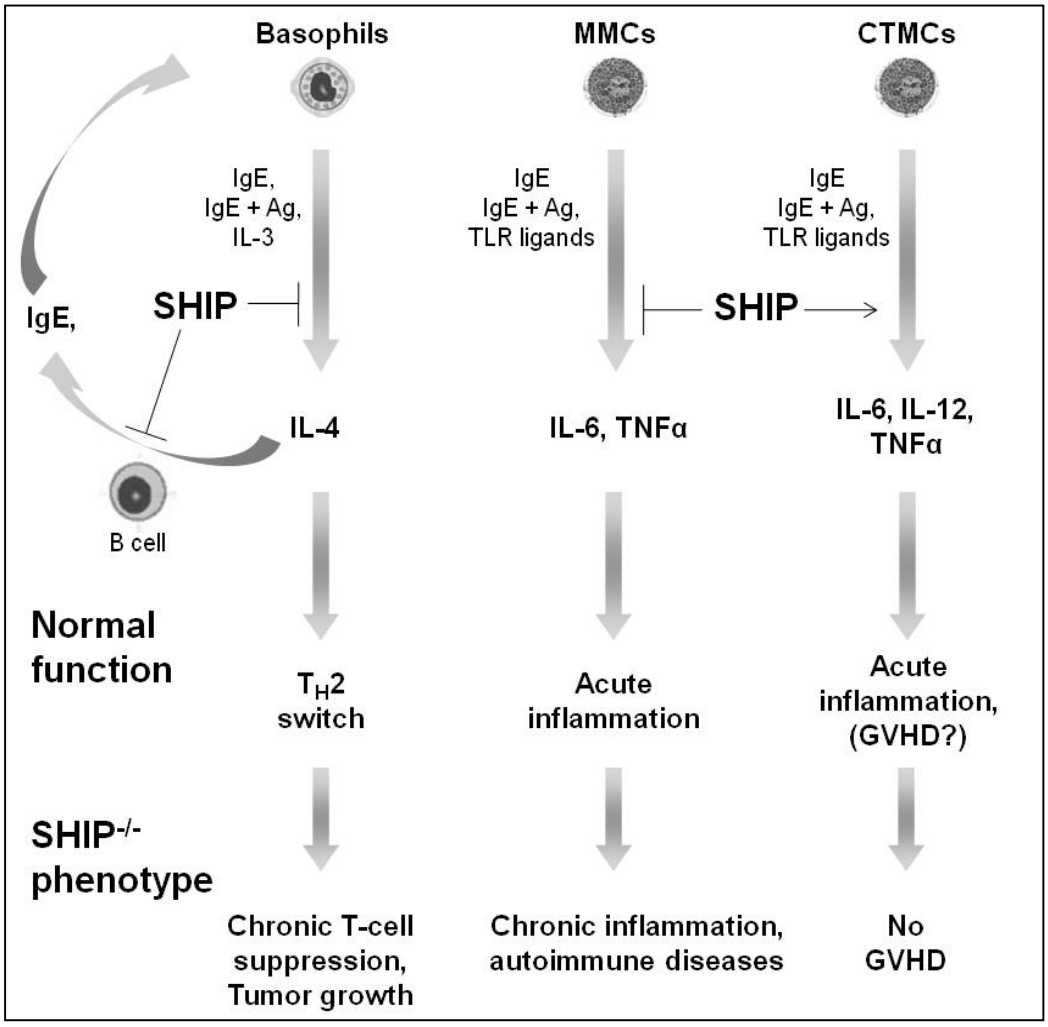


Figure 4.1 Potential role of MCs and basophils in the development of the SHIP$^{-/-}$ phenotype. In CTMCs SHIP acts as a positive regulator of the pro-inflammatory cytokines IL-6, IL-12 and TNFα, which play a key role in tissue homeostasis, as seen in conditions such as GVHD. In MMCs and basophils, SHIP acts as a negative regulator of IL-6, TNFα and IL-4 production. SHIP$^{-/-}$ CTMCs do not produce pro-inflammatory cytokines and this may explain, at least in part, why SHIP$^{-/-}$ mice do not display GVHD. Increased production of IL-4 by SHIP$^{-/-}$ basophils in response to IgE, with or without Ag, or IL-3, combined with an increased sensitivity of MMCs to IgE, induces a positive feedback loop that results in the production of high levels of IL-6 and TNFα by MMCs, resulting in chronic inflammation of mucosal tissues.

