The role of SHIP1 in modulating disease severity in the K/BxN serum transfer model of rheumatoid arthritis

by

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Abstract

SHIP1 (SH2 domain containing inositol-5'-phosphatase) is a negative regulator of the phosphatidylinositol-3 kinase (PI3K) pathway. SHIP1 is expressed in hematopoietic cells, and mediates its effect by hydrolyzing PIP_3, an important second messenger generated by the PI3K pathway. In this way, SHIP attenuates a variety of signaling cascades including those mediating cell survival and proliferation. Due to its importance in regulating immune cell signaling, SHIP1 is an attractive therapeutic target.

In this thesis, I explored the role of SHIP1 in the context of rheumatoid arthritis, an autoimmune inflammatory disease. To this end, I employed the K/BxN serum transfer model of rheumatoid arthritis. This disease model is auto-antibody driven and lymphocyte independent, and thus facilitates characterization of the effector phase of disease, a process that relies on components of the innate immune system. Arthritis was dramatically exacerbated in global SHIP1 knock-out mice, as evidenced by changes in ankle thickness, clinical scoring and histological analysis. Heterozygous SHIP1 mice also displayed increased disease severity in comparison to wild type litter mates, possibly due to an expanded population of circulating neutrophils, that increases with age. Since naive global SHIP1 knock-out mice exhibit a range of hematopoietic abnormalities, to elucidate the cell intrinsic contribution of SHIP1 ablation to the disease phenotype, I induced K/BxN mediated arthritis in mice with lineage restricted deletion of SHIP1. Mice with a neutrophil/macrophage-restricted loss of SHIP1 (LysMcre), like global SHIP1 knock-out mice, displayed an alternatively activated 'M2' biased macrophage phenotype, and developed exacerbated disease. Neutrophil-restricted loss of SHIP1 (GEcre) was also sufficient to exacerbate disease and resulted in earlier disease onset. In order to identify how the loss of SHIP1 in neutrophils results in heightened disease severity, I performed a series of in vitro experiments utilizing polymorphonuclear leukocytes freshly isolated from bone marrow. While we cannot exclude that SHIP1 may be playing additional roles in neutrophil functions, I report that SHIP1 plays a role in attenuating responsiveness of neutrophils to GPCR and FcγR ligation, two families of receptors that are necessary for induction and amplification of rheumatoid arthritis in the K/BxN serum transfer model.

Preface

All experiments, with the exception of those described below, were performed by Alison Hirukawa. I conceived of and designed all of the experiments and analyzed the data, wrote and edited the thesis.

Figure 11 is based on data collected from experiments conducted by Dr. Frann Antignano. Kim Snyder performed the arginase activity assay in Figure 11.

The data in Figure 21 was generated with the help of Dr. Michael Hughes and Kim Snyder.

The microarray in Appendix B was performed by Dr. Jamie Haddon.

Dr. Michael Hughes interpreted some of the data, and Dr. Kelly McNagny edited the thesis.

All animal work was approved by the UBC Animal Care Committee, Certificate #A07-0376.

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List of Abbreviations

-/-	Null genotype
+/-	Heterozygous genotype
+/+	Wild-type genotype
Akt	Alternatively known as PKB
APC	Antigen presenting cell
B6	C57Bl/6
β-ME	Beta-mercaptoethanol
BM	Bone marrow
BSA	Bovine serum albumin
BMMC	Bone marrow derived mast cells
Brd-U	5-Bromo-2-deoxyuridine
BTK	Bruton's tyrosine kinase
C5a	Complement 5a
CO_2	Carbon dioxide
CD	Cluster of differentiation
Dok	Downstream of tyrosine kinase
EDTA	Ethyenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
Erk	Extra-cellular signal related kinase
ES	Embryonic stem
ESMC	ES derived mast cell
F2	C57Bl/6 crossed with 129S, hybrid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcγR	Fc gamma receptor
FITC	Fluorescein isothiocyanate
fl/fl	Floxed alleles
G-CSF	Granulocyte colony stimulating factor
GE	Elane (neutrophil elastase)
GM-CSF	Granulocyte-macrophage stimulating factor
GPCR	G protein coupled receptor
GPI	Glucose 6-phosphate isomerase
HBSS	Hank's balanced salt solution
H&E	Hemotoxylin and eosin

HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
$InsP_4$	Inositol-1,3,4,5-tetrakisphosphate
i.p	Intraperitoneal
i.v	Intravenous
K/BxN	KRN TCR transgene and MHCII A(g7)
kDa	Kilodalton
Lin	Cell surface markers for hematopoietic lineage
LSK	Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$
LTB_4	Leukotriene B4
LysM	Lysozyme M
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MTG	Monothioglycerol
NSG	NOD scid gamma
PBS	Phosphate buffered saline
PerCP	Peridinin chlorophyll protein complex
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PLCγ	Phospholipase C γ
PMN	Polymorphonuclear leukocytes
pNPP	Para-nitrophenylphosphate
PTB	Phosphotyrosine binding domain
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SEM	Standard error measure
SH	Src Hhomology domain
SHIP	SH2-containing inositol 5'-phosphatase
TBS-T	Tween 20 buffered solution
TCR	T-cell receptor
Tie-2	Tyrosine kinase with immunoglobulin and EGF homology domains 2
TNF-α	Tumour necrosis factor alpha

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Much of my work was facilitated by collaboration. I would like to thank Dr David Lee (Harvard Medical School) for the K/BxN serum to perform the preliminary experiments, Dr Paul Kubes, Dr Bjorn Petri, Carla Badick, and Keir Pittman from the University of Calgary, for all work related to the *GEcreSHIP* experiments. I would also like to thank Denis and Marie Demco from Calgary for their generous hospitality.

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Finally, I would like to thank the National Science and Engineering Research Council for their financial support.

Dedication

This work is dedicated to my family: Linda, Tomio and Christina Hirukawa.

1. Introduction

The most conventional understanding of the immune system is that it functions to fight infection, thus protecting the host. Once infection is cleared, immune cell functions are shifted towards tissue repair and healing. However, the failure of cells of the immune system to respond appropriately to external stimuli or an inability to return to homeostasis can result in the perpetuation of a pro-inflammatory condition, culminating in tissue destruction. Often, aberrant immune cell function is rooted in dysfunctional signal transduction pathways- the method by which a cell integrates signals from the external environment with intracellular cell processes. External stimuli interact with extracellular cell surface receptors, resulting in activation of intracellular signal transduction cascades, which in turn specify cellular behavior. Thus, characterizing signal transduction pathways and major intracellular regulators of these pathways in various immune cell types is an area of keen interest and holds promise for the development of pharmaceutical targets. Furthermore, to treat a variety of immune disorders, understanding of cell signal regulation within the context of a specific disease model may reveal novel approaches for therapeutic intervention.

1.1 The innate immune response

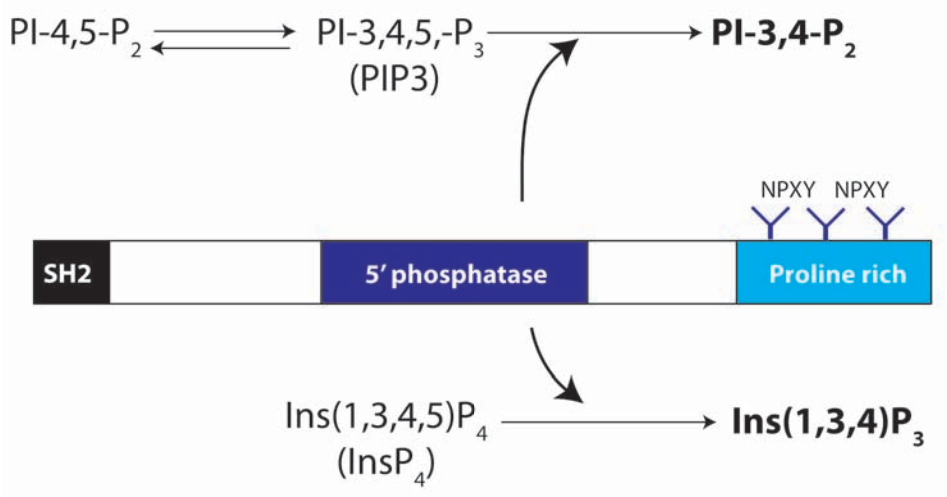
The immune system of vertebrates can be divided into two branches; the innate immune system and the adaptive immune system. A more evolutionarily ancient mode of host defense, the innate immune response constitutes the first line of defense against infection and damage. Major functions of the innate immune system include barrier functions, production of cytokines (specialized chemical mediators) that facilitate immune cell recruitment to sites of infection, identification and elimination of foreign molecules encountered in various tissues, and activation of the adaptive immune system via antigen presentation. The innate immune system is composed of both hematopoietic (blood-derived) and non-hematopoietic cells. Epithelial cells are non-hematopoietic cells that possess toll-like receptors, capable of recognizing features of micro organisms. Members of the hematopoietic lineages include mast cells, neutrophils, basophils, platelets, macrophages, natural killer cells and dendritic cells. Mast cells exert much of their function through the process of degranulation, the localized release of chemical mediators that results in cellular recruitment and

