Evaluation of the Influence of SHIP1-interacting Proteins and TTP on Mast Cell Signaling

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Thomas Hochdörfer

aus Bad Dürkheim

Berichter: Universitätsprofessor Dr. rer. nat. Michael Huber
Universitätsprofessor Dipl. Ing. Dr. Werner Baumgartner

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Zusammenfassung


Es ist bekannt, dass die Kontrolle über die Produktion der wichtigen proinflammatorischen Zytokine TNF-α und IL-6 von vielen mRNS-Bindeproteinen reguliert wird, welche die Stabilität und Translation der Transkripte beeinflussen. Forschung mit Makrophagen hat die Wichtigkeit der p38-MK2-TTP Achse für die Regulierung der Stabilität und Translation von TNF-α mRNS gezeigt. Im ersten Teil dieser Studie haben wir den möglichen Einfluss von p38 und TTP für die LPS-induzierte Zytokin Produktion in MZ untersucht. Mit pharmakologischen Inhibitoren konnten wir eine starke Abhängigkeit der LPS induzierten IL-6, TNF-α und IL-1β Produktion von der p38 Aktivität, aber nicht vom Erk Signalweg nachweisen. Die LPS-induzierte Genexpression von TTP war ebenfalls abhängig von p38. Wir konnten keine signifikanten Unterschiede zwischen LPS stimulierten TTP-defizienten und Wildtyp MZ, die aus dem Knochenmark entsprechender Mäuse differenziert wurden (BMMCs) feststellen, weder in Bezug auf die Genexpression noch die Zytokinproduktion von IL-6, TNF-α und IL-1β. Außerdem wurde die Stabilität der TNF-α mRNS nicht vom Fehlen von TTP in MZ beeinflusst. Im Gegensatz zur TTP Genexpression konnten wir keine TTP Proteinexpression in MZ nachweisen. Während TTP erfolgreich aus Lysaten LPS-stimulierter RAW 264.7 Makrophagen präzipitiert werden konnte, gelang dies aus MZ Lysaten nicht. Ansonsten fanden wir Gen- und Proteinexpression der anderen TIS11 Protein Familienmitglieder BRF1 und BRF2, deren Interaktion mit 14-3-3 Proteinen, und Genexpression von Zfand5, die alle mit der Regulation von mRNS Stabilität in Verbindung gebracht werden.
Diese Daten deuten darauf hin, dass die Kontrolle der Zytokin mRNS Stabilität und Translation in MZ von anderen Proteinen als TTP ausgeübt wird.

Abstract

Belonging to the innate immune system, mast cells (MCs) are at the front line of the immune response. They are equipped with receptors capable of recognizing general pathogen-associated patterns, like the Toll-like receptors (TLRs), but also with the specific receptor for IgE, FcεRI. Under certain circumstances, activation of the latter can lead to allergic reactions, to which MCs contribute by secretion of the powerful mediator histamine via degranulation of the cells. Apart from their role in allergies, they contribute to the immune response by releasing a varied set of mediators, like cytokines or eicosanoids, orchestrating the immune response based on the pathogens they recognize.

Control of production of the important proinflammatory cytokines TNF-α and IL-6 is known to be regulated by various mRNA-binding proteins, influencing stability and translation of the respective transcripts. Research in macrophages has shown the importance of the p38-MK2-TTP axis for regulation of TNF-α mRNA stability and translation. In the first part of this study we examined a possible involvement of p38 and TTP in LPS-induced cytokine production in MCs. Initially, using pharmacological inhibitors we found strong dependence of LPS-induced IL-6, TNF-α and IL-1β production on p38 activation, whereas activation of the Erk pathway appeared dispensable. LPS treatment also induced p38-dependent gene expression of TTP. Unexpectedly, we found no significant differences between TTP-deficient and WT MCs in response to LPS, neither in gene expression or cytokine production of IL-6, TNF-α or IL-1β. Also, stability of TNF-α mRNA was not influenced by TTP deficiency in MCs. In contrast to TTP gene expression, TTP protein expression could not be detected in MCs. While we successfully precipitated and detected TTP from lysates of LPS-stimulated RAW 264.7 macrophages, this was not possible from MC lysates. Otherwise we found gene and protein expression of the other TIS11 family members BRF1 and BRF2, their interaction with 14-3-3 proteins, and gene expression of Zfand5, all of which are connected to regulation of mRNA stability. These data suggest that control of cytokine mRNA stability and translation in MCs is exerted by proteins other than TTP.
Most research in enzymes is in regard to their direct catalytic effector functions. Apart from catalytic domains, many enzymes also possess various domains involved in mediating interactions with other proteins. These interactions may have a big influence on the signaling in the cell, without activating the actual catalytic function of the protein. Previous work in our lab on the lipid phosphatase SHIP1 implied non-catalytic functions of the enzyme in the regulation of MC signaling. In the second part of this study we identified several novel interaction partners of SHIP1 using mass spectrometry. We confirmed interaction of these proteins with SHIP1 via immunoprecipitation. Many identified proteins were implicated in ubiquitin signaling, like CIN85, Nedd4 or STS1. We further concentrated on the interaction of SHIP1 and CIN85 and found that mostly the biggest isoforms of each protein are interacting. This interaction was constitutive and did not change after antigen treatment of MCs. We could confirm interaction of the two proteins in RBL-2H3 MCs using the proximity ligation assay for confocal microscopy.
Chapter 1

Introduction

Allergy as a disease is becoming more and more prevalent in the industrialized world today, often being connected to increased hygiene in everyday life [42, 117]. The major effector cells in the course of allergy, as well as atopy, are mast cells (MCs), which induce powerful reactions in the body by release of their mediators in the matter of minutes. Even though these cells can act destructively, evolution has not gotten rid of them over time. To this day there are no reports of any human being described with a complete absence of MCs [168]. Although there is a possibility that there is no obvious phenotype for humans devoid of MCs and it is simply overlooked so far [137]. Experimentally, MC-deficient mouse models, like the Kit\(^{W/Wv}\) and Kit\(^{W−sh/W−sh}\) mutants, have been established and used for investigation of various diseases for participation of MCs [10, 87]. But, as MCs are not the only cells expressing the receptor tyrosine kinase Kit, mutation of the respective gene to get rid of MCs cannot be seen as a specific MC "knockout". This established model system has recently been challenged by multiple groups independently generating MC-deficient mice without inducing mutations in the Kit gene. In one of the models, overexpression of Cre-recombinase under the carboxypeptidase A3 promoter, which is highly active in MCs, leads to eradication of MCs at early stages of their development [47]. Another group used a similar approach, depleting MCs by Cre-mediated deletion of the apoptosis suppressor gene myeloid cell leukemia sequence 1 (MCL1) [97]. Two other groups made use of diphtheria toxin and its receptor to generate either constitutive or inducible MC deficiency in mice [41, 121]. While confirming the role of MCs in IgE-mediated anaphylaxis, contradictory results have been shown regarding the involvement of MCs in development of
arthritis and experimental autoimmune encephalomyelitis, the mouse model of human multiple sclerosis [47]. As these new MC-deficient mouse models have disadvantages of their own, namely either reduced basophil numbers or only depletion of one subpopulation of MCs, further research is needed to define the actual role of MCs in the immune system [137].

Deriving from myeloid progenitor cells, MCs are thought to belong to the innate immune system. In mice, MCs can be divided into two subtypes, which depend on mediator content and localization, connective tissue-type MCs and mucosal MCs. Mucosal MCs are mainly found in the lamina propria of the respiratory tract and in the mucosa of the gastrointestinal tract. Their proliferation is T cell-dependent, mediated by IL-3, -4, -9 and -10. Connective tissue-type MCs, on the other hand, are T cell-independent and are found in the submucosa of the gastrointestinal tract, the skin and in the peritoneum [166]. MCs are thought to be released as progenitor cells from the bone marrow into the peripheral blood circulation. They then home to the respective tissues where they complete their final maturation dependent on the local microenvironment [4, 167].

Effector functions of MCs can be divided into three categories. The most prominent is the degranulation of the cells, which is exocytosis of insoluble granules which are packed inside the resting cell. In a matter of seconds, powerful preformed mediators inside these granules, such as histamine, TNF-α and proteases, are released into the surrounding environment [57]. The degranulation is usually triggered by multivalent antigen (Ag) being recognized by IgE antibodies bound to the high-affinity IgE receptor FcεRI on the cell surface. Under special circumstances also other substances can induce the exocytosis of granules, as it has been shown for Steel Factor stimulation of SHIP1-deficient MCs [72]. The second effector function, the generation of arachidonic acid metabolites, is slightly slower than degranulation. In this process mediators are produced by a conversion of arachidonic acid to eicosanoids such as prostaglandins and leukotrienes [64]. The slowest of these responses is the de novo production of various cytokines and chemokines over the course of hours. These cytokines include, amongst others, TNF-α, IL-1α, IL-1β, IL-6, IL-3, IL-4 and GM-CSF [23, 57]. Activation of MCs does not necessarily lead to induction of all three effector functions, as in the case for Ag recognition. Stimulation via the Toll-like receptor system will not lead to degranulation of MCs but induces the arachidonic acid metabolism and cytokine production [45, 93].
The following chapters will go into more detail about receptor systems, signaling pathways, and molecules important in MC biology.

1.1 Toll-like receptors - recognition of patterns in pathogens

As the adaptive immune system requires several days to react to pathogens, the innate immune system needs its own set of receptors to sense invading pathogenic microorganisms. Germline-encoded pattern recognition receptors (PRRs) are able to detect a broad spectrum of structures shared by pathogens, also known as pathogen-associated molecular patterns (PAMPs).

Fig. 1: Overview of Toll-like receptor trafficking and signaling. After PAMP engagement, TLRs form hetero- or homodimeric structures and recruit adapter proteins such as MyD88, TIRAP, TRIF and TRAM. TLR2/1, TLR2/6, TLR5, and TLR4 induce signaling from the cell surface, while TLR3, TLR7, TLR8, TLR9 and TLR11 signal from inside intracellular vesicles. This leads to activation of NF-kB and production of interferon (IFN). Adapted from [83].

Toll-like receptors (TLRs) are one of several families of PRRs. They are type I transmembrane proteins and the recognition of PAMPs is mediated by leucine-rich repeats in the
ectodomain while the cytosolic Toll-IL-1 receptor (TIR) domain activates the downstream signaling (see figure 1). To date, 10 human and 12 mouse TLRs have been identified. While TLR11, 12 and 13 are not expressed in humans, the gene encoding a homologue of TLR10 is present in mice, but has been damaged and yields no functional product [83]. TLR1, 2, 4, 5 and 6 are localized on the cell surface. TLR3, 7, 8, 9, 11 and 13 are expressed in intracellular vesicles [14]. PAMPs detected by these receptors include lipoproteins (detected by TLR1, 2 and 6), double-stranded RNA (recognized by TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded RNA (TLR7 and 8) and unmethylated DNA (TLR9) [2]. TLR13 has recently been shown to recognize 23S rRNA of bacteria that are not resistant to erythromycin [118]. Expression in BMMCs has been confirmed for TLR1, 3, 6, 7 and 8 on the mRNA level and for TLR2 and 4 on the protein level [106, 111, 153]. In order for TLR4 to recognize its ligand, lipopolysaccharide (LPS) has to be bound by the secreted protein LPS-binding protein (LBP). Then, it can bind to the complex of TLR4 and MD-2 and induce downstream signaling. Another co-receptor, CD14, is needed for TLR4 to recognize the smooth forms of LPS, containing repetitive units of O-polysaccharide. As BMMCs lack mCD14, they can only recognize the rough forms of LPS without the O-polysaccharides, unless they are incubated with the soluble form of the CD14 receptor (sCD14) [73].

After recognition of the respective ligand, TIR domain-containing adaptor proteins are recruited to the receptors to induce downstream signaling. These adaptor proteins include Myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adapter protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM). With the exception of TLR3, all TLRs signal via MyD88, although TLR2 and 4 require TIRAP as additional adapter to recruit MyD88. Activation of the MyD88 pathway results in NF-κB and MAP kinase activation, which induces production of inflammatory cytokines [83]. Upon TLR4 receptor activation, MyD88 and TIRAP are recruited via interactions of the TIR domains. Then MyD88 interacts with the IL-1R-associated kinase-4 (IRAK-4), which leads to activation of other IRAK family members, like IRAK-1 [96, 114]. Through this cascade, TRAF6, an E3 ubiquitin protein ligase, as well as other E2 ligases, are recruited. The complex consisting of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and TAB3 is then activated [181]. This is followed by the activation of the
IKK-complex, consisting of IKK-α, IKK-β and IKK-γ (NEMO), phosphorylating the NF-κB inhibitory protein IκB-α, which is subsequently ubiquitinated and degraded in the proteasome. Finally the nuclear localization signal of NF-κB is unmasked by the degradation of IκB-α, and NF-κB can then translocate into the nucleus and initiate transcription of its target genes [164]. Activation of TAK1 will also lead to initiation of the JNK and p38 kinase pathways [164].

The TRIF pathway is used by TLR3 and TLR4, the latter requiring TRAM as a bridge. Stimulation of this pathway leads to activation of NF-κB and IRF3 and induces both inflammatory cytokines and type I interferons [83]. Stimulation of TLR4 in BMMCs does not lead to activation of the TRIF pathway and also does not induce IFN production. Also, BMMCs deficient in TRIF show impeded cytokine production in response to LPS while in MyD88-deficient BMMCs LPS-dependent NF-κB activation is completely absent [84]. While the MyD88-dependent signaling is initiated by the receptor at the cell surface, TRIF-mediated signaling, for instance in macrophages, occurs only after internalization of the TLR4 complex into early endosomes [78]. In order for the internalization to take place, mCD14, the co-receptor of TLR4 and MD-2 is required [178]. As BMMCs do not express mCD14, there is no internalization of the TLR4 complex after LPS recognition and thus no TRIF signaling [73, 75].

1.2 TIS11 family of mRNA-binding proteins

Protein production is not solely regulated via the transcription of the corresponding gene. Quite recently, various posttranscriptional mechanisms have been highlighted for their importance in controlling the translation of proteins. One of these mechanisms is conducted by the TIS11 protein family, known for destabilizing mRNAs. So far four different members of this family have been identified. The most prominently researched member is Tristetraprolin (TTP), also known as ZFP36 or TIS11. The other members include the Butyrate-response factor (BRF) 1, also known as ZFP36L1 or TIS11b, and BRF2, also known as ZFP36L2 or TIS11d [8]. Recently a fourth member, ZFP36L3, was discovered expressed only in placental tissue of mice but not in any human tissues [13].

All TIS11 proteins share a highly conserved tandem zinc finger domain that enables them to bind AU-rich elements (AREs) in mRNAs. With the help of computational studies, the
total amount of ARE-containing transcripts of human genes is estimated to fall into the range between 5 - 8% [6]. ARE sequences are made up of repeats of the pentameric sequence AUUUA. Depending on the number of repeats and additional regions, AREs are subdivided into several classes [169]. For TTP, the optimal and minimally required binding sequence has been defined as UUAUUUAUU [170]. ARE-containing transcripts include, amongst others, mRNA of cytokines such as TNF-α, IL-3 and IL-6, growth factors, like GM-CSF and VEGF, and even the ARE-binding proteins themselves as in the case of TTP [33, 144, 158, 180]. ARE-containing mRNAs are regulated in their stability via ARE-binding proteins. While there are proteins promoting destabilization and subsequent decay of ARE-containing transcripts, such as the TIS11 proteins, other proteins are known to stabilize the respective mRNAs via ARE binding, such as HuR or the recently discovered Zfand5 [43, 63]. Both, ARE-containing mRNA destabilizing and stabilizing proteins, may bind to the same binding sites and compete with each other, which leads to a tightly regulated system of protein translation [63, 159].

The mechanism of destabilization of ARE-containing mRNA has been studied intensively for

**Fig. 2:** Schematic representation of the regulation of TNF biosynthesis by the p38-MK2/3 pathway. Both p38 and MK2/3 can directly phosphorylate TTP at multiple phosphorylation sites. Adapted from [140].
TTP and decay of TNF-α mRNA. TTP directly interacts with Ccr4, which is part of a major deadenylation complex with Caf1 and Not, leading to the decay of ARE-containing transcripts [100]. As well, TTP is able to stimulate the activity of PolyA specific ribonuclease (PARN) without directly interacting with it. This also leads to deadenylation and subsequent decay of polyadenylated ARE-containing mRNAs [90]. There are two primary decay pathways and it is not currently known which one is preferentially used by TTP. The first one is the 5′ → 3′ decay in processing (P)-bodies containing enzymes for mRNA decay. The second one is the 3′ → 5′ decay mediated through a complex of exonucleases known as the exosome [52]. Functional regulation of TTP occurs mainly via extensive phosphorylation (see figure 2). The MAP kinase ERK2 phosphorylates TTP at serine 220 [157]. For the p38 MAP kinase pathway it has been shown that p38 itself can phosphorylate TTP [27]. In addition MAPKAP kinase 2 (MK2), a downstream target of p38, has been identified as the kinase phosphorylating serines 52 and 178 of TTP [32]. Unphosphorylated TTP is localized in the nucleus and is actively influencing the stability of ARE-containing transcripts, including mRNA for TTP itself, which leads to a low protein expression of TTP [158]. Upon phosphorylation at the serines 52 and 178, TTP associates with 14-3-3 proteins, which will then lead to translocation of TTP to the cytoplasm [76, 151]. Phosphorylated TTP shows reduced binding affinity to the ARE of the TNF-α mRNA that overall results in increased stability of ARE-containing transcripts [66]. There are also conflicting reports that found no change of affinity dependent on the phosphorylation of the protein [26]. Binding to 14-3-3 proteins protects TTP from dephosphorylation by protein phosphatase 2A [152]. Interaction with the 14-3-3 proteins also leads to stabilization of TTP protein due to the exclusion from proteasomal degradation [38].

Apart from its role in destabilizing mRNAs with ARE sequences, it has recently been proposed that TTP also influences the translation of transcripts. Phosphorylation of TTP by MK2 shifts the localization of TNF-α mRNA from monosomes to polysomes, which leads to a greater translation of transcripts [159]. Cooperation with the translational repressor RCK as well leads to translational inhibition [130].
1.3 The high-affinity IgE receptor FceRI

The FceRI is a high affinity IgE-binding receptor consisting of 4 subunits in its mature form on murine MCs and basophils. The receptor contains one α-chain, one β-chain, and two disulphide-linked γ-chains. The α-chain is comprised of two extracellular immunoglobulin-related domains that bind a single IgE molecule. The short cytoplasmic tail of the α-chain has no known signaling function [89]. Both the β- and γ-chains are not involved in ligand binding but each contains one cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) [134]. Ag-crosslinking of the receptor leads to phosphorylation of the tyrosines in the ITAMs, generating docking sites for SRC homology 2 (SH2) domain-containing proteins that initiate downstream signaling [89].

Fig. 3: Simplified model of FceRI signaling. After allergen recognition by IgE bound to the receptor, FYN and LYN phosphorylate ITAMs of the β- and γ-chains. This leads to binding of Syk to the phosphorylated ITAMs and subsequently to downstream activation of PI3K, MAPK, and calcium signaling, ultimately inducing degranulation or production of eicosanoids and cytokines. Adapted from [89].
CHAPTER 1. INTRODUCTION

Simple binding of the IgE antibody to the receptor can already influence regulation of surface expression. RBL-2H3 cells grown in presence of IgE accumulate FcεRI receptors on their surface [50]. Also for bone marrow-derived and peritoneal MCs (BMMCs and PMCs, respectively), surface expression of FcεRI is enhanced in vitro and in vivo by incubation with IgE [174]. Binding of IgE stabilizes the receptor on the cell surface stopping degradation of surface-expressed receptors, but still maintaining basal synthesis. There is no evidence for an increase in transcription or translation of the FcεRI. Initially the accumulation comes from preformed receptors, that are recycled or recently synthesized [16]. Binding of IgE to the FcεRI also prevents the apoptosis of cytokine-deprived BMMCs and induces multiple phosphorylation events, such as activation of MAP kinases [80].