4.2 Regulation of SHIP Protein Levels

The goal of our studies was to better understand the mechanisms involved in regulating SHIP levels. Previous studies in our lab established that certain TLRs (TLRs 4 and 9) trigger, via the MyD88-dependent pathway, the production of autocrine-acting TGFβ. This cytokine, in turn, stimulates a 10-fold increase in SHIP protein levels and this increased SHIP plays a critical role in preventing chronic bacterial or viral infections from triggering an overly enthusiastic host immune response, which could lead to septic shock and subsequent death (Sly et al. 2004, Sly et al. 2009). On the other hand, downregulation of SHIP levels, for example, in macrophages, appears to be an important step in shifting from an M1, "killer" cell, which is involved in promoting a pro-inflammatory response to kill invading micro-organisms and tumour cells, to an M2 "healer" cell which, unlike their M1 counterparts, suppresses further tissue damaging immune responses and, instead, supports host tissue regeneration and collagen synthesis (scarring). This was initially based on our finding that alveolar and peritoneal macrophages in $SHIP^{-/-}$ mice were M2 while their wild type counterparts were M1. Subsequently, we found that IL-4, which is thought to play an important role in this switch to an M2 phenotype, reduces SHIP levels (Ho and Sly 2009). Interestingly, many solid tumours "highjack" this M1/M2 switching mechanism by secreting factors that cause infiltrating monocytes to become M2-like and actually help tumours grow and avoid immune-mediated killing. Naturally, it is of great interest to understand how this occurs in order to prevent it.

SHIP is only expressed in hemopoietic cells and, at least in CML, its expression is reduced by the causative oncogene, BCR-ABL. This increases the activity of the PI3K pathway and thus gives CML progenitors a selective survival advantage over normal myeloid progenitors.

To better understand how SHIP levels are down regulated by oncogenes and by normal physiological regulators, like IL-4, we studied two different model systems. Specifically, we investigated SHIP degradation in $Ba/F3_{p210\text{-}tetOFF}$ cells, which inducibly expresses BCR-ABL and in IL-4 treated BMmacs.

Discussion: Regulation of SHIP Protein Levels

Our initial studies confirmed that BCR-ABL activity indeed leads to lower SHIP protein levels (Sattler et al. 1999). Typically, Ba/F3 cells are IL-3 dependent but BCR-ABL overcomes this cytokine dependence and it is thought that downregulation of SHIP is important for CML progression (Daley and Baltimore 1988, Jiang et al. 2003). Thus it is tempting to speculate that low SHIP activity helps BCR-ABL transformed cells to escape cytokine dependence. Upon the addition of doxycycline, BCR-ABL was almost undetectable by Western analysis while SHIP levels increased dramatically and reached their maximum within 24 h (figure 3.2.1.2-A). We also showed that SHIP levels increased to the same extent in all cells (figure 3.2.1.2-B) rather than within a subpopulation, reassuring us that $Ba/F3_{p210\text{-}tetOFF}$ cells provided a useful tool to investigate the mechanisms that lead to SHIP breakdown. PTEN and SHIP2, two other important lipid phosphatases that act on PIP_3, did not change upon addition of doxycycline. However, PTEN levels increased in the presence of IL-3. These results demonstrate that SHIP, SHIP2 and PTEN protein levels are regulated, at least in part, by different mechanisms.