increased vascular permeability. Mature neutrophils will exert their function by migrating to the site of injury and destroying pathogens via phagocytosis, degranulation or the production of reactive oxidation species. Macrophages are primarily recognized for their ability to engulf pathogens and stimulate the adaptive immune response, natural killer cells release granules that can induce programmed death of target cells, while dendritic cells have a potent capacity to present antigen.

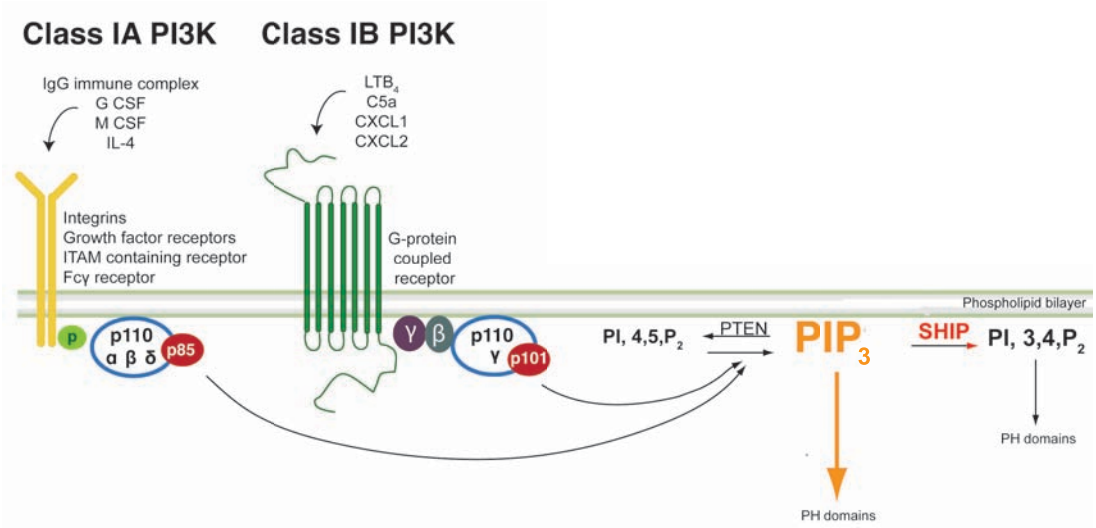
1.2 Src homology 2-containing inositol phosphatase-1 (SHIP1)

1.2.1 The structure of SHIP1

SHIP1 (hereafter referred to as SHIP) is a 145 kDa intracellular protein, comprised of three major functional domains: an N-terminal SH2 domain, a proline-rich C-terminal region, and a central phosphoinositol 5‘-phosphatase region (Figure 1A). The SH2 domain permits association with tyrosine phosphorylated proteins such as Shc, Doks, Gabs, and proteins with immunoreceptor based inhibitor/activation motifs (ITIMs, ITAMs) such as FcγIIB and FcεRI (Sweeney et al., 2005). The proline-rich region confers an ability to bind to a subset of proteins containing SH3 domains, such as Grb2, Src and PLCγ1 (Rohrschneider et al., 2000; Sly et al., 2003; Wisniewski et al., 1999). SHIP also contains amino acid motifs that, when phosphorylated, bind to proteins with phosphotyrosine binding (PTB) domains such as Shc, or SH2 domains. These structural qualities are essential to facilitating localization of SHIP to the plasma membrane; they mediate membrane recruitment in response to activation of receptor tyrosine kinases, immunoreceptors and tyrosine-kinase associated receptors through the phosphotyrosine residues present on receptors or docking proteins (Günzl & Schabbauer, 2008). SHIP can also localize to the plasma membrane in response to G protein coupled receptor (GPCR) engagement, but the mechanism by which this occurs is less clear. While these structural domains play a role in signaling and, importantly, recruitment of SHIP to the plasma membrane, it is the enzymatic function of SHIP, conferred by its central phosphoinositol 5‘-phosphatase region, that is likely responsible for most of its biological effects (Kerr, 2010) (Figure 1B).