Intracellular signaling downstream of the FcεRI is conducted by several signaling molecules and adapter proteins (see figure 3). Src family kinases (SFKs) together with the Syk kinase initiate signaling cascades proximal to the receptor. The SFK Lyn, constitutively bound to the FcεRI β-chain, phosphorylates the ITAMs of the β- and γ-chains after receptor engagement [163]. This leads to Syk binding to the phosphorylated ITAMs of the γ-chain, which stabilizes Syk in an active conformation and triggers amplification of downstream signaling pathways [77]. Among all the substrates for Syk are the adaptor proteins linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein of 76 kDa (SLP76). Adaptor proteins assemble large signaling complexes including other molecules such as the Bruton’s tyrosine kinase (BTK), which together with Syk activates PLCγ and induces calcium signaling [89].

The role of the kinase Lyn in FcεRI signaling is not entirely clear. Throughout literature, there are studies showing that Lyn-deficient BMMCs either show an increased or a decreased degranulation response in comparison to WT BMMCs [115, 175]. Other data suggest a dependence on the strength of the stimulation. While in low intensity FcεRI stimulation Lyn acts as a positive regulator with high intensity stimulations the role of Lyn changes towards a negative regulator [172]. As well, the different genetic backgrounds of the mice used in the various studies regarding the function of Lyn in MCs have also been shown to influence the results. Albeit increasing degranulation response in 129/SV mice, Lyn-deficiency does not have an effect in C57BL/6 mice [175]. However, Ag-triggered cytokine production is enhanced in Lyn-deficient BMMCs independent of genetic backgrounds [175]. Other important Src family
kinases in FcεRI signaling are Fyn and Hck. Both kinases have been shown to have a positive regulatory function in Ag-dependent MC activation in processes such as degranulation and cytokine production [56, 70]. Fyn is known to activate several lipid signaling pathways such as the PI3K pathway described in more detail in chapter 1.4. Interestingly, the three Src kinases can regulate each other in addition to their specific downstream signaling. The Fyn kinase pathway has been shown to be negatively regulated by Lyn, while Lyn itself is negatively regulated by Hck [70, 115].

Activation of the FcεRI also triggers calcium signaling in MCs, that contributes to degranulation and production of both eicosanoids and cytokines [171]. Calcium signaling in MCs consists of two steps, first calcium is released from the intracellular calcium stores in the endoplasmatic reticulum (ER). Depletion of the ER calcium then triggers extracellular calcium influx through store-operated calcium channels [124]. Regulation of intracellular calcium response is regulated by PLCγ, that can generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) from phosphatidylinositol bisphosphate (PIP₂). IP₃ stimulates release of intracellular calcium by binding to its receptor in the ER membrane. DAG and intracellular calcium together are able to activate PKCs ultimately leading to induction of signaling pathways like the NF-κB pathway [124].

1.4 Phosphatidylinositol 3-kinase pathway and its regulation

The phosphatidylinositol 3-kinases (PI3Ks) are part of a central pathway in MC signaling, influencing MC mediator release, chemotaxis, adhesion, and homeostasis of MCs [86]. PI3Ks are a family of lipid kinases that are divided into three different classes differing in their function and structure. One of the traits defining the classes is their substrate recognition. Class I PI3Ks can phosphorylate the unphosphorylated phosphatidylinositol (PI), PI monophosphate (PI(4)P) and PI bisphosphate (PI(4,5)P₂) leading to generation of PI(3)P, PI(3,4)P₂ and PI(3,4,5)P₃ respectively. Class II PI3Ks are able to phosphorylate PI and PI(4)P, while Class III PI3Ks may only phosphorylate PI [162]. Class I PI3Ks can additionally function as serine protein kinases [39]. For cell types that belong to the hematopoietic lineage, like MCs, there is little proof of a role for class II and III PI3Ks making class I PI3Ks the major isoform involved in
cellular processes. Class I PI3Ks are heterodimeric, comprised of a regulatory and a catalytic subunit, and are subdivided into two groups, IA and IB. All Class IA regulatory subunits have a binding region to interact with the catalytic subunits, two SH2 domains, which enables them to bind to phosphorylated tyrosines of various receptors and adaptor proteins, and one SH3 domain. Class IB PI3Kγ is activated by heterotrimeric G-protein-coupled receptors (GPCRs), regulated by the free βγ subunits of G proteins after GPCR activation [86]. MCs express the class IA p85α, p85β, p50α, and the class IB p101 and p84 regulatory subunit isoforms, in addition to the class IA PI3K catalytic subunit isoforms, p110α, p110β and p110δ and the class IB p110γ catalytic subunit [15, 86].

The signaling downstream of phosphorylated PIs occurs via binding of proteins with domains specialized in recognizing PIs, such as the pleckstrin homology (PH) domain [60]. Binding to the PIs leads to changes in localization, conformation, and activity of the PH-domain containing proteins. The PH domains between different proteins vary in their ability to recognize and bind PIs, which leads to a complex system of signaling downstream of the PI3Ks [177]. Apart from the PH domain, other PI binding domains have been characterized, like the FYVE and the PX domain, both able to bind to PI(3)P [53, 173]. One of the PH domain-containing proteins showing great influence in MC signaling downstream of PI3K is the serine/threonine kinase Akt. As it was independently identified as a protein kinase related to PKA and PKC, Akt is also called protein kinase B (PKB) [34]. In mammalian cells there are three closely related isoforms, Akt1, Akt2 and Akt3 (PKBα, PKBβ, and PKBγ, respectively). All isoforms share a similar structure, namely a N-terminal regulatory domain including the PH-domain, a hinge region connecting the PH-domain to the serine/threonine kinase domain and a C-terminal region important for inducing and sustaining the kinase activity [48]. When not bound to PIs, Akt has been shown to stay in an inactive conformation due to the intramolecular interaction of the PH domain with the kinase domain [25]. Once the protein is able to bind to PIs, its conformation opens up and exposes the threonine residue 308 in the kinase domain for phosphorylation by the phosphoinositide-dependent kinase-1 (PDK1) [3]. The second phosphorylation site important for full Akt activity is serine 473, which was shown to be phosphorylated by the mammalian target of rapamycin complex-2 (mTORC2) [145]. Downstream targets of Akt include proteins involved in diverse cellular processes such as GSK3 (glucose regulation, proliferation, migration,
apoptosis), BAD (apoptosis) or BRF1/2 (mRNA stability) and many others, making it a central player in PI3K signaling [9, 48].

Generation of PIs via PI3Ks can also be regulated directly by lipid phosphatases such as the SH2 domain-containing inositol 5’-phosphatase 1 (SHIP1), covered in chapter 1.5, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (see figure 4A). More precisely PTEN is a PI(3,4,5)P3 3-phosphatase, that counteracts the kinase activity of PI3K directly, thereby making it an important influence for the PI homeostasis in the cells [101]. As well this regulation has coined the term ”tumor suppressor” for PTEN, as deregulation of the PI3K pathway can lead to increased Akt signaling which results in proliferation of cells [149].

1.5 SH2 domain-containing inositol 5’-phosphatase 1

In contrast to the aforementioned lipid phosphatase PTEN, SHIP1 dephosphorylates PIs at the D5 position. While PI3Ks generate PI(3,4,5)P3 from PI(4,5)P2, dephosphorylation of PI(3,4,5)P3 by SHIP1 will lead to formation of PI(3,4)P2. This means that any PI(3,4,5)P3 dephosphorylated by SHIP1 are taken out of the PI3K-PTEN homeostasis, leading to a different signaling outcome than the standard PI3K signaling.

The structure of the protein comprises a N-terminal SH2 domain, followed by a centrally positioned 5’-phosphatase domain, including a C2 domain that mediates binding to phospholipids, and a proline-rich region towards the C-terminus of the protein (see figure 4B) [35]. Recently, a PH-R domain with structural and functional similarities with members of the PH domain family has been found N-terminal of the phosphatase domain [108]. At the C-terminus, two NPXY sequences have been identified at Y917 and Y1020 that can be tyrosine phosphorylated and be used for the interactions with other SH2 domain-containing proteins, such as the adaptor protein Shc [92]. We have recently shown that the SH2 domain of SHIP1 itself can interact with the phosphorylated NPXY sequence at Y1020, although it is not clear if this interaction is or is not limited to either intra- or intermolecular interactions [112]. Additionally the proline-rich region contains two PxxxPR or Px(P/A)xxR motifs at R1033 and R1139 that serve as atypical SH3 domain binding sites for the adaptor protein CIN85, covered in chapter 1.6 [22, 88]. Isoforms of various sizes have been identified for SHIP1, that range from the biggest isoform with 145 kDa
to the smallest isoform with 110 kDa. Smaller isoforms lack a part of the C-terminus, which has been attributed to either mRNA splicing or proteolytic cleavage [35, 37]. While SHIP1 is expressed only in the hematopoietic system, the closely related SHIP2 appears to be expressed ubiquitously [126].

Mice devoid of SHIP1 expression were generated in 1998. SHIP1 knockout mice are viable and fertile but show failure to thrive and, after 14 weeks, survival of mice is reduced to 40 % [65]. Mice lacking SHIP1 show massive chronic hyperplasia of myeloid cells leading to splenomegaly, lymphadenopathy, and infiltration of various organs by myeloid cells [99]. The increase in mortality of SHIP1-deficient mice is attributed to infiltration of the lung by myeloid cells [65]. MCs deficient in SHIP1 show a hyperresponsive phenotype and they proliferate and differentiate faster from progenitor cells. Ag-induced degranulation is massively increased in SHIP1 KO BMMCs, even at supraoptimal Ag concentrations, defying the usually bell-shaped response curve observed in WT BMMCs. This coined the term "gatekeeper of mast cell degranulation" for SHIP1 [71]. SHIP1-deficient BMMCs are even able to degranulate after stimulation with Steel Factor in contrast to WT BMMCs [72]. Apart from degranulation, also production of various cytokines such as IL-6 and TNF-α is markedly increased in SHIP1 KO BMMCs in

Fig. 4: A PI3K pathway and regulation by PTEN and SHIP1. PI3K phosphorylates PI(4,5)P₂ to PI(3,4,5)P₃, which is reversed by dephosphorylation by PTEN. On the other hand, SHIP1 dephosphorylates PI(3,4,5)P₃ to PI(3,4)P₂. B Structure of SHIP1 comprised of a N-terminal SH2 domain, a central 5'-phosphatase domain including C2 domain, and a C-terminal proline-rich region including two NPXY sequences.
response to Ag, as well as upon stimulation of Toll-like receptors (TLRs) [68, 79].

Apart from its catalytic function, due to the SH2 domain and the proline-rich region, it has been proposed that SHIP1 also has a non-catalytic function based on its interactions with other proteins. As theoretically interactions between different SHIP1 proteins could occur from the N-terminal SH2 domain of one SHIP1 protein to the C-terminal NPXY sequences of another, one can imagine the possibility of huge scaffolds created by SHIP1 proteins. So far, this is just a hypothesis and still needs to be proven directly [112]. In BMMCs, interaction of SHIP1 with several proteins has been confirmed. It has been shown that after receptor engagement, SHIP1 directly interacts with the ITAM motifs of the FcεRI chains, which might be a possible mechanism for the downregulation of supraoptimal Ag stimulation [55, 120]. Interaction of SHIP1 with p66Shc leads to the recruitment of this complex to the adaptor protein LAT upon FcεRI stimulation. This is thought to translocate SHIP1 to the plasma membrane to exert its catalytic activity [138, 160]. Another study suggested that SHIP1 may mediate the FcγRIIB inhibitory influence on FcεRI signaling via the interaction with p62(dok) and RasGAP [122]. Other studies regarding interaction partners of SHIP1 have been performed in various cell types, but as the network of interacting proteins may differ between different types of cells, they will not be discussed here.

1.6 The adaptor protein Cbl-interacting protein of 85 kDa

The Cbl-interacting protein of 85 kDa (CIN85) belongs to a family of adaptor molecules, that consists of Cas ligand with multiple SH3 domains (CMS), also known as CD2-associated protein (CD2AP), and CIN85 itself. Depending on the organism CIN85 has been found in, there are several names for the adaptor protein. It is called CIN85 in humans, Ruk or SH3 domain-containing gene expressed in tumorigenic astrocytes (SETA) in rats, and SH3 domain-containing kinase binding protein 1 (SH3KBP1) in mice. All of these proteins share between 92 % and 97 % sequence identity [40]. In this thesis, the protein will further on be called CIN85 to avoid confusion.

While CIN85 exists in many different isoforms, resulting from usage of multiple promoters and alternative splicing (see figure 5), CMS has only one isoforms that corresponds to the
biggest isoform of CIN85. Expression of the different CIN85 isoforms varies between cell types from neuronal cells mostly expressing the two biggest isoforms [148], to cell lines of various tissue origins expressing several combinations of isoforms. No two types of cells seem to be completely alike in the expression pattern of the various isoforms [107].

Fig. 5: Depiction of the molecular structure of isoforms of CIN85 starting from the N-terminus with the three SH3 domains (A, B, and C), the proline-rich region (PR), and the coiled-coil domain (CC) at the very C-terminus. Figure adapted from [61]

All isoforms share the same C-terminus comprised of the coiled-coil domain, which has been shown to mediate homodimerization and tetramerization of CIN85 [22, 165]. The coiled-coil domain also enables the protein to bind to phosphatidic acid, possibly converted from lysophosphatidic acid by endophilin, a well known binding partner of CIN85 [150, 179]. Most of the bigger isoforms contain a centrally located proline-rich region. Like for the coiled-coil domain, the proline-rich region is deemed important for multimerization of CIN85 [22]. But also other proteins can bind with their respective SH3 domains to the proline-rich region of CIN85 like the PI3K subunit p85α [17, 58, 165]. Towards the N-terminus, up to three SH3 domains can be found, that enable CIN85 to bind to proline-rich regions of other proteins like SHIP1 [22], SHKBP1 [46], SLP65 [116], MEKK4 [1], and Cbl [156] but also enable binding to its own proline-rich region [17]. While some proteins only seem to be specifically bound by one of these three SH3 domains, there are others that bind to more than one. The SH3 domains of CIN85 recognize an atypical PxxxPR or Px(P/A)xxR motif, and binding of the motifs also depends on preferences for certain amino acids at the "x" positions [88]. Additionally, all SH3 domains of the protein are able to bind ubiquitin with varied affinities, e.g. SH3-C with the strongest and SH3-B with the weakest affinity. The binding motif seems to be a central hydrophobic region
in ubiquitin, that is surrounded by negative potential. The recognition site in the SH3 domain for ubiquitin binding is in the same region that confers binding to proline-rich regions so that competitive binding of the domain is possible [11].

CIN85 plays an important role in downregulation of receptor tyrosine kinase signaling. This downregulation is achieved by interaction of CIN85 with endophilins, regulatory components of clathrin-coated vesicle formation, and the E3 ubiquitin ligase Cbl, which can ubiquitinate the respective receptor tyrosine kinase leading to its degradation [40]. One of the most investigated receptors, in that respect, is the epidermal growth factor receptor (EGFR). CIN85 controls EGFR receptor trafficking along endocytic and recycling pathways [88]. Knockdown of CIN85 by siRNA leads to reduced ubiquitination and delayed degradation of EGFR [135].

Also for receptors in immune cells CIN85 has been shown to have influence over signaling events. In B cells, crosslinking of the B cell receptor increased binding of CIN85 to Cbl over time [22], and it has been shown that there is constitutive interaction of SLP65 with CIN85, possibly necessary for membrane translocation of SLP65 [116]. In human neutrophils, CIN85 and c-Cbl are recruited to FcγRIIα after crosslinking of the receptor, ultimately leading to its proteasomal degradation [104]. In RBL-2H3 MCs, overexpression of CIN85 increased binding to Cbl, that in competition with STS1, leads to enhanced ubiquitination of Syk and reduced FcεRI-mediated activation [125]. In a different study, it was shown that overexpression of CIN85 lead to increased internalization of FcεRI upon Ag stimulation, that also reduced degranulation of RBL-2H3 cells [109]. To our knowledge, so far, there has been no research regarding CIN85 using the model of BMMCs.
Chapter 2

Objectives

The aim of the first part of this study will be to characterize the role of TTP in LPS-induced cytokine production in MCs, as TTP is undeniably an important factor for regulation of TNF-α production in macrophages. Further we will be investigating what additional ARE-containing mRNA-stabilizing and -destabilizing proteins are expressed in MCs. The influence of TTP will be clearly defined by generating MCs from bone-marrow cells of TTP-deficient mice and their WT littermates. TTP KO and WT BMMCs will be checked for differences in gene expression and protein production of several proinflammatory cytokines.

In the second part of this study we were interested in finding novel interaction partners of the lipid phosphatase SHIP1 and define the function of the interaction. We will screen for potential targets via combining mass spectrometry and bioinformatical analysis. Predicted interaction partners will be confirmed with immunoprecipitation and in confocal microscopy with the proximity ligation assay. Several interaction partners already published without looking into the actual function of the interaction will be investigated as well.
Chapter 3

Results

The first part of the results focuses on the influence of the mRNA-destabilizing protein TTP on the p38-dependent induction of IL-6, TNF-α and IL-1β production by LPS stimulation in BMMCs. The second part continues with studies of interaction partners of the lipid phosphatase SHIP1 and in further detail the interaction of SHIP1 with the adapter protein CIN85.

3.1 LPS-induced production of IL-6, TNF-α and IL-1β in MCs is dependent on p38 but independent of TTP

3.1.1 Costimulation of IGF-1 and LPS enhances p38 activity in comparison to LPS alone

In previous work we have shown a great dependence of LPS signaling on the PI3K pathway, enabling IGF-1 as a PI3K-activating factor to modulate the LPS-induced cytokine profile of BMMCs [68]. As well we could show that inhibition of p38 not only diminished the influence of IGF-1 to increase LPS-induced IL-6 and TNF-α production, but also the response to LPS in general. Despite these differences in cytokine production, for stimulation up to 1 h, there was no substantial change in phosphorylation of p38 to be found [67]. As we measure ELISAs after 4 h of stimulation, we were interested if we would see an effect of IGF-1 on LPS-induced p38 activation beyond 1 h of stimulation. WT BMMCs were stimulated with either LPS or LPS at the same time with IGF-1 up to 3 h in 30 min intervals (Fig. 6A). Densitometric analysis
of the Western blot data showed an increase of Akt and p38 phosphorylation when IGF-1 and LPS were used together in comparison to LPS alone. For Erk 1/2 phosphorylation there were no substantial differences between adding IGF-1 to LPS and using LPS alone for stimulation (Fig. 6B). This finding implied that p38 as well as PI3K signaling is involved in the modulation of LPS signaling by IGF-1 in BMMCs.

Fig. 6: IGF-1 increases LPS-induced phosphorylation of Akt and p38, but not Erk 1/2. A WT BMMCs were either left untreated (-) or stimulated with 1 µg/ml LPS or LPS and 1 µg/ml IGF-1 at the same time for the depicted time points. Postnuclear supernatants (PS) were analyzed by immunoblotting with antibodies against p-Akt (top panel), p-Erk 1/2 (second panel from top), p-p38 (third panel from top) and Gapdh (bottom panel). B Densitometric analysis based on the data shown in A. Values were calculated by normalizing the intensities for p-Akt, p-Erk 1/2 and p-p38 on the Gapdh band intensity. Data is representative of three independent experiments using separate cell cultures.