Since the $Ba/F3_{p210\text{-}tetOFF}$ cells take a long time (up to two weeks) to re-express BCR-ABL at high levels after washing out doxycycline it was not possible to measure SHIP's half-life in the presence of BCR-ABL by this method. Thus, we treated $Ba/F3_{p210\text{-}tetOFF}$ cells with different inhibitors to see which ones were able to prevent SHIP breakdown when BCR-ABL was constitutively expressed. We found that the Src kinase inhibitor PP2 prevented degradation of SHIP in the presence of BCR-ABL (figure 3.2.1.3). An interaction between different Src kinases and SHIP has been shown before and that Src kinases (i.e. Lyn) can tyrosine phosphorylate SHIP (Giuriato et al. 2000, Hibbs et al. 2002, Baran et al. 2003, Hernandez-Hansen et al. 2004). However, a link between Src kinase-induced SHIP phosphorylation and SHIP degradation has not been shown as yet.

We then went on to investigate the relationship between total SHIP protein levels and tyrosine phosphorylated SHIP levels (see figure 3.2.1.4-A-C) and found that there was a very nice inverse relationship. This suggested that the phosphorylation of SHIP could be a trigger for SHIP's degradation and this was strengthened by the observation that the pharmacological inhibition of SHIP's phosphorylation by the Src kinase inhibitor PP2 prevented SHIP phosphorylation and its breakdown.

Discussion: Regulation of SHIP Protein Levels

To measure SHIP's degradation rate we first incubated Ba/F3$_{p210\text{-}tetOFF}$ cells with PP2 for 24 h to prevent its breakdown in the presence of BCR-ABL and then washed it out and monitored BCR-ABL, total SHIP and phosphorylated SHIP levels for 6 h (figure 3.2.1.4-C). We found that after washing out PP2, SHIP rapidly became highly phosphorylated and total SHIP levels dropped down to control levels within 3 h, consistent with earlier reports (Sattler et al. 1999).

We also found that SHIP was degraded via the proteasome (figure 3.2.1.5/1) and that the ubiquitin ligases Cbl and/or Cbl-b, based on their interaction with SHIP (figure 3.2.1.5/2), are likely candidates to facilitate SHIP's poly-ubiquitination and subsequent degradation in the proteasome.

Our initial studies with IL-4 treated BMmacs, showed that IL-4 indeed induced SHIP degradation and did so directly, since the addition of IL-4 blocking Ab to IL-4-conditioned BMmac medium diminished SHIP's degradation (figure 3.2.2.2). We also found that IL-4 induced SHIP breakdown through Src kinases since it was greatly reduced by treatment with PP2, a universal Src kinase inhibitor (figure 3.2.2.3-A). Furthermore, SHIP was degraded through the proteasome since it was blocked by treatment with the proteasome inhibitor MG-132 (figure 3.2.2.3-B). Our results further suggest that SHIP may be present in a complex that allows for rapid degradation through the proteasome and that Src kinases might act as a trigger that "releases" SHIP into the proteasome pathway (figure 3.2.2.3-C). We suggest this may be the case because when IL-4-treated BMmacs were lysed with TX-100 and NP-40, instead of SDS sample buffer, with protease inhibitors present during lysis, SHIP levels were substantially lower when the lysis-buffer was not supplemented with MG-132. Such an effect was not seen for GAPDH, suggesting that this was not due to a non-specific degradation effect. Since Cbl$^{-/-}$ BMmacs still displayed IL-4-induced SHIP breakdown we cannot say, at this time, which ubiquitin ligase is involved in SHIP degradation. However, at least in Ba/F3$_{p210\text{-}tetOFF}$ cells, we found that SHIP interacts with both Cbl and Cbl-b, leaving the possibility that Cbl-b either substitutes for Cbl or that Cbl-b actually is primarily responsible for the ubiquitination of SHIP (figure 3.2.2.5).