A.



B.

Figure 1. SHIP structure and role in PI3K signaling

A, SHIP is composed of three major structural domains. The central phosphatase domain is integral to negative regulation of the PI3K pathway, by virtue of its ability to dephosphorylate the PI3K secondary messenger PIP_3.

B, Class IA (p110δ) and Class IB (p110γ) PI3K isoforms expressed in immune cells, are activated by distinct signalling pathways**.** The class I PI3K isoforms p110δ and γ are predominantly expressed in hematopoietic cells. PI3Kδ is typically activated by receptors that use tyrosine kinase signalling pathways (eg, receptor tyrosine kinases (RTK), integrins, immunoreceptors), whereas PI3Kγ is activated by the Gβγ subunits of G protein coupled receptors (GPCRs). In both cases, membrane recruitment and activation of PI3K results in rapid accumulation of $PI(3,4,5)P_3$ (PIP_3), which is degraded by SHIP. GPCRs may also activate PI3Kδ indirectly via Src-family kinases (SFK).

1.2.1 Enzymatic activity of SHIP

SHIP's enzymatic activity is restricted to two target phosphoinositides; PI(3,4,5)P_3 (**PIP_3**) and Ins(1,3,4,5)P_4 (**$InsP_4$**). Through hydrolysis of the 5' phosphate, SHIP converts these substrates to **PI(3,4)P_2** and **Ins(1,3,4)P_3**, respectively. In leukocytes, the hydrolysis of membrane tethered PI(4,5)P_2 is catalyzed by phospholipase Cγ (PLCγ) to produce Ins(1,4,5)P_3, which is in turn phosphorylated by kinases to generate $InsP_4$ (Caffrey et al., 2001; Pesesse et al., 1998). PI(4,5)P_2 is also the precursor for PIP_3, which is generated by class I phosphatidyl-inositide kinase (PI3K) phosphorylation. Translocation of SHIP to the plasma membrane, specifically at sites of PIP_3 and $InsP_4$ synthesis, is essential to its enzymatic effects. Extracellular stimulation (Damen et al., 1996) does not directly alter SHIP's phosphatase activity, but it does play a critical role in its function via dictation of SHIP's localization though its SH2 domain and proline-rich C terminus.

Both substrates of SHIP, **PIP_3** and **$InsP_4$**, function as mediators of intracellular signaling cascades. PIP_3 recruits signaling intermediaries that contain pleckstrin homology (PH) domains to the inner leaflet of the plasma membrane. This translocation, in turn, leads to the activation of the PH domain proteins (either through auto-phosphorylation or phosphorylation by other molecules) such as Akt, which facilitates signaling cascades that mediate important cellular functions such as survival, proliferation, motility, phagocytosis, and cell adhesion (Cantley, 2002; Vanhaesebroeck et al., 2001). The physiological role of $InsP_4$, however, has been far less characterized. In a mast cell line, $InsP_4$ facilitates activation of Ca^{2+} influx, by inhibiting metabolism of $InsP_3$ (Hermosura et al., 2000). However, at higher concentrations, $InsP_4$ can act as an inhibitor of $InsP_3$ receptors. Thus, within some contexts, $InsP_4$ can either facilitate or inhibit Ca^{2+} influx.

With respect to cell signaling, the ramifications of SHIP's enzymatic activities are two-fold; (1) by reducing levels of PIP_3 and $InsP_4$**,** SHIP **negatively** regulates the signaling pathways in which these phospholipid messengers participate, and (2) **PI(3,4)P_2** and **Ins(1,3,4)P_3** (the products of SHIP activity) can play a positive role in cell signaling. Like PIP_3, PI(3,4)P_2 can act as a secondary messenger by recruiting PH domain containing proteins to specific membrane domains (Zhang et al., 2009).