3.1.2 p38 has strong, Erk only moderate influence on LPS-induced cytokine production

In a previous study we have shown while inhibition of p38 activity with BIRB0796 diminished LPS-induced production of IL-6, TNF-α and IL-1β, Erk 1/2 inhibition using the pharmacological inhibitor UO126 only affected IL-6 production [67]. We and others have shown before that the MEK inhibitor UO126 shows cross-reactivity on other target proteins while the MEK inhibitor
PD0325901 shows much greater specificity for its target protein [5, 105, 132]. To confirm the influence of p38 and to reassess the effect of Erk 1/2 on LPS-induced cytokine production, WT BMMCs were preincubated with the respective inhibitors or vehicle (DMSO) and subsequently stimulated with LPS for 4 h.

**Fig. 7:** Inhibition of p38 shuts down LPS-induced cytokine production while inhibition of the Erk pathway only moderately decreases it. **A** WT BMMCs were pretreated for 20 min with either DMSO, 1 µM PD0325901 or 0.1 µM BIRB0796, then left untreated (-) or stimulated with 3 µg/ml LPS for 4 h. Subsequently, the amounts of IL-6 (left panel) and TNF-α in supernatants (middle panel) and IL-1β (right panel) in cell lysates were determined by ELISA. Each bar represents the mean of triplicates ± SD. Comparable results were obtained in several experiments using different BMMC cultures (n ≥ 3). **B** WT BMMCs were pretreated for 20 min with either DMSO, 0.01 µM or 0.1 µM BIRB0796, then left untreated or stimulated with 3 µg/ml LPS for 2 h. IL-6 (left panel), TNF-α (middle panel) and IL-1β mRNA (right panel) expression was analyzed by RT-qPCR. Data is representative of three independent experiments using separate cell cultures.

We found that treatment with PD0325901 only moderately decreased LPS-induced IL-6 and TNF-α production and had no significant influence on IL-1β production, while incubation with BIRB0796 drastically diminished production of all three cytokines (Fig. 7A). The concentration of PD0325901 used was previously shown to be sufficient to inhibit Erk activation in BMMCs [105]. We were interested if p38 inhibition already influences mRNA expression of these cytokines in response to LPS. WT BMMCs were pretreated with increasing concentrations of BIRB0796 or DMSO and subsequently stimulated with LPS for 2 h. Pharmacological inhibition of p38 dose-dependently diminished expression of IL-6, TNF-α and IL-1β mRNA (Fig. 7B). These data demonstrate the importance of the p38 MAPK pathway in regulation of LPS-induced cytokine production in BMMCs.
3.1.3 LPS induces p38-dependent mRNA expression of TTP in BMMCs

For the measured cytokines, extensive research has been performed regarding regulation of mRNA stability and translation via TTP [140, 144, 180]. As the activity of TTP is regulated via the p38 MAPK pathway, we were wondering whether the effects we have seen with costimulation of LPS and IGF-1 on cytokine production might depend on TTP activity. First we checked if TTP mRNA expression was induced after LPS treatment in MCs. WT BMMCs were either left untreated or stimulated with LPS either for 1, 2 or 3 h. The TTP gene transcript was already detectable after 1 h and increased until 3 h of LPS treatment (Fig. 8A and Suppl. Fig. 1). A previous study in macrophages showed that inhibition of the p38 MAPK pathway leads to reduction of TTP gene expression [158]. As we had seen strong influence of p38 on cytokine production in MCs, we investigated the potential p38-dependent regulation of TTP mRNA expression. Indeed, we found strong reduction of TTP mRNA expression already with a low concentration of inhibitor in comparison to the control (Fig. 8B and Suppl. Fig. 2). These data suggest the presence of TTP and its potential regulation via transcription or mRNA stability by p38 in BMMCs.

![Fig. 8](image)

**Fig. 8:** LPS induces TTP gene expression in BMMCs in a p38-dependent manner. A WT BMMCs were left untreated (-) or stimulated with 3 μg/ml LPS for the indicated times. TTP mRNA expression was analyzed by RT-qPCR. Data is representative of three independent experiments using separate cell cultures (see Suppl. Fig. 1). B WT BMMCs were incubated with either DMSO or the indicated concentrations of BIRB0796 for 20 min and subsequently stimulated with 3 μg/ml LPS for 2 h. TTP mRNA was analyzed by RT-qPCR. Data is representative of three independent experiments using separate cell cultures (see Suppl. Fig. 2).
3.1.4 TTP is dispensable for development of BMMCs

To check for the function of TTP in MCs we generated BMMCs from TTP KO mice [28]. After 5 weeks in culture we found surface expression of the markers FcεRI and c-Kit to be comparable between TTP-deficient and WT BMMC cultures (Fig. 9A & B).

![Graph showing differentiation of WT and TTP KO BMMCs over 8 weeks](image)

**Fig. 9:** TTP-deficient BMMCs develop and degranulate comparably to WT BMMCs. A Overview of the differentiation of WT and TTP KO BMMCs over the course of 8 weeks. Starting from week two, cells were measured weekly by FACS analysis for the surface markers FcεRI and c-Kit. Values refer to the amount of double positive cells in %. B Differentiation of WT and TTP-deficient BMMCs after 5 weeks in culture was determined by FACS analysis measuring surface expression of FcεRI and c-Kit. Comparable results were obtained in 5 independent WT and TTP-deficient BMMC cultures. C WT and TTP-deficient (KO) BMMCs were preloaded with IgE overnight. Cells were left untreated (-) or stimulated with either 2, 20 or 200 ng/ml antigen (Ag, DNP-HSA) for 20 min. Degranulation was assessed by relative release of β-hexosaminidase. Each bar represents the mean ± SD of three independent BMMC cultures, each taken from the mean of duplicates.

To test for the general responsiveness of the cells, we performed a degranulation assay comparing TTP-deficient and WT BMMCs. As we have shown in previous publications, degranulation is a fast reaction of the MCs to antigen (Ag) stimulation, occurring in a matter of minutes [44]. As the described functions of TTP are all mRNA related, TTP deficiency should have no influence on the process of degranulation. Indeed measuring β-hexosaminidase activity as readout for degranulation we found no significant differences in Ag-triggered degranulation between...
CHAPTER 3. RESULTS

TTP-deficient and WT BMMCs (Fig. 9C). These data show that BMMCs lacking expression of TTP develop normally and show no impairment in the degranulation response.

3.1.5 TTP-deficient BMMCs show no alterations in LPS-induced cytokine production compared to WT BMMCs

The major readout for loss of function of TTP in macrophages is the increase in both TNF-α transcript and protein expression, which has also been shown for IL-6 and IL-1β [12, 140, 144, 180]. First we checked TTP-deficient and WT BMMCs for differences in gene expression of the cytokines IL-6, TNF-α and IL-1β. TTP-deficient and WT BMMCs were either left untreated or stimulated with LPS for 90 min or 3 h.

![Fig. 10: Gene expression and protein production of IL-6, TNF- and IL-1β is comparable between WT and TTP-deficient BMMCs.](image)

**Fig. 10:** Gene expression and protein production of IL-6, TNF- and IL-1β is comparable between WT and TTP-deficient BMMCs. A WT and TTP-deficient (KO) BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for 90 min or 3 h. IL-6 (left panel), TNF-α (middle panel) and IL-1β mRNA (right panel) expression was analyzed by RT-qPCR. Each bar represents the mean ± SD of three independent BMMC cultures. Comparable results were obtained in several experiments (n ≥ 3). B WT and TTP-deficient (KO) BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for 4 h. Subsequently, the amounts of IL-6 (left panel), TNF-α (middle panel) and IL-1β (right panel) were determined by ELISA. Each bar represents the mean ± SD of three independent BMMC cultures, each taken from the mean of triplicates. Comparable results were obtained in several experiments (n ≥ 5).

Induction of TNF-α mRNA expression did not further increase from 90 min to 3 h (data not shown) and there was no significant difference between TTP-deficient and WT BMMCs (Fig. 10A, middle panel). For expression of IL-6 and IL-1β mRNA we saw an increase from 90 min to 3 h, but also no significant difference between TTP-deficient and WT BMMCs at any time.
point (Fig. 10A, left and right panel). We then looked for differences in cytokine production, as the amount of protein translated from mRNA might differ between TTP-deficient and WT cells. TTP has recently been shown not only to decrease stability but also to block translation of transcripts in two independent studies [130, 159]. Thus the lack of TTP could still lead to increased cytokine production. However, production of IL-6, TNF-α and IL-1β did not show significant differences between TTP-deficient and WT cells after LPS treatment (Fig. 10B). All together the data presented show that TTP-deficiency in BMMCs causes no evident phenotype regarding LPS-induced production of the proinflammatory cytokines IL-6, TNF-α, and IL-1β.

3.1.6 Lack of phenotype of TTP-deficient cells may be due to undetectable protein expression of TTP in WT BMMCs

Since all of the previous results oppose the established function of TTP in LPS signal transduction, we had to make sure that there is a significant difference in TTP expression between TTP-deficient and WT BMMCs. Therefore, TTP-deficient and WT BMMCs were either left untreated or stimulated with LPS for 1, 2 or 3 h. While we could confirm TTP mRNA expression in WT BMMCs, peaking at 2 h of stimulation, we could not detect TTP transcripts in the TTP-deficient BMMCs (Fig. 11A and Suppl. Fig. 3). Several TTP antisera have been developed for detection of TTP by Western blotting. We obtained two antisera raised against the C-terminus of TTP, SAK21A [102], and K2 [146], to check protein expression. TTP-deficient, WT BMMCs and RAW 264.7 macrophages, as a positive control, were either left untreated or stimulated with LPS for 4 h. The BMMCs had to be treated with a higher LPS concentration than the RAW macrophages, as the reaction of BMMCs to LPS is weaker than of macrophages, possibly due to absence of mCD14 and the TRIF signaling pathway [73, 84]. Detection with both antisera showed strong expression of TTP in RAW 264.7 macrophages after LPS treatment, which was absent in LPS-stimulated WT BMMCs (Fig. 11B). To be sure that neither our method of lysis nor our blotting procedure is the reason for not being able to detect TTP in BMMCs, we tested hot lysis of cell pellets as well as wet blotting in contrast to the previously used semi-dry blotting. None of these changes led to detection of TTP protein after LPS treatment in WT BMMCs (data not shown). To exclude that the C-terminus containing the recognition site for the previously used antisera is not available for antibody binding in BMMCs,
we used an antiserum raised against the N-terminus of TTP, SAK20A [102]. As in the previous result, LPS-treated WT and TTP KO BMMCs were indistinguishable from each other (Fig. 11C).

**Fig. 11:** Despite TTP gene expression no TTP protein is detectable in WT BMMCs. **A** WT and TTP KO BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for the indicated times. TTP mRNA was analyzed by RT-qPCR. Data is representative of three independent experiments using separate cell cultures (see Suppl. Fig. 3). **B** WT BMMCs, TTP-deficient BMMCs, and RAW 264.7 macrophages were left untreated (-) or stimulated with LPS for 4 h (3 µg/ml for BMMCs, 0.1 µg/ml for RAW macrophages). Postnuclear supernatants (PS, 40 µg protein for BMMCs, 20 µg protein for RAW macrophages) were analyzed by immunoblotting with antisera raised against the C-terminus of TTP (SAK21A [102] top panel, K2 [146] middle panel) and an antibody against Gapdh (bottom panel). **C** WT and TTP KO BMMCs were treated with 3 µg/ml LPS for 3 h. PS were analyzed by immunoblotting with an antiserum against the N-terminus of TTP (SAK20A [102]). **D** WT, TTP-deficient BMMCs and RAW 264.7 macrophages were left untreated (-) or stimulated with LPS for 3 h (3 µg/ml for BMMCs, 0.1 µg/ml for RAW macrophages). Pellets were lysed and PS were subjected to anti-TTP immunoprecipitation. Precipitates (IP, third and fourth panel from top) as well as PS (first and second panel from top) were analyzed by anti-TTP (first and third panel from top) and anti-14-3-3 (second and fourth panel from top) immunoblotting. HC, heavy chain.

Since the levels of TTP protein may simply be too low for detection via Western blotting, we looked at TTP protein upon immunoprecipitation using the N-terminal antiserum. RAW 264.7 macrophages were used as positive control. Postnuclear supernatants (PS) again showed
induction of TTP protein expression only in LPS treated RAW 264.7 cells but not in WT BMMCs (Fig. 11D, first panel from top). As well, TTP protein could only be precipitated from unstimulated and LPS-stimulated RAW 264.7 macrophages (Fig. 11D, second panel from bottom). As the signal of the heavy chain (HC) of the precipitating antibody can superimpose the signal of proteins around 50 kDa size, as in the case for TTP, we used protein A conjugated with HRP for detection as described [91]. While we still saw a strong signal of the HC, we could readily detect TTP in LPS treated RAW 264.7 cells and weakly in untreated RAW cells, but not in WT and TTP-deficient BMMCs (Fig. 11D, third panel from top). As the signal detected from the HC after immunoprecipitation may still mask modified TTP protein running at a larger size, we also checked for interaction of TTP with 14-3-3 proteins. It has been shown before that LPS-induced TTP expression also increases binding of 14-3-3 proteins to TTP [32]. Confirming our previous results we only found precipitated 14-3-3 proteins in untreated and LPS-stimulated RAW 264.7 macrophages with the expected increase in interaction in response to LPS stimulation. In both TTP-deficient and WT BMMCs we were not able to detect 14-3-3 proteins in the anti-TTP precipitates (Fig. 11D, fourth panel from top).

The lack of TTP protein expression in BMMCs suggests that there should not be any difference regarding stability of TNF-α mRNA between TTP-deficient and WT BMMCs. To investigate this, we treated LPS stimulated TTP-deficient and WT BMMCs with the transcription inhibitor Actinomycin D (AcD) and harvested AcD-treated cells at several time points. In agreement with the previous data we found no significant difference between TTP-deficient and WT BMMCs regarding stability of TNF-α mRNA (Fig. 12A). To exclude a fast degradation of newly synthesized TTP protein by the proteasome, we preincubated WT BMMCs with the proteasomal inhibitor MG-132 before stimulation with either LPS or Ag alone or both of them together at the same time. We have shown before that simultaneous treatment of a TLR stimulus and Ag dramatically increases cytokine production in BMMCs [45, 68]. By combining Ag and LPS stimulation we wanted to exclude that LPS-treatment alone is too weak to induce TTP protein expression in BMMCs. Up to 6 h of stimulation, no TTP protein could be detected in MG-132 treated WT BMMCs in any of the stimulations (Fig. 12B). Surprisingly, even though we were not able to show TTP protein expression in BMMCs, we readily found destabilization of LPS-induced TTP mRNA after AcD treatment (Fig. 12C). In addition to the previously
found general destabilization of TNF-α mRNA, this data suggests expression of ARE-mRNA destabilizing proteins other than TTP in BMMCs.

**Fig. 12:** Lack of TTP still leads to destabilization of TTP mRNA and TTP protein is not stabilized by inhibition of the proteasome. [A] WT and TTP-deficient BMMCs were treated with 3 μg/ml LPS for 2 h. Subsequently, 5 μg/ml Actinomycin D (AcD) were added. After 0 min (-), 30 min, 1 h and 2 h cells were harvested and TNF-α mRNA was analyzed by RT-qPCR. All samples were normalized to the respective control. Each bar represents the mean ± SD of three independent BMMC cultures. Statistical significance refers to the differences between WT and TTP-deficient BMMCs. [B] WT BMMCs were pretreated for 1 h with either DMSO or 100 μM MG-132, then left untreated (-) or stimulated with 5 μg/ml LPS, 20 ng/ml Ag or both at the same time for 3 h or 6 h. RAW 264.7 macrophages were stimulated with 0.1 μg/ml LPS for 3 h as positive control. PS were analyzed by immunoblotting with anti-TTP antiserum (top panel) and an antibody against Gapdh (bottom panel). [C] WT BMMCs were treated with 3 μg/ml LPS for 2 h. Subsequently, 5 μg/ml AcD were added. After 0 min (-), 30 min and 1 h cells were harvested and TTP mRNA was analyzed by RT-qPCR. All samples were normalized to the respective control. Each bar represents the mean ± SD of two independent BMMC cultures. Statistical significance refers to the difference between control (-) and the respective time point.

### 3.1.7 BMMCs and RAW macrophages show differential mRNA expression of TIS11 family members

As the previous results showed great differences of protein expression of TTP between BMMCs and RAW macrophages, we were interested if mRNA expression of TTP would show the same trends. We found higher TTP mRNA expression in RAW 264.7 macrophages in comparison to WT BMMCs already in unstimulated cells. The discrepancy in TTP mRNA levels between the two cell types increased further with LPS treatment. The greatest disparity was found after
1 h, with the RAW macrophages showing a 6 times higher mRNA expression of TTP than WT BMMCs (Fig. 13A). TTP is part of the TIS11 protein family and two other members of this family, BRF1 and BRF2, have been shown to be comparable in their function and regulation [144]. We investigated if mRNA expression of the other TIS11 family proteins was regulated in the same way as TTP mRNA expression. In contrast to TTP, mRNA expression of both BRF1/2 in WT BMMCs was higher than in RAW macrophages at all times. While LPS stimulation upregulated mRNA expression of BRF1/2 over time in BMMCs, only a minor regulation occurred in RAW macrophages (Fig. 13B/C). Apart from TIS11 family proteins we also investigated mRNA expression of the recently discovered Zfand5, a protein stabilizing ARE-containing transcripts [63]. While we found a higher expression of Zfand5 transcript in RAW macrophages than in WT BMMCs that were either untreated or stimulated with LPS for 1 h, after 3 h of LPS treatment mRNA expression of Zfand5 was comparable between the different cell types (Fig. 13D). This data shows that the transcripts for TIS11 family proteins and Zfand5 are differentially expressed in WT BMMCs and RAW 264.7 macrophages.

**Fig. 13:** Differential expression of TTP and BRF1/2 transcripts in BMMCs and RAW 264.7 macrophages. RAW 264.7 macrophages and WT BMMCs were left untreated (-) or stimulated with LPS (3 µg/ml for BMMCs, 0.1 µg/ml for RAW macrophages) for 1 h or 3 h. TTP (A), BRF1 (B), BRF2 (C) and Zfand5 mRNA (D) was analyzed by RT-qPCR. Each bar represents the mean ± SD of three independent RAW 264.7 or BMMC cultures.
3.1.8 The TIS11 family proteins BRF1 and BRF2 are expressed in BMMCs and may compensate for lack of TTP

Next, we were interested whether TTP-deficient BMMCs show differences in mRNA expression of BRF1 and BRF2 in comparison to WT BMMCs. Deficiency of TTP did not lead to a significant difference in mRNA expression of either BRF1 or BRF2 when compared to wildtype cells (Fig. 14A, top and middle panels). As well expression of the transcript for Zfand5 showed no significant difference over the course of LPS stimulation between TTP-deficient and WT BMMCs (Fig. 14A, bottom panel).

![Graph A](image-url)

**Fig. 14:** Other TIS11 family proteins as well as the ARE-mRNA stabilizing protein Zfand5 are expressed in BMMCs. A WT and TTP-deficient BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for 90 min or 3 h. BRF1 (left panel) and BRF2 mRNA (right panel) was analyzed by RT-qPCR. Each bar represents the mean ± SD of three independent BMMC cultures. Comparable results were obtained in several experiments (n ≥ 3). B WT BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for 2 h. Pellets were lysed and PS were subjected to anti-14-3-3 immunoprecipitation. Precipitates (IP, lower panels) as well as PS (upper panels) were analyzed by anti-BRF1/2 (respective top panels) and anti-14-3-3 (respective bottom panels) immunoblotting. Densitometry was performed and relative expression levels (normalization on precipitated 14-3-3) are indicated. C WT and TTP-deficient BMMCs were left untreated (-) or stimulated with 3 µg/ml LPS for 90 min or 3 h. c-Fos (left panel) and Zfand5 mRNA (right panel) was analyzed by RT-qPCR. Each bar represents the mean ± SD of three independent BMMC cultures.