In order to elucidate which Src kinase was important for inducing SHIP degradation we derived BMmacs from mice deficient in different members of the Src kinase family that

have been implicated in SHIP signalling, i.e. Lyn, Fyn and Hck. Interestingly, we observed different effects for each Src family member in their ability to influence SHIP phosphorylation (figure 3.2.2.4/1). For example, while SHIP phosphorylation was reduced in $Lyn^{-/-}$ BMmacs, it was not in $Fyn^{-/-}$ BMmacs and was actually elevated in $Hck^{-/-}$ BMmacs, compared to their WT counterparts. However, Upon IL-4 treatment, SHIP was degraded to the same extent as WT BMmacs. The same was true for $Fyn^{-/-}/Lyn^{-/-}$ and $Lyn^{-/-}/Hck^{-/-}$ BMmacs. Although SHIP phosphorylation in BMmacs derived from these mice was lower compared to WT BMmcas, treatment with IL-4 still induced SHIP breakdown. Importantly, though, treatment with PP2 reduced the phosphorylation of SHIP even further and prevented SHIP degradation (figure 3.2.2.4/2). Thus we conclude that either other Src kinase members are able to substitute for the ones we knocked out in phosphorylating critical tyrosine residues in SHIP that induce its degradation. We cannot, however, rule out the possibility that PP2 inhibits a non-Src family tyrosine kinase that phosphorylates these critical tyrosines. At the same time our results suggest a more complicated picture for the effects of SHIP phosphorylation. The lack of Lyn or Lyn plus a second Src kinase resulted in lower phosphorylation levels of SHIP but did not prevent its degradation. This observation in combination with a recent report showing that $Lyn^{-/-}/Hck^{-/-}$ mice, which have a very similar phenotype to $SHIP^{-/-}$ mice, can be rescued by expression of a membrane-bound form of SHIP suggests that the phosphorylation of SHIP by Lyn plus Hck can also be a trigger for SHIP's relocation (Xiao et al. 2008). This is supported by an earlier report showing that SHIP translocates to the actin cytoskeleton upon Src-mediated tyrosine phosphorylation in activated human platelets (Giuriato et al. 2000, Giuriato et al. 1997). Thus it seems possible that, depending on which of SHIP's tyrosines become phosphorylated, the effects can be different. Tyrosine phosphorylation, however, is also involved in triggering SHIP degradation since this process can be blocked by PP2. Our results also demonstrate a clear role for Stat6 in IL-4-induced SHIP breakdown since this process is abrogated in $Stat6^{-/-}$ BMmacs (figure 3.2.2.6).

Discussion: Regulation of SHIP Protein Levels

4.2.1 Model and Future Perspectives

Our results suggest a model in which the tyrosine phosphorylation of SHIP leads to its degradation via the proteasome (see figure 4.2). In Ba/F3$_{p210\text{-}tetOFF}$ cells, tyrosine phosphorylated and total SHIP levels display an inverse relationship and SHIP's phosphorylation status depends on the presence of BCR-ABL. Treatment with PP2 prevents both SHIP phosphorylation and degradation. Several observations also suggest that SHIP gets degraded through the proteasome. First, in Ba/F3$_{p210\text{-}tetOFF}$ cells we showed that SHIP interacts with the ubiquitin ligases Cbl and Cbl-b. These ligases have been shown to form a complex with the scaffolding protein CIN85 and this protein complex is involved in the negative regulation of several activated RTKs by ubiquitinating and targeting them for proteasomal degradation or by inducing their internalization and recycling (Soubeyran et al. 2002, Szymkiewicz et al. 2002, Take et al. 2000, Molfetta et al. 2005). This downregulation of RTK activity has been shown to have an important physiological impact in several cell types including MCs. We also found that SHIP interacts with CIN85 in MCs (unpublished data) and this interaction has also been shown in HEK293T cells overexpressing both proteins (Kowanetz et al. 2004). Furthermore, we have found that lysis of BMmacs with NP-40 and TX-100 instead of SDS-containing lysis buffer results in rapid degradation of SHIP (but not the loading control, GAPDH). This could be blocked by adding the proteasome inhibitor MG-132 to the lysis buffer. Together, these results clearly demonstrate that SHIP can be rapidly degraded via the proteasome. As mentioned earlier, the tyrosine phosphorylation of SHIP, depending on its site and levels, can have different effects. There are two well established NPX**Y** motifs (Y917 and Y1020) where SHIP becomes tyrosine (Y) phosphorylated upon extracellular stimulation and these phosphorylations have been shown to be involved in SHIP's function (Damen et al. 1996, Damen et al. 2001). However, in a more recent study several new phosphorylation sites within SHIP, upon FcεRI aggregation in BMMCs, have been described, namely serine(S)934, Y867 and Y944 (Cao et al. 2007). The Ab that we used in our studies to detect SHIP phosphorylation preferentially detects phospho-Y1020 but might cross-react with phospho-Y917 (see Material and Methods for derivation of the Ab). The new

phosphorylation sites are likely not detected by this Ab, so we were not able to analyze these phosphorylations and their possible effects on SHIP.

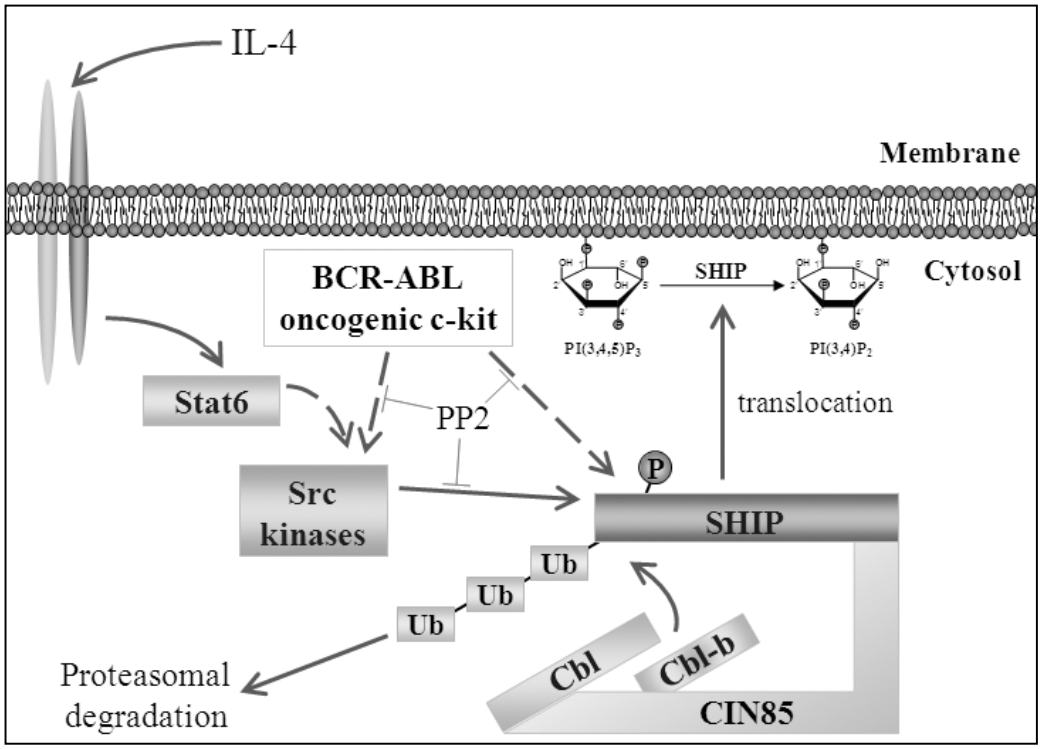


Figure 4.2 Differential effects of phosphorylation on SHIP stability and function. The model shows the different effects SHIP phosphorylation can have. Dashed Arrows indicate uncertain pathways. IL-4 treatment leads to SHIP phosphorylation and subsequent degradation in BMmacs. SHIP's degradation is mediated through Stat6 and a tyrosine kinase since IL-4 treated Stat6$^{-/-}$ BMmacs do not result in SHIP breakdown and PP2 blocks both SHIP phosphorylation and degradation. BCR-ABL and other oncogenic tyrosine kinases lead to high levels of phosphorylated SHIP and low levels of total SHIP. The low levels of total SHIP are linked to its high phosphorylation status since PP2 reverses this effect. Upon phosphorylation, SHIP gets poly-ubiquitinated, possibly by Cbl or Cbl-b, and degraded via the proteasome. Phosphorylation of SHIP can also lead to its translocation to the plasma membrane, where SHIP dephosphorylates PI(3,4,5)P_3. The phosphorylations that determine whether SHIP becomes activated or degraded are still to be elucidated.