As we have seen TTP transcript but could not detect TTP protein, we wanted to investigate protein expression of BRF1 and BRF2. In PS of untreated and LPS stimulated BMMCs we could not detect protein at the predicted sizes of BRF1 and BRF2 (Fig. 14B, top panel). It has been shown before that both BRF1 and TTP bind to 14-3-3 proteins when phosphorylated.
due to activation of Akt or p38 MAPK signaling pathways [32, 147]. In 14-3-3 precipitates we could detect protein at the predicted sizes of both BRF1 and BRF2, although, according to densitometry normalizing on precipitated 14-3-3, interaction did not change between untreated and LPS-stimulated WT BMMCs (Fig. 14B, bottom panels). As the used antibody detects both BRF1 and BRF2 and their predicted sizes for their murine isoforms are similar, we were not able to determine if only one of the two or both are binding to 14-3-3. We also checked for precipitation of TTP with anti-14-3-3 antibodies, but as implied by previous data, no interaction could be detected (data not shown).

The transcription factor c-Fos has also been shown to be a target for TTP-mediated mRNA destabilization [21]. To confirm our findings for a target other than proinflammatory cytokine mRNA, we investigated c-Fos mRNA expression in WT and TTP KO BMMCs. In agreement with the previous data we also found no significant differences between WT and TTP-deficient BMMCs for c-Fos mRNA expression (Fig. 14B, left panel). Taken together these data indicate that while we found no protein expression of TTP, the TIS11 family proteins BRF1 and BRF2 are both expressed at mRNA and protein level in BMMCs and may compensate for the lack of TTP expression.
3.2 The inositol 5'-phosphatase SHIP1 interacts with the adapter protein CIN85

In this section, novel interaction partners of the phosphatase SHIP1 are shown and the nature of the interaction with the adapter protein CIN85 is investigated in more detail.

3.2.1 Precipitation versus degradation

As SHIP1 is a protein of many isoforms, there are also various antibodies commercially available, each detecting a specific set of isoforms. Throughout this study, 4 different antibodies for detection of SHIP1 protein will be shown. The antibody M14 detects the largest SHIP1 isoform (Fig. 15A, top panel, PS), recognizing the last 16 amino acids at the far C-terminus, as we have shown before [94]. The binding site of the antibody P1C1 is further N-terminal from the C-terminus but still in the proline-rich region of SHIP1. Thus the two biggest isoforms can be detected (Fig. 15A, middle panel, PS). The antibodies N1 and G11 supposedly bind the same target region and only differ in their species. Both bind SHIP1 towards the N-terminus, enabling detection of a third, smaller isoform (Fig. 15A, bottom panel, PS). In this study we will refer to the isoform detected by the M14 antibody as isoform 1, the smaller isoform additionally detected by the P1C1 antibody as isoform 2 and the smallest isoform only detected with the N1/G11 antibodies as isoform 3 (See arrows in fig. 15A). Apart from the M14 antibody, exact binding sites are unknown as all antibodies are commercially available and the manufacturer does not disclose the details about their generation. Most immunoprecipitations (IPs) for SHIP1 in this study were performed with the M14 antibody, as from our experience, the greatest yields in precipitated protein are achieved with the M14 antibody. As the M14 antibody only recognizes isoform 1 and all smaller isoforms lack the M14 recognition site, one would expect only isoform 1 to appear in IPs with the M14 antibody. We have shown before that SHIP1 seemingly interacts with itself or other SHIP1 molecules [112]. Thus even when using the M14 antibody for precipitation of the biggest isoform, all smaller isoforms can be found in IP samples as well (Fig. 15A, IP).

In a previous study we have shown that certain stimuli lead to a fast increase of SHIP1 isoform 1 protein levels on Western blots and this was not due to newly synthesized protein [94].
We can confirm this behavior of SHIP1 especially for antigen (Ag) stimulation (Fig. 15A & D). It is not part of this study to investigate this phenomenon any further.

**Fig. 15:** SHIP1 isoforms and their C-terminal degradation in BMMCs. A WT BMMCs were left untreated (-) or stimulated with either 20 ng/ml Ag or 100 ng/ml Steel Factor (SF) for 1 min. Pellets were subjected to anti-SHIP1 (M14) immunoprecipitation. PS and IP samples were analyzed by anti-SHIP1 (M14/P1C1/N1) immunoblotting. Numbers correspond to the different sized isoforms of SHIP1: 145 kDa (1), 135 kDa (2) and 110 kDa (3). B WT BMMCs were either left untreated or cultured for 2 weeks in growth medium (see table 1) with TGF-β. Pellets were lysed in either standard lysis conditions (S, see table 6), Logopharm lysis buffer with standard protease inhibitor concentrations (LB), Logopharm lysis buffer with increased protease inhibitor concentrations (LB+, see table 7) or with hot lysis (1X SDS-loading buffer at 95°C). PS were analyzed by immunoblotting with antibodies against SHIP1 (M14/P1C1) and actin. C Same as B, additionally lysis conditions were supplemented with either 0.1 mM or 0.5 mM 3,4-dichloroisocoumarin (Dci). D WT BMMCs were left untreated (-) or stimulated with 20 ng/ml Ag for 5 min. Pellets were lysed in either S or LB+ conditions and lysates were subjected to SHIP-1 (M14) immunoprecipitation. PS and IP samples were analyzed by anti-SHIP1 (M14) immunoblotting.

Depending on our BMMC culture conditions, we found a great variety of protease gene expression according to multiple transcriptome analyses (data not shown). During cell lysis the C-terminus of SHIP1 seemed to be sensitive to the high protease content of the BMMCs, as with some cell cultures isoform 1 could not be detected in standard lysis conditions, while being readily detectable using hot lysis (Fig. 15B, compare lanes S and HL). Altering lysis buffer composition, e.g. increasing EDTA and EGTA content (Compare table 6 with table 7), improved detection of isoform 1, even more so with increased protease inhibitor contents (Fig. 15B, right side, compare lanes LB with LB+). Adding TGF-β to BMMCs growth medium has been reported to keep MCs from fully maturing, possibly reducing their protease contents.
Using BMMCs grown in TGF-β containing growth medium, detection of isoform 1 was further enhanced in LB+ lysis conditions (Fig. 15B, left side). Apart from hot lysis, no other lysis condition had any influence on the amounts of other SHIP1 isoforms or other proteins detected (Fig. 15B, middle and lower panel and data not shown). As we had seen substantial differences of transcription of granzymes between BMMC cultures, we also tested the addition of the granzyme inhibitor 3,4-dichloroisocoumarin (Dci) to our lysis buffer. We found a dose-dependent improvement of detection of isoform 1, when adding Dci to the lysis conditions (Fig. 15C). In cultures where the standard lysis conditions were sufficient (Compare fig. 15D with B and C, S lanes), yields of precipitated SHIP1 protein using the M14 antibody were comparable between lysis conditions (Fig. 15D). To avoid the possibility of degradation of isoform 1, all IPs in this study were performed using the LB+ lysis conditions.

3.2.2 SHIP1 interacts with various proteins related to ubiquitin signaling

In cooperation with the company Logopharm (Freiburg, Germany) we performed mass spectrometry in Ag and Steel Factor stimulated WT BMMCs to identify novel interaction partners of SHIP1. The M14 antibody and an antiserum against a C-terminal peptide of SHIP1, described in [36], were used for precipitation of SHIP1 and SHIP1 KO BMMCs served as negative control to confirm specificity of the identified proteins. In agreement with previously published data an exemplary 2D-plot shows that SHIP1 displays the greatest interaction with SHIP1 itself, while there are other proteins repeatedly identified as interaction partners in multiple experiments (Fig. 16A, red and green dots, green dot represents CIN85).

We concentrated on confirming interaction of SHIP1 with the respective proteins via the method of co-immunoprecipitation. WT BMMCs were either left untreated (-) or stimulated with either 20 ng/ml Ag or 100 ng/ml SF for 1 min. Subsequently, anti-SHIP1 (M14) immunoprecipitation was performed. We found weak interaction of SHIP1 with the E3 ubiquitin ligase Nedd4 (Fig. 16B, third panel from bottom). Nedd4 has been implicated in downregulation of growth factor receptors like the EGFR or VEGF-R2 [176]. Strong interaction, especially with Ag stimulation, was observed between SHIP1 and CIN85, of which mostly the biggest isoforms were detected in IP samples (Fig. 16B, second panel from bottom). CIN85 is an adapter protein that regulates via interaction with endophilin and the E3 ubiquitin ligase Cbl, internalization
and ubiquitination of receptor tyrosine kinases, such as the EGFR or c-Kit [40]. We also found a strong interaction after Ag stimulation for the adapter protein STS1 (Fig. 16B, bottom panel).

Fig. 16: Characterization of novel SHIP1 interaction partners. A 2D-plot of protein abundance ratios (rPVs) in affinity purifications with anti-SHIP1 (M14, y-axis) and with anti-SHIP1 antiserum (M.Huber, x-axis, described in [36]) from WT versus SHIP1 KO BMMCs. rPV is calculated as median of peak volume ratios (PV = summed m/z signal intensities integrated over time as determined from LC-MS spectra and assigned to an individual peptide) of the top 6 correlating peptides for each protein, symbolized by dots; red: proteins specifically and consistently enriched versus KO control; green: CIN85; grey lines represent specificity thresholds as suggested by distribution of rPV values. B WT BMMCs were either left untreated or stimulated with 20 ng/ml Ag or 100 ng/ml SF for 1 min. Pellets were subjected to anti-SHIP1 (M14) immunoprecipitation. PS and IP samples were analyzed by immunoblotting with antibodies against Talin1, SHIP1 (M14), Cbl and Nedd4, and with antisera raised against CIN85 (CT, [150]) and STS1 (CT, [69]). C WT and SHIP1 KO BMMCs were treated with 20 ng/ml Ag for 5 min. Pellets were subjected to SHIP1 (M14) immunoprecipitation. PS and IP samples were analyzed by immunoblotting with antibodies against SHIP1 (P1C1) and Grb2, and CIN85 (CT) antiserum. D Lysates of WT BMMCs and homogenized whole mouse brain tissue (WMB) were subjected to immunoblotting with an antiserum and an antibody against CIN85 (CT and H300 respectively).

Interestingly it has been shown before that STS1 can negatively regulate Cbl activity by competing with CIN85 for Cbl binding [125]. Apart from these ubiquitin signaling related
proteins we also found weak interaction of the cytoskeletal protein Talin1 with SHIP1 (Fig. 16B, top two panels). Talin1 is known to bind the cytoplasmic β-tail of integrins in the final step of their activation. As both the mass spectrometry data and the immunoprecipitations indicated that from all identified and confirmed target proteins, CIN85 showed the most consistent and strongest interaction with SHIP1, we focused on the nature of the interaction between these two proteins. Using SHIP1 KO BMMCs we could show that our IP data is indeed specific, since both CIN85 and the well-established SHIP1 interacting protein Grb2 were detected in IP samples of WT BMMCs but not in samples of SHIP1 KO cells (Fig. 16C).

CIN85 is known to be expressed in many different isoforms depending on the cell type [107]. In whole mouse brain lysates the two biggest isoforms are the most prominently expressed isoforms [148]. Comparing whole mouse brain with WT BMMC lysates, we found, using an antiserum raised against the C-terminus of CIN85 [150], correlation between the two biggest isoforms present in brain lysates and the two biggest isoforms present in BMMCs (Fig. 16D, left panel, arrows B & C). Using a commercially available antibody raised against amino acids 366 to 665 of CIN85, which represents the C-terminal half of the full length protein, an additional bigger isoform appeared in BMMCs, while detection of the biggest CIN85 isoforms in mouse brain cells was almost completely diminished (Fig. 16D, right panel, arrow A). The three largest isoforms of CIN85 will further on be called isoform A, B and C (Fig. 16D). This data shows strong interaction of the biggest isoforms of the adapter protein CIN85 with SHIP1 in BMMCs.

### 3.2.3 The biggest isoforms of both SHIP1 and CIN85 interact constitutively with each other

So far we precipitated SHIP1 via its C-terminus. As we saw from all the isoforms of CIN85 mostly the largest ones interacting with SHIP1, we were interested to see if there is also a preference for a specific isoform when precipitating SHIP1 via its N-terminus. IP with the SHIP1 (N1) antibody in lysates of untreated or Ag stimulated WT BMMCs only precipitated moderate levels of SHIP1 when compared with the previous M14 antibody IPs (Compare fig. 15A and fig. 17A). Thus only low levels of precipitated CIN85 or Grb2 could be detected (Fig. 17A, bottom two panels). IP with the SHIP1 (G11) antibody resulted in precipitation of mostly isoform 2 and to a slightly lesser extent isoform 1 of SHIP1 in contrast to the previous SHIP1
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(M14) IPs, which preferentially precipitated isoform 1 (Fig. 17B). Of all CIN85 isoforms almost exclusively isoform B precipitated with SHIP1, isoform A was only detected in PS samples (Fig. 17B, bottom two panels).

**Fig. 17:** Immunoprecipitation of SHIP1 from the N-terminal side and via CIN85. **A - C** WT BMMCs were either left untreated (-) or stimulated with 20 ng/ml Ag for 1 min. Pellets were subjected to immunoprecipitation with either **A** anti-SHIP1 (N1), **B** anti-SHIP1 (G11) or **C** anti-CIN85 (H300) antibody. PS and IP samples were analyzed by immunoblotting with antibodies against SHIP1 (P1C1), Cbl, Grb2 or CIN85 (H300) and an antiserum versus CIN85 (CT).

In summary the previous results showed that CIN85 isoform B and to lesser extent isoform C preferentially interacted with SHIP1, independent of precipitation via the N- or C-terminus of SHIP1. We wanted to see, if there is a similar result regarding SHIP1 isoforms when precipitating CIN85. We performed IPs in untreated or Ag stimulated WT BMMCs using the CIN85 (H300) antibody. We found similar ratios between isoforms of CIN85 in IP samples as detected in PS samples, isoform B being the most prominent (Fig. 17C, third panel from top). Interestingly, even though we used the H300 antibody that is capable of detecting isoform A in PS samples, no evident precipitation of isoform A in IP was observed (Fig. 17C, bottom panel). Isoform 1 of SHIP1 was preferentially precipitated with CIN85, especially in the Ag treated cells. To lesser
extent, isoform 2 was detectable as well, but isoform 3 could not be detected in any IP sample (Fig. 17C, top panel). This data suggests that preferentially isoform 1 and 2 of SHIP1 interact with isoform B and C of CIN85.

**Fig. 18:** Interaction of CIN85 and SHIP1 does not change over time. A WT BMMCs were either left untreated (-) or stimulated with 20 ng/ml Ag for 5 min or 10 min. Pellets were subjected to anti-SHIP1 (M14) immunoprecipitation. PS and IP samples were analyzed by immunoblotting with an antibody against SHIP1 (M14) and an antiserum against CIN85 (CT). Two representative experiments are shown. Comparable results were obtained in several experiments using different BMMC cultures (n ≥ 4). B Densitometric analysis based on the data derived from the experiments in A from 4 different BMMC cultures.

In the previous experiments we only checked for 1 min of Ag stimulation. We were interested to see if the interaction between SHIP1 and CIN85 changed during a longer time frame. WT BMMCs were either left untreated or stimulated with Ag for 5 min and 10 min. Pellets were subjected to SHIP1 (M14) IP and analyzed by immunoblotting for SHIP1 and CIN85. For both SHIP1 and CIN85 we found levels of protein increasing over the time of stimulation (Fig. 18A). We already investigated this behavior of SHIP1 in a previous study [94]. We referred to analysis by densitometry to find out if interaction between CIN85 and SHIP1 is changing over time, normalizing band intensity of the signals for CIN85 to the signals for SHIP1. Densitometry analysis showed no significant change of interaction between 5 min and 10 min of Ag stimulation. This data implies a constitutive and steady interaction of SHIP1 and CIN85 after Ag stimulation.

### 3.2.4 Interaction of Cbl with SHIP1 is not entirely clear

We have shown before that SHIP1 directly interacted with the E3 ubiquitin ligase Nedd4. As SHIP1 also interacts with two proteins, CIN85 and STS1, that both interact with a different
E3 ubiquitin ligase, Cbl, we investigated if SHIP1 is also part of a bigger CIN85/STS1/Cbl-complex. In all previous IPs performed with the SHIP1 (M14) antibody, we could not detect Cbl in the IP samples (Fig. 16B, third panel from top, fig. 17A,B & C, second panel from top). In contrast, when precipitating SHIP1 with its N-terminus using the G11 antibody, we could detect Cbl protein in SHIP1 precipitates (Fig. 17B, second panel from top). In addition to the 130 kDa Cbl protein, in IP samples we could also detect a slower migrating protein around 160 kDa, which we also found when precipitating CIN85 (Fig. 17B & C, second panel from top).

**Fig. 19:** Nature of interaction of Cbl with SHIP1 is uncertain. A & B WT BMMCs were either left untreated (-) or stimulated with either 20 ng/ml Ag or 100 ng/ml SF for the indicated time points. Pellets were subjected to anti-Cbl immunoprecipitation. PS and IP samples were analyzed by immunoblotting with antibodies against SHIP1 (M14) and Cbl and an antiserum against CIN85 (CT). C same as A & B additionally SHIP1 KO BMMCs were used for stimulation and immunoprecipitation.

To confirm the interaction between SHIP1 and Cbl, we precipitated Cbl itself. While in several IPs of Ag and SF treated WT BMMCs, we were not able to detect precipitation of SHIP1 with Cbl, we also had results where we could readily show precipitation of SHIP1 with Cbl (Compare fig. 19A & B with C, top panels). In agreement with previous studies we could detect CIN85 in samples of precipitated Cbl protein (Fig. 19A, B & C, bottom panels). This data suggests that there is a potential interaction between SHIP1 and Cbl, although the requirements for binding are not entirely clear.
3.2.5 Internalization of FcεRI, but not c-Kit, is augmented in SHIP1-deficient BMMCs

CIN85 has been implicated in the regulation of internalization of several RTKs, like c-Kit, which is expressed by BMMCs [40, 155]. As to our knowledge no full knockout of CIN85 has been established, we investigated internalization of c-Kit comparing WT and SHIP1 KO BMMCs to see if the missing constitutive interaction of SHIP1 and CIN85 influences the process of internalization. We are aware that any difference observed might as well be due to SHIP1 missing and this is an indirect way of defining a function for the interaction between SHIP1 and CIN85. WT and SHIP1-deficient BMMCs were either left unstimulated or treated with SF for either 1 min, 10 min and 20 min or 30 min and 1 h. Internalization was assessed via measuring surface expression of c-Kit by FACS analysis. While we found a fast internalization of c-Kit with 40 % reduction of surface expression after 1 min of stimulation, at no time point we observed any substantial differences between WT and SHIP1 KO BMMCs (Fig. 20A).

Fig. 20: Internalization of c-Kit is not dependent on SHIP1, internalization of FcεRI depends on Ag concentration. A WT and SHIP1 KO BMMCs were either left untreated (-) or stimulated with 100 ng/ml SF for 1 min, 10 min, 20 min (left panel) or 30 min and 1 h (right panel). Subsequently c-Kit surface expression was checked by FACS analysis. B WT (left panel) and SHIP1 KO BMMCs (right panel) were left untreated (-) or stimulated with 20, 200 or 2000 ng/ml Ag for 1 min, 15 min, 30 min and 1 h. Surface expression of FcεRI was examined using FACS analysis (Experiment was performed by Marlies Kauffmann).

As it has been shown before that CIN85 is able to enhance ligand-induced receptor inter-
nalization of the FcεRI, we checked for differences between SHIP1-deficient and WT cells in FcεRI internalization [109]. WT and SHIP1 KO BMMCs were left untreated or stimulated with either 20, 200 or 2000 ng/ml Ag for 1 min, 15 min, 30 min or 1 h. Surface expression of FcεRI was assessed via FACS analysis. The pattern for internalization was quite different between WT and SHIP1-deficient cells. While in WT BMMCs, surface expression was reduced to about 70% after 1 min, SHIP1 KO BMMCs showed reduction in surface levels of FcεRI to 50% of control levels (Fig. 20B). Surprisingly we found a recovery of surface expression of FcεRI in WT BMMCs, dose-dependently increasing with the Ag concentration used for stimulation. Treatment with 2000 ng/ml Ag showed a surface expression of FcεRI after 1 h of stimulation similar to the untreated control (Fig. 20B, left panel). In contrast, SHIP1 KO BMMCs only showed a slight recovery of FcεRI surface expression from time points 1 to 15 min, after which surface expression declined again up to 1 h of stimulation. Similar to WT BMMCs, SHIP1 KO BMMCs also showed the greatest recovery of FcεRI surface expression with the highest amount of Ag used (Fig. 20B, right panel).

Taken together the data suggest that the interaction between CIN85 and SHIP1 seems not to be important for c-Kit internalization, while it might have a role in FcεRI internalization. Still more research is necessary to define if the results obtained are not simply due to lack of SHIP1 rather than due to lack of interaction of CIN85 and SHIP1.

### 3.2.6 Confirmation of IP data using confocal microscopy

Previous data showing interaction of CIN85 and SHIP1 was based entirely on the method of immunoprecipitation. Quite recently a new method, the proximity ligation assay (PLA), was developed with the specific purpose of detecting interaction of proteins by confocal microscopy [95]. Briefly described, secondary antibodies, directed against the respective species of the primary antibodies, are conjugated with oligonucleotides. If the proteins of interest are close enough, the oligonucleotides can be ligated to form a circle. Rolling-circle amplification of the oligonucleotide sequence is initiated by adding a polymerase. In the last step fluorescently labeled oligonucleotides will hybridize to the rolling-circle products (RCPs). Washing steps ensure that dot-shaped fluorescence will only appear where the DNA was amplified, which designates the location of the interaction. Using this method we were hoping not only to confirm the previous
IP data, but also gain information about where in the cell the interaction of SHIP1 and CIN85 takes place.

Fig. 21: Proximity ligation assay is established in RBL-2H3 cells via detection of the interaction between SHIP1 and Grb2. **A** Immunofluorescence of Grb2 (top panels) and SHIP1 (P1C1, bottom panels) antibodies in untreated RBL-2H3 cells using Cy3-labeled secondary antibodies. From left to right pictures show Cy3 staining, DAPI and merge of the previous two pictures. **B** Untreated RBL-2H3 cells were analyzed by PLA using Grb2 and SHIP1 (P1C1) antibodies. From left to right pictures show RCPs (PLA), DAPI, phase contrast and merge of the previous three pictures.

To establish this method, we switched the cell system to the adherent mast cell line RBL-2H3. As target proteins we first selected SHIP1 and Grb2, as their interaction was already published [35] and confirmed by earlier experiments (Fig. 16C). First we checked if our primary antibodies are functional for confocal microscopy in RBL-2H3 cells. Untreated RBL-2H3 cells were fixed and permeabilized. Respective primary antibodies were detected with Cy3-conjugated secondary antibodies and then analyzed by confocal microscopy. We found that immunofluorescence of the SHIP1 (P1C1) antibody was restricted to the cytoplasm of the cells, confirmed by DAPI staining of the nucleus. Immunofluorescence of the Grb2 antibody showed a strong stain of the cytoplasm but also weaker in the nucleus, when compared with the DAPI staining (Fig. 21A). This is in
agreement with reports that show cytoplasmic and weak nuclear localization of Grb2 with the method of cell fractionation [139]. Next PLA was performed with untreated RBL-2H3 cells. The primary antibodies that were used were again SHIP1 (P1C1) and Grb2. We could detect multiple fluorescent dots, representing the RCPs, dispersed throughout the cells (Fig. 21B, left panel). Localization was exclusively cytoplasmic, as could be determined from the DAPI staining of the nucleus and the phase contrast view of the cells (Fig. 21B).

![Fig. 22: Proximity ligation assay in RBL-2H3 cells confirms interaction of SHIP1 and CIN85. A Immunofluorescence of SHIP1 (P1C1, upper panels) antibody and CIN85 (CT, lower panels) antiserum in untreated RBL-2H3 cells using Cy3-labeled secondary antibodies. From left to right pictures show Cy3 staining, DAPI and merge of the previous two pictures. B RBL-2H3 cells were either left untreated (-, top picture) or stimulated with 20 ng/ml Ag for 1 h (bottom picture). Pictures show phase contrast view. C RBL-2H3 cells were either left untreated or stimulated with 20 ng/ml Ag for 1 min, 5 min, 10 min or 1 h. Subsequently a proximity ligation assay (PLA) was performed, using anti-SHIP1 (P1C1) and anti-CIN85 (CT) as primary antibodies. Pictures show the merge of the PLA and the DAPI signal.]

We were now interested if we can detect RCPs when performing PLA with the SHIP1 (P1C1) antibody and the CIN85 (CT) antiserum. First the CIN85 (CT) antiserum was checked in immunofluorescence. Untreated RBL-2H3 cells were fixed and permeabilized. The primary antiserum, CIN85 (CT), was detected with Cy3-conjugated secondary antibody and then analyzed by confocal microscopy. We found that immunofluorescence of the CIN85 (CT) antiserum was mostly detected in the cytoplasm of the cells, confirmed by DAPI staining of the nucleus (Fig. 22A). Next, PLA was performed with RBL-2H3 cells either left untreated or stimulated...
with 20 ng/ml Ag for 1 min, 5 min, 10 min or 1 h. As primary antibodies we used SHIP1 (P1C1) and the CIN85 (CT) antiserum. Already in untreated cells we could detect RCPs representing interaction of SHIP1 and CIN85, located throughout the cytoplasm (Fig. 22C, left panel). Up to 10 min of stimulation no apparent change in amount, intensity or localization of the RCPs was observed (Fig. 22C). After 1 h of stimulation the RCPs appeared to be more spread out around the nuclei of the cells, but without any defined localization to a specific site or compartment (Fig. 22C, right panel). We found that spreading of the signal over a wider area was attributed mostly due to spreading of the RBL-2H3 cells themselves over the course of Ag stimulation.

**Fig. 23:** Immunofluorescence, but not RCPs, can be detected in BMMCs. **A** Immunofluorescence of SHIP1 (P1C1, left panels) antibody and CIN85 (CT, right panels) antiserum in untreated WT BMMCs using Cy3-labeled secondary antibodies. From left to right pictures show Cy3 staining, DAPI and merge of the previous two pictures. **B** Untreated WT (left panels) and SHIP1 KO BMMCs (right panels) were analyzed by PLA. Top left picture shows DAPI staining, top right picture shows the RCPs, bottom left picture shows phase contrast view, bottom right picture shows merge of the previous three pictures.

While in untreated cells, using the phase contrast view of the confocal microscope, the plasma membrane can be identified, in cells treated for 1 h with Ag we could not focus on
plasma membrane structures (Fig. 22B). In fact the structures observed for the 1 h Ag treated cells are the boundaries of the nuclei and not the plasma membrane (Compare fig. 22B, lower panel, with fig. 22C, right panel). Taken together, we successfully established PLA in RBL-2H3 cells and were able to confirm interaction of SHIP1 with Grb2 and CIN85, without observing any specificity in localization of the RCPs.

We intended to establish PLA in BMMCs, as all previous IP data were generated in this model system. First we tested the SHIP1 (P1C1) antibody and CIN85 (CT) antiserum for functionality in immunofluorescence in BMMCs. Untreated WT BMMCs were incubated with the respective primary antibodies and subsequently stained with Cy3-labeled secondary antibodies. Slides were analyzed with confocal microscopy. As previously shown in RBL-2H3 cells, we also found immunofluorescence for both antibodies to be mainly cytoplasmic (Fig. 23A). Next we performed PLA using the SHIP1 (P1C1) antibody and CIN85 (CT) antiserum with the respective secondary PLA probes in untreated WT BMMCs, following the same protocol as used for the previous experiments with the RBL-2H3 cells. We only detected diffuse signals without formation of any dot structures in the cytoplasm of WT BMMCs (Fig. 23B, left side). Similar signals were observed using SHIP1 KO BMMCs with the same experimental setup, implying only background staining using PLA in WT BMMCs (Fig. 23B, right side). Several changes to the original PLA protocol were tested, summarized in the following list:

- Comparing methanol with paraformaldehyde for permeabilization of cells
- Switching to different target proteins (mostly SHIP1 and Grb2)
- Increasing primary antibody concentration from 1:100 up to 1:10
- Using PMCs instead of BMMCs
- Testing PLA for FACS analysis
- Using Fc-receptor blocking agent
- Increasing incubation time for amplification step
- Stimulate BMMCs with Ag
None of the changes led to detection of RCPs as observed in the RBL-2H3 cells (data not shown). The data shows that although immunofluorescence signals can be detected in BMMCs, RCPs cannot be detected.
Chapter 4

Discussion

4.1 LPS-induced production of IL-6, TNF-α and IL-1β in MCs is dependent on p38 but independent of TTP

Correct regulation of cytokine production is vital for an adequate immune response, limiting damage to the host and preventing the development of chronic diseases. Overexpression of both TNF-α and IL-6 was reported, amongst other syndromes and diseases, to cause cachexia, rheumatoid arthritis, and atherosclerosis [24, 74, 85, 129].

In previous studies, we have investigated modulation of TLR/TIR-ligand response by activation of PI3K via a second stimulus [68]. Much of this work was focused on the costimulation with IGF-1. While IGF-1 stimulation alone did not activate cytokine production in BMMCs, it modulated the LPS-induced cytokine profile. IL-6 and TNF-α expression was upregulated, whereas IL-1β production was downregulated [67]. We found a great dependence on PI3K activity for these effects, as inhibition of PI3K abolished the influence of IGF-1 [68]. As well we found that the p38 MAPK pathway was important for the effect of IGF-1 on LPS-induced IL-6 and TNF-α production. Using a specific pharmacological inhibitor we already found at low concentrations of inhibitor no difference in cytokine production of IL-6 and TNF-α between LPS alone and LPS in combination with IGF-1 [67]. In the previous study we could not show enhanced p38 phosphorylation as a result of costimulation with IGF-1, mostly because of the short time frame of stimulation and because we did not evaluate the results with the help of densitometry. In the current study we were able to see a reproducible increase in p38 phos-
phorylation when combining IGF-1 and LPS in comparison to just LPS alone, supporting our previous cytokine ELISA data (Fig. 6B, right panel). This prompted us to look into regulatory proteins influencing the production of TNF-α, but also IL-6, downstream of p38 signaling.

Especially for production of TNF-α, intensive research has shown that stability and rate of translation of TNF-α mRNA is tightly regulated by various proteins [43, 63, 130, 140, 159]. Some of these pathways were also confirmed to play roles for mRNA stability in regulation of IL-6 and also IL-1β production [30, 180]. Central to regulation of TNF-α production in macrophages is the mRNA-binding protein TTP. So far, most of the research on TTP was based on macrophage cell systems, in particular in the context of LPS stimulation. To our knowledge only one study has been published investigating TTP in BMMCs, but in contrast to our study the authors concentrated on signaling induced by antigen (Ag) stimulation and induction of TTP by IL-4 [154]. This prompted us to investigate the involvement of TTP in LPS-induced cytokine production in BMMCs.

The principal finding of this part of the thesis was that in response to LPS stimulation only the TTP transcript was produced but no TTP protein in BMMCs was detectable. Instead we found mRNA and protein expression of two other TIS11 family proteins BRF1/2 and mRNA expression of a recently described mRNA-stabilization promoting protein, named Zfand5. These results imply that proteins other than TTP may partake in balancing the response of BMMCs to LPS treatment. Also, the lack of TTP protein expression suggests that the modulation of LPS-induced cytokine production by IGF-1 we have seen in a previous report is not depending on TTP activity.

Initially, we wanted to confirm the results of our previous study and show that LPS-induced cytokine production in BMMCs is indeed regulated via p38 MAPK activity, as this is the main pathway involved in regulation of TTP [19, 102, 140, 159]. We could show that LPS-induced IL-6, TNF-α and IL-1β mRNA expression and protein production are suppressed when p38 activity is inhibited (Fig. 7). Together with the results showing upregulation of TTP gene expression after LPS treatment and decrease of LPS-induced mRNA expression of TTP in the presence of the p38 inhibitor BIRB0796 (Fig. 8 and Suppl. Figs. 1 & 2), we have confirmed basic results about function and regulation of TTP in macrophages [19, 102] in the model system of BMMCs. In contrast to p38 inhibition, we only found weak effects on LPS-
induced cytokine production of IL-6 and TNF-α when inhibiting the Erk pathway (Fig. 7A, left and middle panel). For IL-1β production there was no significant effect observed (Fig. 7A, right panel). This was in line with results published about the substances imperatorin and roxatidine, which inhibit LPS-induced p38 but not Erk activity in RAW 264.7 macrophages. These substances were shown to downregulate IL-6, TNF-α and IL-1β production in LPS-stimulated macrophages, implying dispensability for the Erk pathway in this respect [31, 59]. Interestingly, TACE-mediated processing of pre-TNF was demonstrated to be dependent on Erk signaling in LPS-stimulated macrophages, indicating cell type-specific regulation of TNF-α production [141].

In an attempt to more precisely investigate the role of TTP in LPS-induced signaling in BMMCs, we generated BMMCs from TTP-deficient and WT mice. TTP-deficient and WT BMMCs proliferated and developed comparably in terms of surface expression of FcεRI and c-Kit. While in the first weeks of differentiation, double positivity for FcεRI and c-Kit varied to certain extents between cultures, all cultures reached similar levels after 4 to 5 weeks (Fig. 9A & B). While compared to WT BMMCs, TTP-deficient cells showed a slightly lower surface expression of FcεRI (Fig. 9B), we have not found any differences in degranulation in thorough Ag dose-response studies (Fig. 9C).

Surprisingly we found no significant differences between TTP-deficient and WT BMMCs in LPS-induced IL-6, TNF-α and IL-1β mRNA and protein expression or stability of TNF-α mRNA (Fig. 10 & fig. 12A). Since all TTP targets we checked so far belong to the class of proinflammatory cytokines, we also checked mRNA expression of the transcription factor c-Fos, another reported target of TTP [21, 144]. In agreement with the previous results we also found no significant differences in mRNA expression of c-Fos between LPS stimulated WT and TTP-deficient BMMCs (Fig. 14C, left panel).

As well TTP protein was not detectable after LPS treatment up to 8 h in WT BMMCs while we could readily detect TTP in RAW 264.7 macrophages stimulated with LPS (Fig. 11B and data not shown). Concurrently, immunoprecipitation with an N-terminal anti-TTP antiserum only precipitated TTP in the RAW 264.7 macrophages, but not in any of the BMMCs (Fig. 11D). TTP protein expression has been shown to be severely diminished in cells devoid of MK2/3 activity [140]. We found this not to be the reason for the absence of TTP protein in
BMMCs, as we could detect LPS-induced phosphorylation of MK2 in BMMCs (Suppl. Fig. 4). Also, inhibition of the proteasome has been shown to stabilize TTP protein expression in RAW 264.7 macrophages and in several melanoma cell lines [18, 38]. In contrast to these reports, we could not detect TTP protein LPS-stimulated WT BMMCs pretreated with the proteasomal inhibitor MG-132 (Fig. 12B). Thus, we conclude while we can reproducibly confirm expression of transcripts for TTP in WT BMMCs, there is no detectable expression of protein occurring. This finding may explain why we could not find any significant differences between TTP-deficient and WT BMMCs in response to LPS.

As we had to assume from our results that LPS does not induce TTP protein expression in BMMCs, we set out to analyze expression of other TIS11 family members, BRF1 and BRF2, in BMMCs, which might compensate for the lack of TTP. The fourth member of the TIS11 family of proteins, Zfp36l3, is restricted in expression to placental and extraembryonal mouse tissue [13]. Indeed we found enhanced mRNA expression of BRF1 and BRF2 after LPS treatment that was comparable between TTP-deficient and WT BMMCs (Fig. 13B/C & 14A). Similar to TTP, we found no protein expression in total cell lysates but could confirm presence of BRF1/2 protein via co-immunoprecipitation with 14-3-3 (Fig. 14B), a known interaction partner of phosphorylated TTP and BRF1 [32, 103]. For the stimulation time point chosen we were not able to see an increase in interaction of BRF1/2 and 14-3-3. As the used antibody does not distinguish between BRF1 and BRF2 and both of them are similar in predicted size, 36 and 37 kDa for the murine proteins respectively, we cannot say if either one exclusively or both of them together interact with the 14-3-3 proteins. To our knowledge published data only confirmed interaction of TTP and BRF1 with 14-3-3 [32, 103]. So far no study specifically investigated BRF2 for that matter. Given that TTP and BRF1/2 share over 70 % amino acid identity and TTP and BRF1 have been shown to be regulated in a similar fashion, it is well possible that BRF2 may also interact with 14-3-3 proteins [144].

In our 14-3-3 precipitates we were only able to detect a single band for BRF1/2 (Fig. 14B, IP samples). Similar to TTP, previous studies often find multiple bands when detecting either BRF1 or BRF2, ranging in size from 36 to 62 kDa indicating strong modification of the proteins [98]. For all TIS11 proteins, several studies have shown that the modifications are primarily phosphorylations, as phosphatase treatment could reduce the occurrence of multiple bands to
CHAPTER 4. DISCUSSION

a single band [20, 98]. This implies that in BMMCs, BRF proteins are only phosphorylated at few or even just a single site. It has been shown before, that both serine 92 and 209 need to be mutated to abolish binding of 14-3-3 to BRF1 [9]. Both sites have been shown to be phosphorylated by Akt, a kinase downstream of PI3K [9, 147]. As we have shown the importance of PI3K signaling for LPS-induced cytokine production in BMMCs before [68], we can assume that BRFs in MCs may only be phosphorylated at these two serines. This might explain why protein expression of BRFs is low in BMMCs, since previous studies suggest that phosphorylation of BRF1 and TTP leads to their stabilization [9, 20]. Since in BMMCs BRFs are only phosphorylated at a low level, this might influence their stability, leading to a low protein expression.

Apart from mRNA-destabilizing proteins, we also checked for gene expression of a recently discovered ARE-containing mRNA-stabilizing protein, Zfand5 [63]. Similar to the results of that study we found expression of Zfand5 mRNA after LPS treatment of BMMCs (Fig. 14C, right panel). This lead to the conclusion that apart from TTP, other mRNA-stabilizing and -destabilizing proteins are expressed in BMMCs in response to LPS treatment.

This difference in LPS-signaling between macrophages that strongly express TTP, and MCs that according to our results do not express detectable amounts of TTP protein, may lie in the disparity of TNF-α production between these two cell types. Macrophages are frequently cited to be the major source of TNF-α during inflammatory processes [28, 123, 131]. In a previous study Keck et al. have shown in a direct comparison that bone marrow-derived macrophages produced more than hundred-fold greater amounts of TNF-α than BMMCs relatively to the Re-LPS concentration used for stimulation [84]. For such a massive cytokine production, strong negative regulatory mechanisms are needed to keep the response in check. Heavy upregulation of TTP ensures fast control over stability and translation of TNF-α transcripts, as TTP accumulates when inactivated by phosphorylation via the TLR4-induced p38-MK2 axis [158]. As soon as the response to LPS diminishes, activity of p38 and MK2 is reduced and the phosphatase PP2A can dephosphorylate the accumulated TTP protein, which should then be able to rapidly destabilize and inhibit further translation of the TNF-α mRNA [152]. As MCs produce much lower amounts of TNF-α, low expression of the BRF proteins may be enough to control the amount of TNF-α transcripts in these cells without the help of TTP.