Of note, both BCR-ABL and oncogenic c-kit are constitutively active tyrosine kinases that lead to very high constitutive phosphorylation of SHIP and its subsequent degradation (Vanderwinden et al. 2006, Sattler et al. 1999). This opens up the possibility that multiple

phosphorylations of SHIP might lead to its degradation while a lower phosphorylation level, perhaps on a selective subset of tyrosines and/or serines and/or threonines, serves as a translocation signal. To elucidate the effects of different SHIP phosphorylations, one possible strategy is to apply mass spectroscopy to our Ba/F3$_{p210\text{-}tetOFF}$ and BMmacs systems in order to identify which sites become phosphorylated. If SHIP gets immunoprecipitated before subjecting samples to mass spectrometry, it is also possible to identify binding partners at the same time, similar to the studies in our lab with BMMCs that showed an interaction between SHIP and CIN85. Once phosphorylation sites are identified, site directed mutagenesis might provide a useful strategy to assess the importance of each phosphorylation site. This in combination with the identification of interaction partners can be valuable in the generation of new drugs that are able to target certain properties of SHIP. Given the central role SHIP plays in the regulation of the innate immune system, it is a good target for drug development and a small molecule activator of SHIP has already been described (Ong et al. 2007).

5 Summary/Zusammenfassung

5.1 Summary

The 145 kDa protein, SHIP, is a hemopoietic-restricted negative regulator of the PI3-Kinase pathway and plays a central role both in hemopoiesis and the activation of innate immune cells. Our goal in this thesis was to extend the well established role that SHIP plays in BMMC development and activation to other MC subsets. We found that SHIP affected MC properties in many different ways. For example, in the absence of SHIP, MCs generally matured more quickly and were more prone to degranulation. Interestingly, survival in the absence of growth factors was not affected by SHIP in most MC subsets but, in the presence of IgE, WT BMMCs survived far better than SHIP$^{-/-}$ BMMCs. However, there was no difference between WT and SHIP$^{-/-}$ MMCs. In addition, SHIP appeared to be a positive regulator of TLR-mediated cytokine production and a negative regulator of FcεR1-mediated activation. However, in WT BMMCs there was synergy between TLR and FcεR1 activation, which was not seen in WT MMCs, suggesting that even MCs from the same subset can acquire distinct properties during their differentiation. Also, the synergistic effects were the strongest in SHIP$^{-/-}$ cells, indicating an important role for SHIP in the integration and processing of different signals. We also found that SHIP was a potent negative regulator of IL-4 production. However, this likely was of more importance in basophils than MCs.

Since SHIP activity is regulated in large part by its protein levels and since several cancers down-regulate SHIP to increase their survival we also explored how SHIP protein levels are down-regulated. Our studies with inducible BCR-ABL expressing cells and with IL-4-induced BMmacs, revealed that SHIP was degraded through the proteasome since treatment with the proteasome inhibitor MG-132 prevented SHIP protein levels from declining. We also found that the ubiquitin ligases Cbl and Cbl-b interacted with SHIP, suggesting they are involved in ubiquitinating SHIP. Studies in which we lysed BMmacs

with non-ionic detergents revealed that SHIP was “set up” for rapid proteasomal degradation since MG-132 prevented lysis-induced SHIP degradation. Tyrosine phosphorylation of SHIP seemed to be required for its breakdown in both cell systems since its degradation was prevented by inhibiting Src kinases with PP2. Also, at least in BCR-ABL expressing cells, there was a strong inverse relationship between total and phosphorylated SHIP protein levels. We also found that induction of SHIP degradation required Stat6 signalling, at least in IL-4 treated BMmacs, since this effect was abrogated in Stat6$^{-/-}$ BMmacs.