This is supported by our results showing that RAW macrophages express more than 6 times
higher amounts of TTP transcript than WT BMMCs (Fig. 13A). A recent study has proposed a model in which microRNAs (miRNAs) establish a threshold for mRNA translation, below which protein expression is highly repressed. Close to the threshold production of protein is sensitive to the amount of mRNA available [113]. A potential miRNA involved in this kind of regulation of TTP expression could be miR-29a, which has been shown to downregulate TTP protein in polarized Ha-Ras-transformed mammary epithelial cells [54]. Thus we hypothesize that LPS treatment of WT BMMCs does not lead to the amount of TTP transcript needed for its translation to protein. The opposite situation was found for BRF1/2 mRNA expression. While WT BMMCs already show a higher expression of BRF1/2 mRNA in control cells, almost no regulation occurs in RAW macrophages (Fig. 13B/C).

While we were not able to detect TTP protein, we have frequently found destabilization of TTP transcripts in experiments with Actinomycin D-treated cells (Fig. 12C). One possibility could be that the BRF proteins influence the stability of the TTP transcript. It has been shown before that TTP can destabilize its own mRNA and several studies pointed out overlapping binding targets within the TIS11 family members [144, 158]. But so far no study has shown direct binding of BRFs to TTP transcript and thus we can only hypothesize that this might be the reason for destabilization of TTP mRNA in BMMCs. Interestingly, an additional mechanism for silencing of translation has been proposed for both TTP and BRF1. It was shown that both proteins are able to localize ARE-containing transcripts into processing bodies (PBs), promoting translational silencing. While proteins involved in mRNA decay are also associated with the PBs, it is not entirely clear whether they are already active in the PBs, or kept inactive until mRNA and enzymes are released from the PBs [49]. This suggests the possibility of transcriptional silencing of TTP mRNA in MCs, as the TTP transcript itself contains AREs.

Taken together, while the impact of TTP on cytokine production in macrophages is undeniable, it cannot be concluded that this has to be the case for all other cytokine producing cell types as well. More and more enzymes regulating mRNA stability and translation have been discovered recently, implying that different cell types may use differential expression of these proteins to serve their needs in controlling cytokine production. For treatment of diseases caused by cytokine overproduction it might be worthwhile to look into target proteins other than TTP, depending on which cell type is the cause of the damaging cytokine response and on
which proteins are expressed in that particular cell type.

As for the initial idea of an involvement of TTP in IGF-1 mediated modulation of LPS-induced cytokine production in BMMCs, the lack of protein expression suggests no influence of TTP in these pathways. It may well be possible that alternatively the BRFs are involved in this particular signaling, as they also have been shown to be regulated via Akt and p38 MAPK pathways. Both pathways we already found to be essential for the effects of IGF-1 on LPS signaling in BMMCs. Further research is needed to define the specific influence of BRFs in MC signaling.
4.2 The inositol 5’-phosphatase SHIP1 interacts with the adapter protein CIN85

Most proteins bearing a catalytic function, are primarily defined in the various studies investigating them by this effector function. If a newly discovered enzyme is found, researchers will first try to define potential targets for modification by its catalytic domain. Apart from proteins with obvious effector functions, there are also proteins not featuring any kind of direct effector function. Instead they serve as scaffolds or adaptors for enzymes, either bringing together several proteins of one signaling cascade, as in the case of β-arrestins and the Erk MAPK pathway [110], or localizing proteins to specific compartments, each representing a different potential downstream signaling pathway. An example of the latter are the 14-3-3 proteins, mentioned in the previous section. By binding and relocating TTP they influence its downstream effector functions [76].

Classifying proteins as either enzymes or adapter proteins is obviously an oversimplification established by scientists over the years to make it easier to categorize and to study them. But if something is to be learned from nature, then it is that actually there are no strict categories. If there are enzymes and adapter proteins, it is not unlikely that there are proteins with these combined characteristics. This may be overlooked if an enzyme possesses a potent effector function which can be easily defined and makes it hard to investigate a function independent of this catalytic activity.

This section of the thesis is part of a bigger project investigating non-catalytic functions of the lipid phosphatase SHIP1 in MCs. In previous work our lab has looked extensively into the direct function of SHIP1 in BMMCs. Amongst other findings, we showed that SHIP1 influences Ag-mediated degranulation at supraoptimal IgE or Ag levels, and that the SH2 domain of SHIP1 is capable of interacting with the C-terminus of SHIP1 [55, 112]. Unpublished data hinted to a non-catalytic function of SHIP1 as well. While the other part of this project is concerned with the influence of a phosphatase dead mutant of SHIP1 in mast cell effector functions, the aim of this part was to find novel interaction partners of SHIP1 and characterize the nature of their interaction.

Collaborating with the biotech company Logopharm (Freiburg, Germany) we employed mass
spectrometry combined with bioinformatic analysis. Immunoprecipitation of SHIP1 with two different antibodies in lysates of WT BMMCs stimulated with SF or Ag for 1 min were compared to lysates of SHIP1 KO BMMCs as negative control. The found proteins were analyzed with the bioinformatic tools of Logopharm. Intrinsic positive controls were the interaction with SHIP1 itself, and the interaction with the already known interaction partners Grb2 and Dok3 [37, 112, 136], which for Grb2 we again confirmed in this study (Fig. 16C). Novel interaction partners for which we confirmed interaction with SHIP1 in immunoprecipitation and subsequent detection on Western blot, were the E3 ubiquitin ligase Nedd4 and the adapter protein STS1 (Fig. 16B). For the latter the interaction was published during the course of this study by another group, finding that STS1, like SHIP1, is a negative regulator for FcεRI signaling in MCs [29]. As well the strong interaction of SHIP1 and CIN85 was also published before our study, but only in B cells or cell lines [22, 62, 88]. The interaction of SHIP1 with Nedd4 was very weak as we could only precipitate detectable levels of Nedd4 with SHIP1 when performing immunoprecipitation in lysates with over 5000 µg protein content and even then precipitated protein was very weak in comparison to PS and other interaction partners (Fig. 16B and data not shown). STS1 was readily detectable when precipitating SHIP1, but in controls using only beads we could also precipitate STS1 protein, albeit less than in samples with SHIP1 antibody (data not shown). This made it difficult to investigate either Nedd4 or STS1, which is why we mostly concentrated on the very strong and specific interaction between SHIP1 and CIN85 (Fig. 16C).

Both SHIP1 and CIN85 come in various isoforms. Isoforms of SHIP1 are C-terminally truncated and have been shown to be quite similar in size between different cell types [112]. CIN85 isoforms, on the other hand, all share the same C-terminus and differ in between their N-terminal domains. As many as 12 isoforms have been described, which are products of different promotors or splicing events [61, 148]. A comparison of various cell lines has shown that no two cell types are alike in CIN85 expression patterns [107]. In BMMC lysates we predominantly found the bigger isoforms, termed isoform B and C, of CIN85 at around 85 kDa in size. Depending on the cell culture we could detect up to 4 of the largest CIN85 isoforms (Compare fig. 16B and D), possibly representing all isoforms that contain at least one of the up to three SH3 domains [148]. In PS samples we could also detect various smaller proteins using the two CIN85 antibodies for
detection (Fig. 16D). Due to the heterogeneity of the CIN85 isoforms between cell types we could not make any assumptions which of the described smaller isoforms were present in BMMCs. It has been shown before that the interaction between SHIP1 and CIN85 is facilitated by the SH3 domains of CIN85 and the proline-rich region of SHIP1 [22, 88]. As we could only precipitate the bigger isoforms of CIN85 with SHIP1 and none of the smaller ones, we could conclude that only the largest isoforms detectable in BMMCs contained the SH3 domains (Fig. 16B). Using the CIN85 (H300) antibody we were able to detect the additional isoform A, slightly larger than 100 kDa, but this isoform seemed not to interact with SHIP1 as we could not detect it in IP samples (Fig. 17B, bottom panel). Because we were not able to detect it with the C-terminal antiserum CIN85 (CT), we can exlude that this was the actual largest isoform of CIN85, as the C-terminus is the same for all isoforms. We can only suspect that this was an isoform modified at the site of recognition of the CIN85 (CT) antiserum, but we cannot say anything about the nature of the modification.

While precipitation of the C-terminal end of SHIP1 lead to multiple isoforms of CIN85 being detectable in IP samples, precipitation of the N-terminal side of SHIP1 almost exclusively lead to detection of isoform B of CIN85 (Compare fig. 16B and fig. 17B). This would suggest that isoform C and smaller isoforms interacting with SHIP1 might bind at the N-terminus of SHIP1 as precipitation with the N-terminal antibody could occupy the site of interaction at the N-terminus. From the structure of SHIP1 the only known domain for interaction with other proteins at the N-terminus is a SH2 domain [35]. According to the database PhosphoSitePlus® (see http://www.phosphosite.org for more details) there is only one phosphorylatable tyrosine known for CIN85 at amino acid 109 (Y109), located in the region of the second SH3 domain of CIN85, SH3B. The second largest isoform, in our case possibly isoform C, of CIN85 would still contain SH3B and thus also Y109 (Fig. 5, RukΔA/SETA), but as we can only see the largest isoform B, we can exclude interaction of CIN85 with the SH2 domain of SHIP1.

Precipitation of CIN85 itself predominantly lead to detection of SHIP1 isoform 1 and much lesser extent isoform 2, but not isoform 3 (Fig. 17C, top panel). As isoforms of SHIP1 are C-terminally truncated, only isoform 1 and 2 still contain the proline-rich region, confirming the findings of other groups that the proline-rich region of SHIP1 and the SH3 domains of CIN85 interact [22, 88]. We can assume that SHIP1 isoform 2 misses a part of the proline-rich region
as it was precipitated in lesser amounts than the complete isoform 1 (Fig. 17C, top panel). Another possibility is that CIN85 only interacted with SHIP1 isoform 1 while this isoform then interacted via its SH2 domain with phosphorylated NPXY sequences of SHIP1 isoform 2. Taken together the results so far suggest that only the biggest isoforms of each SHIP1 and CIN85 interact with each other, as only these isoforms still contain the necessary proline-rich region and SH3 domains, respectively.

We were interested if we can see a change in interaction of SHIP1 and CIN85 over time after Ag stimulation, which might give us a hint towards the purpose of the interaction. Ag stimulation up to 10 minutes did not result in any significant change of interaction between the two proteins, also there was no indication of specific isoforms interacting to a greater or lesser extent with each other (Fig. 18). The data had to be normalized on SHIP1 levels, as detected SHIP1 protein increases in both PS and IP samples, a phenomenon we already addressed in a previous study [94]. The fact that we could see CIN85 following this behavior underlined the strength of interaction between the two proteins. The data suggests constitutive interaction between SHIP1 and CIN85. A similar behavior was found for the interaction of CIN85 and SLP65 after B-cell receptor (BCR) stimulation. CIN85 and SLP65 constitutively interacted and this interaction was necessary for phosphorylation of SLP65 and its translocation to the plasma membrane [116]. A comparable mechanism may apply for SHIP1 and CIN85 in BMMCs.

Earlier we have addressed the weak interaction of the E3 ubiquitin ligase Nedd4 with SHIP1 (Fig. 16B, fourth panel from top). Both SHIP1 interaction partners CIN85 and STS1 have been reported to interact with the E3 ubiquitin ligase Cbl [125]. We were interested to see if there is also an interaction between SHIP1 and Cbl, even though our mass spectrometry data did not suggest this. It has been shown before that SHIP1 and Cbl interact weakly in vitro when both proteins are overexpressed in 293T cells. The interaction strengthened when CD2AP, which belongs to the same protein family as CIN85, was co-expressed with SHIP1 and Cbl [7]. Immunoprecipitation with the anti-SHIP1 (M14) antibody did not result in detection of Cbl in IP samples (Fig. 16B, third panel from top). In contrast Cbl was precipitated when using the SHIP1 (G11) antibody, as there were two bands detected in IP samples, one of the size of Cbl in PS samples at 130 kDa and another one at 160 kDa (Fig. 17B, second panel from top). Precipitation of CIN85 as a positive control resulted only in detection of the slower migrating
band of Cbl at 160 kDa (Fig. 17C, second panel from top). As the two SHIP1 antibodies used for precipitation gave us mixed results for a possible interaction of SHIP1 and Cbl in BMMCs, we precipitated Cbl and checked for interaction with SHIP1 and, as a positive control, CIN85. While we were able to detect CIN85 in Cbl-IP samples in varying amounts between experiments, SHIP1 could only be detected when precipitation of CIN85 with the Cbl antibody was strong (Compare fig. 19A & B with C). The amounts of precipitated Cbl were constant through the treatments used in any of the experiments, still both SHIP1 and CIN85 follow a similar association pattern with Cbl during stimulation. Both are weakly associated with Cbl in unstimulated samples, increase interaction after 1 min of Ag treatment, which is slightly reduced again after 10 min. CIN85 seems to interact directly, and not via SHIP1, with Cbl, as we found comparable association of CIN85 and Cbl in SHIP1 KO BMMCs (Fig. 19C). The data would suggest, that we can only precipitate SHIP1 with Cbl if there is strong interaction with CIN85 in a trimeric complex of SHIP1, CIN85 and Cbl. But our own data obtained precipitating with the SHIP1 (M14) antibody contradicted that theory as we saw strong precipitation of SHIP1 and CIN85 without being able to detect Cbl (Fig. 17B). The only domains known in Cbl that would fit for interaction with SHIP1 would be the C-terminally located SH2 domain, possibly binding to the phosphorylated NPXY sequences of SHIP1, or the stretch of phosphorylatable tyrosines towards the N-terminus, which could in turn be bound by the SH2 domain of SHIP1 [142]. The two closely related proteins SHIP2 and c-Cbl have been shown to interact with each other [161]. Further analysis of SHIP2 mutants with either a defective SH2 domain or mutated NPXY sequences showed that interaction with Cbl occurred via the SH2 domain of SHIP2 [128]. This seemed not to be the case in BMMCs, as precipitation via the C-terminus of SHIP1 (M14 antibody) should theoretically not interfere with the N-terminally located SH2 domain of SHIP1, not taking into account intramolecular interactions of the SH2 domain with phosphorylated NPXY sequences [112]. Further complicating the picture it has also been shown that Cbl, just like SHIP1, also interacts with the SH3 domains of Grb2, opening up another possibility for a protein to bridge an indirect interaction between SHIP1 and Cbl [143]. Taken together we were not able to reveal how Cbl and SHIP1 are able to interact, although there is enough data speaking for the interaction of both proteins, possibly not directly but via CIN85 or another common binding partner.
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As it has been shown that Cbl and CIN85 interact to mediate internalization and degradation of receptor tyrosine kinases, like c-Kit [155], the constitutive interaction of SHIP1 with CIN85 and the possible interaction with Cbl may hint towards a role of this interaction in c-Kit internalization in BMMCs. To indirectly test this hypothesis, we compared internalization of c-Kit surface expression after SF stimulation in WT and SHIP1 KO BMMCs. Surface expression of c-Kit was reduced to less than 70 % after 1 min already, declining further as stimulation went on up to around 15 % after 1 h of treatment with SF (Fig. 20A). We did not find reproducible and substantial differences between WT and SHIP1 KO BMMCs in c-Kit internalization at the investigated time points. This is in agreement with previous data, showing the interaction of CIN85 and Cbl in both WT and SHIP1 KO BMMCs (Fig. 19C), ruling out a requirement for SHIP1 in c-Kit internalization.

The other main surface receptor on MCs is the IgE receptor FcεRI. In RBL-2H3 cells it was shown that together with Cbl, CIN85 drives ligand-induced FcεRI internalization, dampening degranulation when overexpressed [109]. As before with c-Kit internalization, we looked into the differences of WT and SHIP1 KO BMMCs in internalization of FcεRI. For this we used three Ag concentrations, as the response of WT BMMCs to Ag stimulation always follows a bell-shaped dose response curve, which is not present in SHIP1 KO BMMCs [55]. While the WT BMMCs showed a dose-dependent recovery of FcεRI surface stain up to 1 h, the SHIP1 KO BMMCs only showed a recovery between early time points, followed by further reduction in surface expression (Fig. 20B). Loss of SHIP1 thereby lead to a similar trend as overexpression of CIN85, a faster internalization of FcεRI, which may hint to a negative influence of SHIP1 on CIN85 mediated internalization of the FcεRI. This may of course not only be attributed to the loss of interaction between CIN85 and SHIP1, but as well to either the absence of SHIP1 protein or its catalytic function. Taken together the data suggests involvement of SHIP1 in FcεRI, but not c-Kit, internalization in BMMCs.

So far all our data about the interaction between SHIP1 and CIN85 in BMMCs has been established performing immunoprecipitation of proteins and detecting them on Western blots. While we had performed experiments with the proper controls to ensure specificity of the interaction, we are still not in the clear regarding the function of the interaction. Recently a new method for confocal microscopy has been developed, designed specifically to visualize interac-
CHAPTER 4. DISCUSSION

tronation of proteins in the confocal microscope, the proximity ligation assay (PLA) [95]. Basically, secondary antibodies, specific for the species of the primary antibodies, are conjugated with oligonucleotides, which, when ligated, are forming a circle. Using a polymerase, a rolling-circle amplification is started and the DNA is amplified at the location of interaction of the two proteins of interest. Fluorophores conjugated to oligonucleotides designed to recognize sequences of the rolling-circle products (RCPs) can then bind the amplified DNA. The method is not restricted to detection of protein interactions, anything where two antibodies will bind closer than 40 nm distance can be detected by this method, like protein modifications with one antibody versus the protein and another one versus the modification. Using this method we hoped to gain more insight on the location of the interaction between SHIP1 and CIN85 inside the cells, which may hint towards a specific function if we can see association to distinct compartments.

First we established the method using the RBL-2H3 cell line. In contrast to the BMMCs, RBL-2H3 cells are adherent cells which makes handling for microscopy easier. While we could successfully detect RCPs staining for SHIP1 and CIN85, already in unstimulated cells, we found no distinct relocalization of RCPs into any compartments over the course of 1 h of Ag stimulation (Fig. 22C). Notable was a spreading of the RCPs over a wider area over the time course of stimulation, especially evident when comparing the 10 min and 1 h time points (Compare fig. 22C, last two panels to the right). We found that this was due to the cells themselves spreading over the cover slips when treated with Ag. In the phase contrast view, the structure formed by the plasma membrane is clearly distinguishable in untreated cells, while after 1 h of Ag treatment the only structure that could be focused was the nuclear membrane of the cells (Compare fig. 22B with C, right panel). Thus it was impossible to define a specific compartment where the interaction between SHIP1 and CIN85 could have localized.

We then continued to establish PLA also in the model system of BMMCs. While immunofluorescence could be readily detected in BMMCs for our proteins of interest, SHIP1 and CIN85, we were not able to detect RCPs in BMMCs (Fig. 23). We applied several changes to the original protocol, after consulting with Irene Weibrecht from the Söderberg group, who developed PLA [95], but none of them lead to a successful detection of RCPs in BMMCs (summarized at the end of chapter 3.2.6). Thus we have to conclude so far, that we cannot successfully perform PLA in BMMCs.
To summarize the findings of this part of the thesis, we showed interaction of SHIP1 with the novel interaction partner Nedd4, and with the already known interaction partners STS1 and CIN85. The interaction between SHIP1 and CIN85 was the strongest of all interaction partners investigated. We found that predominantly the largest isoforms of both proteins interact, as only those still contain SH3 domains (CIN85) and the proline-rich region (SHIP1), necessary for the interaction. The proteins interacted constitutively and there was no change in interaction over time in Ag stimulation. Possibly the E3 ubiquitin ligase Cbl is included in a trimeric complex of Cbl, CIN85 and SHIP1, although further experiments need to verify this hypothesis. Using the indirect approach of analyzing SHIP1 KO BMMCs, we suggest an involvement of SHIP1, and thus possibly CIN85, in mediating the internalization of the FcεRI, but not c-Kit. We could confirm interaction of SHIP1 and CIN85 with PLA in confocal microscopy, but only in the RBL-2H3 cell line and not in BMMCs.
Chapter 5

Perspectives

5.1 LPS-induced production of IL-6, TNF-α and IL-1β in MCs is dependent on p38 but independent of TTP

As our results indicate that treatment with LPS, but also Ag, did not induce protein expression of TTP in BMMCs, but still gene expression could be detected, there is the possibility that another stimulus is needed to induce the translation of TTP protein. Stimulation of other receptor systems than TLR4 or FcεRI may result in protein production. TTP is often characterized as an anti-inflammatory protein, as many known targets are mRNAs of proinflammatory cytokines. Anti-inflammatory molecules, like IL-4 or IL-10, may be able to induce TTP protein synthesis in BMMCs.

Apart from TTP, BRF1/2 and Zfand5, there are other mRNA-stability influencing proteins. The protein HuR has been shown to compete with TTP for binding of TNF-α mRNA. Transcriptome analysis of BMMCs revealed high constitutive expression of HuR mRNA. As we did not find TTP protein expression, it would be interesting to see if MCs lack HuR protein expression as well. Also while we could show protein expression of BRF1/2, further research is need to define their actual function in BMMCs. Knockout mice for both BRF1 and BRF2 die in the first two weeks after birth, so future experiments will have to be based on siRNA approaches or conditional knockouts.
5.2 The inositol 5’-phosphatase SHIP1 interacts with the adapter protein CIN85

In this study we were not able to clarify the function of the interaction between CIN85 and SHIP1. So far we also could not specify the general function of CIN85 in MCs. To our knowledge there have been no complete CIN85 knockout mice generated yet. Thus, further experiments could be performed using siRNA to knock down CIN85 expression in BMMCs. It would be interesting to differentiate BMMCs from bone-marrow cells of the established CIN85\[^{\Delta Exon2}\] mice. These mice only lack the two biggest isoforms, which are the main SHIP1-interacting isoforms in BMMCs. This would give us a better insight of the impact of interaction between the two proteins, especially when comparing respective results with data obtained from siRNA-mediated general knockdown of CIN85. Both knockdown of CIN85 with siRNA and BMMCs generated from CIN85\[^{\Delta Exon2}\] mice should again be checked for differences in internalization of Fc\(\epsilon\)RI, as the results we obtained in SHIP1 KO BMMCs hint for a role of CIN85 in this process. As most SHIP1-interaction partners are connected to ubiquitin-related signaling pathways, it may be interesting to look into the ubiquitination status of SHIP1 and its interaction partners.

Finally, results from the second study belonging to this project, which investigated the phenotype of BMMCs expressing the phosphatase-dead mutant of SHIP1, should be checked for a possible influence of CIN85. The question would be if the interaction between the two proteins is influenced by the lack of phosphatase activity of SHIP1 and if differences to the WT BMMCs may be accountable to changes in this interaction.
Chapter 6

Materials and Methods

Materials and Methods are described according to standard protocols used in the Institute of Biochemistry and Molecular Biology, RWTH Aachen University, and modified regarding individual differences in experimental procedures.

6.1 Lipopolysaccharide preparations

Lipopolysaccharide preparations from S. Minnesota were a gift from Marina Freudenberg and Chris Galanos, purification of which is described in literature [51]. Different preparations over the years would show varied strength of stimulation in the BMMCs, also dependent on the different types of FBS used for differentiation of the cells.

![Comparison of different LPS preparations](image)

**Fig. 24:** Comparison of different LPS preparations. WT BMMCs were either left untreated or stimulated with 0.3, 1 or 3 µg/ml of one of two LPS preparations for 4 h. Subsequently amount of IL-6 was determined by ELISA.

This is the reason for varying LPS concentrations used in various experiments. A representative comparison is shown in figure 24. LPS #1 is already in plateau phase at 0.3 µg/ml while
LPS #2 only induces comparable amounts of IL-6 at a concentration 10 times higher. With each new aliquot obtained, its strength in stimulation was tested with titration of LPS and measuring IL-6 production of BMMCs. For further experiments the highest concentration inducing IL-6 production, without being in the plateau phase of response, was chosen.

### 6.2 Differentiation of bone marrow-derived mast cells

Bone marrow-derived mast cells (BMMCs) can be differentiated in vitro from bone marrow cells in the presence of IL-3 [133]. For the isolation of bone marrow cells, femurs of 6-8 weeks old mice (C57BL/6 x 129/SV) were prepared and flushed under sterile conditions with 5 ml growth medium (Table 1) per femur.

<table>
<thead>
<tr>
<th>Table 1: Growth medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 with L-Glutamine (Gibco)</td>
</tr>
<tr>
<td>FBS 12% (Lonza/PAA)</td>
</tr>
<tr>
<td>HEPES 1 M (Gibco)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (Lonza)</td>
</tr>
<tr>
<td>β-mercaptoethanol 0.5 M</td>
</tr>
<tr>
<td>IL3 supernatant</td>
</tr>
</tbody>
</table>

For culturing, bone marrow cells were plated on polystyrene cell culture dishes in a concentration of 1 x 10^6 cells/ml and incubated at 37 °C in a water-saturated atmosphere of 5 % CO₂. IL-3 supernatants were harvested from X63Ag8653 cells according to protocol [81]. Differentiation of progenitor cells to BMMCs takes 4 - 6 weeks. While the cultures start out very heterogeneous with both suspension and adherent cells, cultures become more and more homogeneous during the differentiation process. Status of differentiation is checked using FACS staining for markers FceRI and c-Kit. Cells were used for experiments after gaining a double positivity of both markers of at least 95 %.

### 6.3 Preparation of mast cells for stimulation

Unless stated otherwise, prior to the experimental procedure BMMCs were counted the day before stimulation using either a hemocytometer or the CASY ® Technology Cell Counter. Required amounts of cells were spun down in a cooled centrifuge (Beckman Coulter Allegra X-
15R) at 335 rcf (1200 rpm) for 5 min at 4 °C. Pellets were resuspended at a concentration of 1 x $10^6$ cells/ml in starvation medium (Table 2) and incubated at 37 °C and 5 % CO$_2$ overnight. In case of antigen (Ag) stimulations cells were loaded with 0.15 µg/ml SPE7 (IgE directed against DNP-HSA).

**Table 2: Starvation medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 with L-Glutamine (Gibco)</td>
<td>500 ml</td>
</tr>
<tr>
<td>FBS 10 % (Lonza/PAA)</td>
<td>50 ml</td>
</tr>
<tr>
<td>HEPES 1 M (Gibco)</td>
<td>5 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (Lonza)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>β-mercaptoethanol 0.5 M</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The next day, cells were spun down at 335 rcf for 5 min at 4 °C, the pellets were washed with PBS and counted again. After another centrifugation step, cell pellets were resuspended in stimulation medium (Table 3) at a concentration of 1 x $10^6$ cells/ml and distributed to tubes, plates or wells respectively.

**Table 3: Stimulation medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640 (Gibco)</td>
<td>500 ml</td>
</tr>
<tr>
<td>BSA 10 % (Lonza/PAA)</td>
<td>5 ml</td>
</tr>
<tr>
<td>HEPES 1 M (Gibco)</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

### 6.4 Cell lines and their preparation for stimulation

Two cell lines have been used for several experiments in this thesis. RAW 264.7 macrophages are a murine leukaemic monocytic macrophage cell line. RBL-2H3 mast cells are a basophilic leukemia cell line. Both cell lines are adherent and were cultured in BMMC starvation medium (Table 2). In regular intervals cells were passaged before they reached confluency. Medium was taken off and cells were washed with PBS. After PBS was taken off, 1 ml trypsin (for a 10 cm plate) was added and the culture dish then incubated for 5 min at 37 °C. Cells were then flushed off carefully with BMMC starvation medium, then spun down in a cooled centrifuge (Beckman Coulter Allegra X-15R) at 335 rcf (1200 rpm) for 5 min at 4 °C. Pellets were resuspended and then distributed to new plates according to the dilution factor. Dilutions depended on individual cell growth and ranged from 1:10 to 1:20.
The day prior to stimulation, confluent cells were washed with PBS, then starvation medium with 1 % FBS was put on the plates to starve the cells. The next day, cell cultures were washed with PBS, then stimulation medium (Table 3) was added. The cells were then incubated for 20 min at 37 °C before the respective stimulations. Cell lysis after stimulation was performed directly on the plate.

6.5 Degranulation

Protocol is based on the method established by Ortega et al. [119]. BMMCs were resuspended in either stimulation medium (Table 3) or Tyrode’s buffer (Table 4) and distributed in duplicates on a round-bottom 96-well plate, 0.2 x 10⁶ cells/well. Cells were incubated for 20 min in an incubator at 37 °C and subsequently stimulated for further 20 min.

Table 4: Tyrode’s buffer (sterile filtered)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.6 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.4 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>5 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>130 mM</td>
</tr>
<tr>
<td>HEPES, pH 7.4</td>
<td>10 mM</td>
</tr>
<tr>
<td>BSA 0.1 % in dH₂O</td>
<td>fill up</td>
</tr>
</tbody>
</table>

Plates were then spun down in a cooled centrifuge (Beckman Coulter Allegra X-15R) at 335 rcf (1200 rpm) for 10 min at 4 °C. Supernatants were carefully taken off and stored on a new 96-well plate. Pellets were then lysed in 0.2 ml stimulation medium or Tyrode’s buffer supplemented with 0.5 % IGEPAL for 30 min on ice. Subsequently lysates were spun down in a cooled centrifuge (Eppendorf, 5415R) for 5 min at 4 °C. 10 µl of either supernatant or lysate was added to 50 µl substrate solution (1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M Na-citrate, pH 4.5; stored at -20 °C) on a flat-bottom 96-well plate, incubated for 90 min at 37 °C. Reaction was stopped with 150 µl glycine (0.2 M, pH 10.7). Absorbance was measured at a wavelength of 405 nM in an ELISA reader.

Percentage of degranulation was calculated by the ratio of enzyme activity in the supernatant in relation to the lysates, using the following formula:
%\text{(degranulation)} = 100 \times \frac{\text{Absorption}^{\text{supernatant}} \times \text{volume}}{\left(\text{Absorption}^{\text{supernatant}} \times \text{volume}\right) + \left(\text{Absorption}^{\text{lysate}} \times \text{volume}\right)}

### 6.6 Enzyme-linked immunosorbent assay

In this study the cytokines IL-6, TNF-\(\alpha\) and IL-1\(\beta\) were measured. Cells were stimulated in 96-well plates in triplicates of \(0.2 \times 10^6\) each for 4h at 37 °C and 5 % CO\(_2\).

For measurement of IL-1\(\beta\) the ”Mouse IL-1\(\beta\) ELISA kit OptEIA” (Cat. #559603)” from BD Biosciences was used according to protocol of the kit. As BMMCs do not secrete IL-1\(\beta\) after stimulation, cell lysates were subjected to ELISA. For IL-6 and TNF-\(\alpha\) cell supernatants were applied to ELISA plates. The plates were coated the day before stimulation with the respective coating antibody diluted in PBS (for IL-6 ELISA 2 µg/ml, BD Biosciences #554400; for TNF-\(\alpha\) ELISA 0.5 µg/ml, R&D Systems, #AF-410-NA) and 50 µl antibody dilution was applied to each well. Plates were incubated at 4 °C over night.

Next day the coated 96-well plates were washed 3 times with washing buffer (0.01 % Tween 20 in PBS), then blocked with 2 % BSA in PBS (for IL-6 ELISA) or 1 % BSA and 5 % Sucrose in PBS (for TNF-\(\alpha\) ELISA) for 2 h at room temperature (RT). Plates were washed again 3 times with washing buffer, then either 50 µl (for IL-6 ELISA) or 100 µl (for TNF-\(\alpha\) ELISA) of cell supernatants were added to the wells, together with a recombinant protein standard in duplicates. The highest standard contained either 2500 pg/ml (for IL-6 ELISA) or 2000 pg/ml (for TNF-\(\alpha\) ELISA), six more 1:2 serial dilutions of the highest standard were applied to the plate. Empty wells were filled up with stimulation medium. The plates were incubated at 4 °C over night.

The day after, the 96-well plates were washed 3 times with washing buffer. Respective biotinylated detection antibody diluted in 2 % BSA in PBS (for IL-6 ELISA 1 µg/ml, BD Biosciences, #554402; for TNF-\(\alpha\) ELISA 0.2 µg/ml, R&D Systems, #BAF410) was added at 50 µl per well and incubated for 1 h at RT. Plates were washed again 3 times with washing buffer. Streptavidine coupled alkaline phosphatase was diluted in 2 % BSA in PBS to 1 µg/ml and 50 µl of dilution was applied to each well. Plates were incubated for 30 min at RT, then
washed again 3 times with washing buffer. P-nitrophenyl-phosphate substrate (Sigma, #S0942) was dissolved in 5 ml of sodium carbonate buffer (2 mM MgCl\(_2\) in 50 mM Na-carbonate, pH 9.8) at 1 mg/ml and 50 µl of solution was added to each well. The colorimetric reaction was subsequently measured in an ELISA reader at 405 nm wavelength until the optical density of the highest standard was in between 0.8 and 1.0.

### 6.7 Quantitative polymerase chain reaction

#### 6.7.1 RNA isolation

For each point of stimulation between 3 to 4 x 10\(^6\) cells were stimulated to obtain sufficient amounts of RNA with good quality. Cells were stimulated at 2 x 10\(^6\) cells/ml in 2 ml tubes or at 1 x 10\(^6\) cells/ml in 6-well plates. Stimulation was stopped by either spinning down the 2 ml tubes, taking of the supernatant and freezing the pellets in liquid nitrogen, or in the case of 6-well plates, putting the plates on ice. Cell suspensions on 6-well plates were transferred to 15 ml tubes and spun down in a Beckman Coulter Allegra X-15R centrifuge at 335 rcf (1200 rpm) for 5 min at 4°C. Subsequently pellets were lysed in RLT buffer from the Qiagen RNeasy Mini Kit, supplemented with 10 µl β-mercaptoethanol per ml of RLT buffer. During stimulation cells would also adhere to the 6-well plates, so before lysing the pellets in the 15 ml tubes, RLT buffer was applied to the 6-wells to lyse off adherent cells.

Subsequently protocol of the Qiagen RNeasy Kit was followed. Eluted RNA was measured for concentration and quality (260/230 nm and 260/280 nm values around 2.00) using the Nanodrop spectrophotometer (Thermo Scientific).

#### 6.7.2 Reverse transcription

The protocol of the Qiagen Omniscript RT Kit was followed to reverse transcribe 1 µg of RNA per sample. After reverse transcription the samples were diluted 1:4 in high purity water (Licrosolv, Merck). To test efficiencies of primers, samples were diluted 1:2 first, followed by 5 sequential 1:5 dilutions.
6.7.3 qPCR

Quantitative PCR measurements were performed using the "SensiMix SYBR No-ROX Kit (Bioline, Cat #QT650-02)" on the Rotor-Gene Q 2plex (Qiagen, Cat #9001550), according to the manufacturers protocol. For each sample 2 µl of cDNA generated in the reverse transcription described before, and forward and reverse primers (see chapter 5) for the gene of interest at a concentration of 10 pmol/µl were used.

<table>
<thead>
<tr>
<th>Murine gene</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRF1</td>
<td>GAC CTT CAC GAC ACA</td>
<td>CGC TGG GAG TGC TGT</td>
<td>2.02463</td>
</tr>
<tr>
<td></td>
<td>CCA GAT</td>
<td>AGT TG</td>
<td></td>
</tr>
<tr>
<td>BRF2</td>
<td>CAC AAC TTT CCG TCC</td>
<td>TTC TGG GTC CTG TAA</td>
<td>1.99879</td>
</tr>
<tr>
<td></td>
<td>CTC CTT</td>
<td>TGG TCG</td>
<td></td>
</tr>
<tr>
<td>c-Fos</td>
<td>ACT TTA TCC CCA CGG</td>
<td>TGA CAC GGT CTT CAC</td>
<td>1.85016</td>
</tr>
<tr>
<td></td>
<td>TGA CAG C</td>
<td>CAT TCC C</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>ACT CAA GAT TGT CAG</td>
<td>TGG TCA TGA GCC CTT</td>
<td>1.9483</td>
</tr>
<tr>
<td></td>
<td>CAA TGC A</td>
<td>CCA CAA</td>
<td></td>
</tr>
<tr>
<td>Gusb</td>
<td>QuantiTect Primer Assay</td>
<td>(Qiagen #QT00176715)</td>
<td>2.01478</td>
</tr>
<tr>
<td>IL-6</td>
<td>TCC AGT TGC CTT TTT</td>
<td>GTG TAA TTA AGC CTC</td>
<td>2.00934</td>
</tr>
<tr>
<td></td>
<td>GGG AC</td>
<td>CGA CTT G</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>AAC CTG CTG GTG TGT</td>
<td>CAG CAC GAG GCT TTT</td>
<td>1.99029</td>
</tr>
<tr>
<td></td>
<td>GAC GTT C</td>
<td>TTG TTG T</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGC ACA GAA AGC ATG</td>
<td>TGC CAC AAG CAG GAA</td>
<td>2.1854</td>
</tr>
<tr>
<td></td>
<td>ATC CGC</td>
<td>TGA GAA G</td>
<td></td>
</tr>
<tr>
<td>TTP</td>
<td>GAG GGC CGA AGC TGC</td>
<td>GGT GGC GAT TGG CTT</td>
<td>1.92195</td>
</tr>
<tr>
<td></td>
<td>GGC TGG GT</td>
<td>GGC GAA G</td>
<td></td>
</tr>
<tr>
<td>Zfand5</td>
<td>AGC CAG TTG TCA CTC</td>
<td>CCA CAT CGG CAG TCA</td>
<td>2.05232</td>
</tr>
<tr>
<td></td>
<td>AGC CCA</td>
<td>AAC CCT GT</td>
<td></td>
</tr>
</tbody>
</table>

For determination of primer efficiencies, the sequential dilutions described before were entered as a standard in % in the Rotor-Gene Q software (Qiagen). Primers were only used for further experiments if three qPCR runs with independent standard dilutions resulted in an efficiency calculated between 0.85 and 1.15. Fold increases in gene expression based on the C\textit{t} values measured were calculated with the Pfaffl method [127].
6.8 SDS-PAGE

6.8.1 Lysis conditions

Lysis of cells was performed for 10 to 30 min on ice using either the standard lysis conditions (Table 6) or the improved lysis conditions (LB+, Table 7). As stated in the results for some immunoprecipitations and detections of special proteins the improved lysis conditions were necessary to prevent degradation of proteins, depending on the differentiation of the cells (see chapter 3.2.1).

<table>
<thead>
<tr>
<th><strong>Table 6</strong>: Standard lysis conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGEPAL</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>Aprotinin</td>
</tr>
<tr>
<td>Leupeptin</td>
</tr>
<tr>
<td>PMSF</td>
</tr>
<tr>
<td>PSB buffer (50 mM HEPES, 100 mM NaF, 10 mM Na₄P₂O₇ x10 H₂O, 2 mM Na₃VO₄, 2 mM EDTA, 2 mM Na₂MoO₄ x2 H₂O, in dH₂O, pH 7.35) fill up</td>
</tr>
</tbody>
</table>

Lysates were then spun down at maximum speed for 10 minutes at 4 °C in a cooling microcentrifuge (Eppendorf, 5415R). Supernatants were then transferred to new tubes and subsequently boiled with respective amounts of 5X SDS loading buffer (300 mM Tris, 46 % glycerol, 10 % SDS, 12.5 % β-mercaptoethanol, bromphenol blue in dH₂O) for 5 min at 95 °C.