Thus, in this study we confirmed SHIP’s central role in MC development and FcεR1-mediated activation and extended these studies to TLR-mediated activation. We also identified key factors involved in inducing and mediating SHIP breakdown. This might be important in designing new therapeutic strategies that exploit SHIP’s central role in regulating immune responses.

5.2 Zusammenfassung

Das hämatopoetisch exprimierte Protein SHIP ist ein negativer Regulator des PI3-Kinase Signalweges und ein wichtiger Regulator der Blutzellenbildung sowie der Aktivierung von Zellen des angeborenen Immunsystems. Ein Ziel dieser Arbeit war es, die gut erforschte Rolle von SHIP in primären, vom Knochenmark (bone marrow, BM) differenzierten Mastzellen (MCs) auch in anderen MCmodellen zu analysieren.

Es konnte gezeigt werden, dass SHIP die Eigenschaften von MCs auf verschiedene Art und Weise prägt. SHIP erwies sich als ein negativer Regulator der Differenzierung und Degranulierung von MCs, hatte aber keinen signifikanten Einfluss auf das Überleben von MCs in der Abwesenheit von Wachstumsfaktoren. Für die TLR Aktivierung war SHIP ein positiver und für die FcεR1 Aktivierung ein negativer Regulator. In BMMCs and MMCs, zwei verschiedenen Modellsystemen für den gleichen MCsubtyp, wurden zuweilen auch unterschiedliche Einflüsse von SHIP beobachtet. Dies könnte darauf Hinweisen, dass MCs

vom gleichen Subtyp während ihrer Differenzierung unterschiedliche Eigenschaften erlangen können. SHIP hatte auch einen negativen Effekt auf die IL-4 Synthese, was aber wichtiger in Basophilen zu sein schien.

SHIPs Phosphataseaktivität wird überwiegend durch dessen Expressionslevel reguliert, und einige Krebsarten regulieren SHIP herunter, um sich dadurch einen Überlebensvorteil zu verschaffen. Daher war es eine weitere Zielsetzung dieser Arbeit, mechanistische Einblicke, die zur Runterregulierung von SHIP führen, zu erlangen. Es konnte sowohl in BCR-ABL exprimierenden Ba/F3 Zellen, als auch in IL-4 behandelten, vom BM differenzierten, Makrophagen (BMmacs) gezeigt werden, dass SHIP durch das Proteasom abgebaut wird. Dieser Abbau wurde durch den Proteasominhibitor MG-132 unterdrueckt. Die Ubiquitinligasen Cbl und Cbl-b wurden als Kandidaten für die Ubiquitinierung von SHIP identifiziert. Lysierungsexperimente mit nichtionischen Detergenzien in BMmacs zeigten, dass SHIP sehr rapide durch das Proteasom abgebaut werden kann, da dies ebenfalls durch MG-132 inhibiert wurde. SHIPs Tyrosinphosphorylierung wurde in beiden untersuchten Zellsystemen als essentiell für dessen Abbau identifiziert, da der Src Kinaseinhibitor PP2 SHIPs Phosphorylierung und Abbau inhibierte. Zudem wurde in BCR-ABL exprimierenden Ba/F3 Zellen eine disproportionale Beziehung zwischen absoluten und phosphorylierten SHIP beobachtet. In BMmacs wurde der Stat6 Signalweg als essentiell für den IL-4 induzierten SHIP Abbau identifiziert.

Im Rahmen dieser Arbeit haben wir die wichtige Funktion von SHIP in der Entwicklung und FcεR1 induzierten Aktivierung von MCs bestätigt und auf die TLR induzierte Aktivierung ausgeweitet. Es wurden auch erste Einblicke in die molekularen Mechanismen, die zum Abbau von SHIP führen, erlangt. Die Ergebnisse dieser Studie leisten somit einen wichtigen Beitrag in der Entwicklung von neuen Tumortherapien, die die zentrale Funktion von SHIP als Ansatzpunkt haben.