<table>
<thead>
<tr>
<th><strong>Table 7</strong>: Improved lysis conditions (LB+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGEPAL</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>Aprotinin</td>
</tr>
<tr>
<td>Leupeptin</td>
</tr>
<tr>
<td>PMSF</td>
</tr>
<tr>
<td>Iodacetamide</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>EGTA</td>
</tr>
<tr>
<td>3,4-Dichloroisocoumarine</td>
</tr>
<tr>
<td>PSB buffer (see table 6) fill up</td>
</tr>
</tbody>
</table>

In some occasions, hot lysis was performed. 5X SDS loading buffer was diluted 1:5 in dH₂O, then boiled at 95 °C. Frozen cell pellets were directly mixed with 20 - 30 µl boiling 1X SDS loading buffer and then further boiled for 5 min on a shaking heating block at 95 °C.
6.8.2 Gel electrophoresis

Table 8: Recipes for resolving gels

<table>
<thead>
<tr>
<th>Component</th>
<th>12 %</th>
<th>10 %</th>
<th>8 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>2.8 ml</td>
<td>3.4 ml</td>
<td>3.9 ml</td>
</tr>
<tr>
<td>4X lower buffer (1.5 M Tris, 0.4% SDS in dH$_2$O, pH 8.8)</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide (30/0.8)</td>
<td>3.2 ml</td>
<td>2.7 ml</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 µl</td>
<td>7.5 µl</td>
<td>7.5 µl</td>
</tr>
</tbody>
</table>

Boiled samples were then loaded on gels cast with various percentages of resolving gels (Table 8) with a stacking gel on top (Table 9) together with the protein marker “PageRuler™ Prestained Protein Ladder (Fermentas, Cat. #SM0672)”. Gels were run with 1X SDS running buffer (250 mM Tris, 2 M glycine, 10% SDS, in dH$_2$O for 10X buffer) in a Mini Protean II electrophoresis chamber at constant 120 V for about 2 h.

Table 9: Recipe for stacking gel

<table>
<thead>
<tr>
<th>Component</th>
<th>4.45 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td></td>
</tr>
<tr>
<td>4X upper buffer (0.5 M Tris, 0.4% SDS in dH$_2$O, pH 6.8)</td>
<td>1.9 ml</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide (30/0.8)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>75 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5 µl</td>
</tr>
</tbody>
</table>

6.9 Western blotting

6.9.1 Semi-dry transfer

For blotting of proteins from SDS-PAGE gels onto a PVDF membrane the protocol for semi-dry transfer was followed. To prepare the transfer, 4 pieces of Whatmann paper were soaked in anode 1 buffer (0.3 M Tris, 20% methanol, in dH$_2$O), 2 pieces of Whatmann paper were soaked in anode 2 buffer (0.025 M Tris, 20% methanol, in dH$_2$O) and 4 pieces of Whatmann paper were soaked in cathode buffer (0.04 M aminocaproic acid, 20% methanol, 0.01% SDS, in dH$_2$O). The PVDF membrane was activated by incubation in ethanol (100%) for 20 sec, then washed in dH$_2$O for 5 min and subsequently soaked in anode 2 buffer for 10 min. The gel was incubated in cathode buffer for 10 min.

With the anode of the blotting chamber at the bottom and the cathode at the top, the blot
was assembled as follows: 4 pieces of Whatmann paper soaked in anode 1 buffer at the bottom, then 2 pieces of Whatmann paper soaked in anode 2 buffer on top, then the membrane, then the gel, then 4 pieces of Whatmann paper soaked in cathode buffer. Air bubbles were removed while placing each layer. During the blotting process a weight of 1 kg was put on top of the chamber. Blots were run for 1 h 20 min with an amperage depending on the size of the whole blot area. Required current for the blot was calculated with the following formula:

\[
X = \text{height(cm)} \times \text{length(cm)} \times 0.8 \frac{mA}{cm^2}
\]

6.9.2 Wet transfer

The PVDF membrane was activated by incubation in ethanol (100 %) for 20 sec, then washed in dH\textsubscript{2}O for 5 min. The membrane was then incubated for 15 min in transfer buffer (25 mM Tris, 192 mM glycine, 10 % MeOH, in dH\textsubscript{2}O for 1X buffer, buffer is stored as 10X buffer without MeOH). Whatman paper (3 pieces) was incubated in transfer buffer as well and placed on the anode side of the clamp for the transblot tank. Then the membrane, the gel, and again Whatman paper (3 pieces), all pre-incubated in transfer buffer, were placed on top. Air bubbles were removed and the cathode-clamp placed on top. The transfer was performed in a transblot tank (BioRad) in transfer buffer at constant 350 mA either 1 hour in the small transblot chamber or 2 hours in the big transblot chamber.

6.9.3 Detection

After blotting, the blot was disassembled and the membrane incubated for 30 min in 10 % milk powder in PBS for blocking. Subsequently the membrane was washed 3 times for 5 min in washing buffer (0.01 % Tween 20 in PBS). For detection of proteins, antibody for the protein of interest was diluted at the indicated concentration (see table 10) in washing buffer with 1% BSA and 0.02 % NaN\textsubscript{3}. Membranes were incubated with the respective primary antibody bath either over night at 4°C or for 1 h at RT on a shaker.

Blots were washed 3 times for 5 min with washing buffer at RT on a shaker. The membrane was then incubated with a secondary antibody bath consisting of washing buffer and a 1:10000 dilution of the respective HRPO-coupled anti-species antibody (mouse, rabbit or goat) for 45
### Table 10: List of non-conjugated / primary antibodies

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Size (kDa)</th>
<th>Species</th>
<th>Source</th>
<th>Concentration</th>
</tr>
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<td>α-Actin (I-19)</td>
<td>43</td>
<td>goat α-human,</td>
<td>sc-1616 SCBT</td>
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<tr>
<td>α-p-Akt (Ser473)</td>
<td>60</td>
<td>mouse α-mouse,</td>
<td>#4051 CST</td>
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<tr>
<td>α-BRF1/2</td>
<td>36 - 62</td>
<td>rabbit α-human,</td>
<td>#2119 CST</td>
<td>WB 1:1000</td>
</tr>
<tr>
<td>α-Cbl (C-15)</td>
<td>100</td>
<td>rabbit α-human,</td>
<td>sc-170 SCBT</td>
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<tr>
<td>α-CIN85 (CT)</td>
<td>various</td>
<td>rabbit α-rabbit,</td>
<td>I. Dikic [150]</td>
<td>WB 1:1000, IF 1:100</td>
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<tr>
<td>α-CIN85 (H300)</td>
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<td>rabbit α-human,</td>
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<td>α-p-ERK1/2 (Thr202/Tyr204)</td>
<td>44 / 42</td>
<td>rabbit α-human,</td>
<td>#9101 CST</td>
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<tr>
<td>α-Gapdh (6C5)</td>
<td>36</td>
<td>mouse α-rabbit,</td>
<td>sc-32233 SCBT</td>
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<td>α-Grb2</td>
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<td>#1584-1 Epitomics</td>
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<td>sc-1964 SCBT</td>
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<td>T3287 Sigma</td>
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<td>α-TTP (K2)</td>
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<td>P. Kovarik [146]</td>
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<td>rabbit α-mouse,</td>
<td>A. Clark [102]</td>
<td>WB 1:1000</td>
</tr>
<tr>
<td>α-TTP (SAK21A)</td>
<td>36 - 50</td>
<td>rabbit α-mouse,</td>
<td>A. Clark [102]</td>
<td>WB 1:1000</td>
</tr>
</tbody>
</table>
min at RT on a shaker. Blots were subsequently washed 3 times for 5 min with washing buffer at RT. Before detection, the membrane was incubated for 3 min with PCA-ECL (5 ml of 250 mM Luminol (C8H7N3O2) in DMSO, 1.1 ml 90 mM p-Coumaric acid solution (C9H8O3) in DMSO, fill up to 500 ml with 100 mM Tris/HCl pH 8.8; use with 1:2500 H2O2, fresh for each detection). Signals were detected using the LAS-4000 Mini Luminescent Image Analyser (Fujifilm). Densitometric analysis was performed with ImageJ software.

6.10 Immunoprecipitation

Depending on the target and the antibodies used for precipitation, for each point of stimulation between 20 and 40 x 10^6 cells were used. For short time points up to 20 min, cells were resuspended in 1 ml stimulation medium (Table 3) and stimulated in 1.5 ml tubes on the heating block. For longer stimulations, cells were resuspended at 1 x 10^6 cells/ml on 15 cm cell culture dishes and incubated in an incubator during stimulation.

Pellets were lysed in 1 ml of the respective lysis buffer and kept on ice for 20 to 30 min. Samples were then spun down at maximum speed for 10 minutes at 4 °C in a cooling microcentrifuge (Eppendorf, 5415R). Supernatants were then transferred to new tubes. Protein content of lysates was measured with "BCA Protein Assay Kit (Pierce, Cat #23227)”, according to the manufacturers protocol. 30 µg of protein was taken off to be boiled with 5X SDS loading buffer for 5 min at 95 °C for the postnuclear supernatant (PS). Protein amounts of samples were then normalized and the respective antibody added at 1:250 dependent on protein concentration, e.g. for 3000 µg of protein 12 µl of antibody (0.2 µg/ml) was used. The samples were then incubated over night at 4 °C on a rotator.

Next day, 25 µl of a 1:5 dilution of Protein G-sepharose beads (GE Healthcare, 17-0618-01) in PBS was added to the samples and incubated for 1 h at 4 °C on a rotator. To wash the beads, samples were then spun down in a cooled microcentrifuge (Eppendorf, 5415R) at 300 rcf for 1 min at 4 °C, supernatant was carefully taken off without disturbing the pellet. Beads were then resuspended with 1 ml bead washing buffer (PSB buffer with 1:300 10 % Na-DOC and 1:60 10 % IGEPAL). Two more washing steps were performed, after the last one as much supernatant as possible was taken off, beads were then boiled for 5 min at 95 °C with approximate amounts of
5X SDS loading buffer.

### 6.11 Confocal microscopy

To prepare suspension cells for confocal microscopy, glass cover slips were coated with poly-L lysine for 30 min at RT in a 12-well plate (1:10 of a 0.1 mg/ml stock, 1 ml per well or cover slip). Solution was taken off and cover slips were washed with sterile PBS. Suspension cells resuspended in stimulation medium (Table 3) were then added at 1 - 2 x 10⁶ cells/ml per well. In the case of adherent cells, resuspended cells were seeded directly onto the cover slips without any preparation.

Cells were then stimulated on the 12-well plate, stimulation was stopped by carefully removing supernatant and washing two times with PBS⁺/⁺ (1 mM MgCl₂, 0.1 mM CaCl₂, in PBS). After the second washing, cells were fixed with 2.7 % paraformaldehyde for 20 min at RT in the dark. Then cells were permeabilized with PBS⁺/⁺ (0.1X TritonX 100, in PBS⁺/⁺) for 5 min at RT. Quenching was performed using NH₄Cl (50 mM, in PBS⁺/⁺) for 5 min at RT. Subsequently cells were washed in PBS⁺/⁺.

For the proximity ligation assay (PLA), the protocol of the Duolink II kit (Olink, Detection Reagents FarRed 92013-0100, PLA Probe Anti-Mouse MINUS 92004-0100, PLA Probe Anti-Rabbit PLUS 92002-0100) was followed. At all times, incubation with various solutions was performed on parafilm with the cover slips put upside down on a drop of solution (40 µl). The washing steps were performed by placing the cover slips with the cells facing upwards into six-wells containing the respective washing buffers.

For immunofluorescence measurements, basically the same protocol and the same substances of the PLA kit (blocking reagent, washing buffers) were used, the only exception being that only one secondary antibody conjugated with the respective fluorophore was used, incubated for 45 min at RT. Slides were evaluated with the LSM 710 confocal microscope (Zeiss).

### 6.12 Receptor internalization assay

To measure surface levels of receptors, cells were prepared as described earlier, and then distributed at 0.3 to 0.5 x 10⁶ cells/reaction tube. Cells were incubated on a heating block at
37 °C for 20 min, subsequently stimulated for the indicated time points. To stop stimulation, cell suspension was transferred to FACS tubes on ice containing pre-cooled (4 °C) FACS buffer (3 % FBS, 0.1 % NaN₃ in PBS, pH 7.4). Cell suspensions were kept on ice at all times for the following steps. Cells were spun down in a cooled centrifuge (Beckman Coulter Allegra X-15R) at 335 rcf (1200 rpm) for 5 min at 4 °C, pellets were then washed with FACS buffer. Washing was repeated once more, then cell pellets were resuspended in 30 µl of the respective antibody dilution (see table 11) and incubated in the dark for 15 min at 4 °C. After two more washing steps, pellets were taken up in 100 - 150 µl FACS buffer. Samples were measured using the FACS Canto II (BD Biosciences). Data was analyzed by FlowJo software (Treestar Inc.).

Table 11: List of conjugated antibodies

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Dye</th>
<th>Species</th>
<th>Source</th>
<th>Concentration</th>
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<tr>
<td>α-CD117 (2B8)</td>
<td>PE</td>
<td>rat α-mouse, monoclonal</td>
<td>BD Bioscience</td>
<td>FACS 1:100</td>
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<tr>
<td>α-FcεRI (MAR-1)</td>
<td>FITC</td>
<td>armenian hamster α-mouse, monoclonal</td>
<td>eBio-science</td>
<td>FACS 1:100</td>
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</tbody>
</table>

6.13 Statistical analysis

P values were calculated by the paired two-tailed Student’s t test. P values of * < 0.05, ** < 0.005, and *** < 0.0005 were considered statistically significant. Values higher than a p value of 0.05 were regarded as not significant (n.s.).
Chapter 7

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results in extreme lipopolysaccharide sensitivity in an otherwise minimal phenotype.

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CIN85 regulates ubiquitination and


Appendix A

Supplementary figures

**Suppl. Fig. 1:** LPS induces TTP gene expression in BMMCs. WT BMMCs were left untreated (con) or stimulated with 3 μg/ml LPS for the indicated times. TTP mRNA expression was analyzed by RT-qPCR. Data obtained by analyzing cells from independent cultures are shown (the result obtained with BMMCs from culture #1 is already depicted in Fig. 2A). The relative expression ratios including primer efficiencies were calculated by the Pfaffl method [127].
Suppl. Fig. 2: LPS-induced production of TTP mRNA is dependent on p38. WT BMMCs were incubated with either DMSO or the indicated concentrations of BIRB0796 for 20 min and subsequently stimulated with 3 µg/ml LPS for 2 h. TTP mRNA was analyzed by RT-qPCR. Data obtained by analyzing cells from independent cultures are shown (the result obtained with BMMCs from culture #1 is already depicted in Fig. 2B). The relative expression ratios including primer efficiencies were calculated by the Pfaffl method [127].
Suppl. Fig. 3: TTP gene expression is detectable in WT BMMCs. WT (•) and TTP-deficient (KO) BMMCs (◦) were left untreated (con) or stimulated with 3 µg/ml LPS for the indicated times. TTP mRNA was analyzed by RT-qPCR. Data obtained by analyzing cells from independent cultures are shown (the result obtained with BMMCs from culture #1 is already depicted in Fig. 5A). The relative expression ratios including primer efficiencies were calculated by the Pfaffl method [127].

Suppl. Fig. 4: Kinetics of MK2 activity in LPS-treated WT BMMCs. WT BMMCs were left untreated (con) or stimulated with either 3 µg/ml LPS or LPS and 100 ng/ml IGF-1 together for the indicated time points. IGF-1 was added because of investigations in a previous project, see [68] for more details. Postnuclear supernatants (PS) were analyzed by immunoblotting with antibodies against phospho-MK2 (top panel) and GAPDH (bottom panel).
Appendix B

Publications

Hochdörfer T., Tiedje C., Stumpo D.J., Blackshear P. J., Gaestel M., Huber M., LPS-induced production of TNF-α and IL-6 in mast cells is dependent on p38 but independent of TTP, submitted


Hochdörfer T., Kuhny M., Zorn C. N., Hendriks R. W., Vanhaesebroeck B., Bohnacker T., Krystal G., Huber M., Activation of the PI3K pathway increases TLR-induced TNF- and IL-6 but reduces IL-1 production in mast cells, Cellular Signaling, 23(5):866-875, 2011
Appendix C

Acknowledgements

First I would like to thank my boss, Michael Huber, for the opportunity to work on these projects and for his guidance over the years. Even when results were not very promising he always had an idea in which to direction to go. I would like to thank my second advisor (Zweitgutachter), Werner Baumgartner, for supervising my projects in addition to his own work and his input during our meetings. I want to thank my parents for all their support, which made my life much easier, especially while I was still studying.

From the lab I would like to thank Marcel Kuhny, for his great support during the years, as well for reading through and correcting just about anything I sent him. I want to thank Oindrilla Mukherjee, my partner in SHIP1...I mean crime, for the long discussions about anything, experiments, life, and why Germans just are like that. I would like to thank Alison Hagemeister for brightening up any day with her humor and singing...and scientific discussions! Also I want to thank Carolin Zorn for the correction of the introduction and for all her expertise and help she gave me over the years. Further thanks go out to our technicians Marlies Kauffmann, Tanja Nöcker and Karin Maschke-Neuß. Let’s face it, nothing would work without them and in addition they have to put up with annoying PhD students and know-it-all postdocs (no offense Marcel, Caro and Willi). I would as well like to thank all other former and current members from the Huber lab for their support, discussions, humor or just being there (take your pick). In no special order that would be Sascha Yoseffi, Julia Binz, Julia Marschall, Thomas Wilhelm (a.k.a. Willi), Ole Kläner, Katrin Ciecielski, Philipp Rogalski, Frank Faßbender, Magdalena Poplutz, Susana Marina Nunes de Miranda, Katrin Knoll and others who I probably just forgot.
to write down, sorry. Thanks FIFA-crew (Nico, Dirk, Dieter, Tamas, Tobi, Caspar, Consti) for awesome action on the consoles! In general, thanks to all people of the institute of biochemistry, third and fifth floor and everything between the two (and above).

Thanks go out to the SPP Mastzelle SummerSchool crowd for an amazing time in Tübingen and also Berlin. Thanks to Christopher Tiedje for all his support regarding the TTP KO femurs, for the help with the manuscript of the TTP paper, and of course also for the beer he invited me to in Weimar. I would like to thank Noriaki Shimokawa from Gunma University, Japan, for all his patience and enthusiasm regarding the transport of mice femurs to us. Despite his great efforts we were unfortunately not able to work with them, see the last paragraph for more details. Also I want to thank Uwe Schulte and the people at Logopharm for performing the mass spectrometry analysis for us. Further thanks go to Bernd Denecke and his lab for performing the transcriptome analysis for us.

Furthermore a rather unusual addition to the acknowledgements section, but I really feel the need to get this of my chest. Just knowing there is the slight possibility that a bigger audience than just the institute I work in will read about this, is quite enough for me. That said I definitely do not want to thank Fedex, more specifically whoever from Fedex is involved in transporting packages from Japan to Germany. Three times we tried to get the CIN85ΔExon2 mouse femurs over to Aachen. The second and the third time they arrived frozen at our lab, rendering them completely useless for us. While at the first time we just did not have the necessary documents ready to have them pass the controls at the veterinary office, it probably would not have mattered at all. I believe firmly that those femurs were also frozen somewhere on the way. The third time was especially ridiculous, Noriaki had ”4 °C do not freeze” written in big letters on every possible empty space on that box. It even had a sticker on it, which asked not to put it somewhere warm. It arrived frozen regardless of all precautions. Not only did this cost a lot of money, it also cost me a substantial part of my thesis. Not to mention the time invested getting the cells in culture, hoping they might make it somehow. They never did. No one at Fedex was able to figure out how this could have happened repeatedly.
Appendix D

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Ag</td>
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<td>Butyrate response factor</td>
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<td>Bovine serum albumine</td>
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<td>CIN85</td>
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<td>Fetal bovine serum</td>
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<td>Steel Factor</td>
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<td>Zfp</td>
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