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7 List of Abbreviations

Abs	antibodies
Ag	antigen
aka	also known as
AP	activator protein
APCs	antigen presenting cells
APS	ammonium peroxo-disulfate
AS	antisense
BFU	burst forming unit
BM	bone marrow
BMCP	basophil mast cell progenitor
BMMCs	bone marrow derived mast cells
BP	bullous pemphigoid
BSA	Bovine Serum Albumin
CFU	colony forming unit
CPA	carboxypeptidase
CTLMCs	connective tissue like MCs
CTLs	cytotoxic T cells
CTMCs	connective tissue mast cells
DCs	dendritic cells
DD	death domain
DMSO	dimethyl sulfoxide
DNP	2,4-Dinitrophenol
Doks	downstream of tyrosine kinases
dsRNA	double-stranded RNA
EAE	experimental allergic/autoimmune encephalomyelitis
ECL	enhanced chemiluminescence
EGTA	Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	erythropoietin
ETP	early T lineage progenitor
FACS	Fluorescence activated cell sorting
FcεR1	high affinity IgE receptor
Fig	figure
FSMCs	fetal skin derived MCs
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

List of Abbreviations

G-CSF	granulocyte colony stimulating factor
Gm^-	gram negative
Gm^+	gram positive
GM-CSF	granulocyte monocyte colony stimulating factor
GMP	granulocyte macrophage progenitor
HBSS	Hanks' balanced salt solution
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HOCl	hypochlorite
HRP	horseradish peroxidase
HSA	Human Serum Albumin
HSC	hemopoietic stem cell
IFN	interferon
IKK	inhibitor of κB kinase
IL-1R	interleukin-1 receptor
IMDM	Iscove's modified dulbecco's medium
IRAK	IL-1R-associated kinase
IRF	IFN regulatory factor
ITAMs	immunoreceptor tyrosine based activation motifs
ITIMs	immunoreceptor tyrosine based inhibitory motifs
IκB	inhibitor of κB
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
Mal	MyD88-adaptor-like
MAPKs	mitogen activated protein kinases
MCs	Mast cells
M-CSF	monocyte colony stimulating factor
MEP	megakaryocyte erythrocyte progenitor
MLMCs	mucosal tissue like MCs
MMCPs	mouse mast cell proteases
MMCs	mucosal tissue mast cells
MS	multiple sclerosis
MTG	α-Monothioglycerol
NAP-1	NF-κB-activating kinase-associated protein-1
O_2^-	superoxide
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen associated molecular patterns
PAR	protease-activated receptor
PBS	phosphate buffered saline

List of Abbreviations

PCR	polymerase chain reaction
PDK1	PIP_3-dependent protein kinase 1
PGN	peptidoglycan
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
PMSF	phenylmethylsulfonyl fluoride
p-NAG	p-Nitrophenyl-N-acetyl-β-D-glucosaminidine
Pro-rich	proline rich
PRRs	pathogen recognition receptors
PSB	phosphorylation solubilisation buffer
PTEN	phosphatase/tensin homolog deleted on chromosome 10
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
rbcs	red blood cells
RIP-1	receptor-interacting protein-1
RPMI	Roswell Park Memorial Institute
SB	sample buffer
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SHIP	SH2-containing inositol 5′-phosphatase 1
$SHIP^{-/-}$	SHIP knockout
sSHIP	stem cell SHIP
ssRNA	single-stranded RNA
Stat	signal transducer and activator of transcription
TAMs	tumour associated macrophages
TANK	TRAF family-associated NF-κB binding kinase
TBK-1	TANK binding kinase-1
TEMED	N,N,N',N'-Tetramethylethylendiamin
TFs	transcription factors
TICAM	TIR domain-containing adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll like receptors
TMT	Transmembrane tryptase
TNF	tumour necrosis factor

List of Abbreviations

TPO	thrombopoietin
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFNβ
TX100	Triton-X-100
wbcs	White blood cells
WT	wild-type

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