Interestingly, some PH domain proteins preferentially associate with $PI(3,4)P_2$ over PIP_3, suggesting that relative concentrations of these phospholipids can result in different signaling outputs. Furthermore, within certain contexts, optimal activation of pathways requires both $PI(3,4)P_2$ and PIP_3 (Kerr, 2010; Scheid et al., 2002).

1.2.3 SHIP's role in the immune system is dependent on cell type and cell stimulus

SHIP is expressed in all hematopoietic cells, yet the protein levels of SHIP vary considerably between different lineages and at different stages of cellular maturity. Furthermore, different cell types can employ different methods to regulate SHIP expression, resulting in variable outcomes of SHIP signaling. For example, expression of SHIP increases during T cell development (Liu, et al., 1998) yet shows a bimodal expression pattern during B cell development (Geier et al., 1997), and is significantly upregulated in activated B cells (Brauweiler et al., 2000). The nature of the external stimulus applied, in addition to the identity of the cell itself, determines the biological significance of SHIP signaling, as determined by genetic approaches for studying SHIP's function.

The employment of genetic studies has been useful in the elucidation of SHIP's role in cellular functions of various hematopoietic lineages. This has involved the generation and comparison of cells in which SHIP has been 'knocked out' *(SHIP-/-*) to non-manipulated 'wild-type' cells (*SHIP+/+*). Studies examining the effects of SHIP ablation have emphasized that the nature of the cell stimulus and cell type dictates whether SHIP's influence on cellular activation is negative or positive. For example, in B cells, clustering of the B cell receptor (BCR) with FcγRIIB results in tyrosine phosphorylation of intracellular immunoreceptor tyrosine-based inhibition motif (ITIM) region of FcγRIIB, thus triggering recruitment of SHIP to the plasma membrane. This decreases PIP_3 levels, BTK recruitment, and calcium flux (Bolland et al., 1998; Okada et al., 1998; Scharenberg et al., 1998). In *SHIP-/-* B cells, PIP_3 levels are elevated and calcium flux is increased. A similar mechanism has also been observed in *SHIP-/-* mast cells, where loss of SHIP prevents restriction of PIP_3, resulting in increased intracellular calcium flux and increasing sensitivity to the mediators which facilitate cytoskeletal changes necessary for degranulation (Huber et al., 2002). In macrophages, ablation of SHIP enhances phagocytosis, a PI3K-

dependent activity, following ligation of Fcγ receptors and complement receptor 3 (CR3, also known as integrin $\alpha_M\beta_2$) ligation (Cox, 2000). Localization of PI3K derived lipids play a crucial role in cell polarization and migration of neutrophils, and *SHIP-/-* neutrophils isolated from bone marrow display delayed migration towards GPCR agonists (Nishio et al., 2007). Hyper-responsive phenotypes have also been observed within the context of inflammatory stimuli. One study demonstrated a role for SHIP in regulating neutrophil response to pathogen-associated agonists. The authors observed that SHIP-deficient neutrophils were hyper-responsive to peptidoglycan, a Toll like receptor 2 (TLR2) agonist, resulting in an increased release of certain cytokines (Strassheim et al., 2005). These data further substantiate SHIP's role in attenuating immune cell activation.

Other groups, have suggested that SHIP's role in negatively regulating PI3K and Akt may not always inhibit cellular activation. Recent studies in endotoxin stimulated macrophages demonstrated that SHIP can promote NF-κB-dependent transcription of genes, with resulting downstream effects such as cytokine release (Fang et al., 2004). This is in contrast to other studies that showed that SHIP inhibits macrophages activation following immune receptor or growth factor receptor ligation (Tridandapani et al., 2002) . Other studies have also found that *SHIP-/-* mice are more sensitive to endotoxin-induced death and, at a cellular level, that *SHIP-/-* macrophages and mast cells are hyper-responsive to endotoxin, as evidenced by increased release of cytokines such as IL-6 and IL-1 (Sly et al., 2004).

1.2.4 Phenotype of global SHIP knock-out mice

A great deal of insight into the role of SHIP in the immune system *in vivo* has been gained through the generation of viable *SHIP-/-* mice. These mice were generated by two independent groups (Helgason et al., 1998; Q. Liu et al., 1999) by excising the first exon of SHIP. Although the mice are fertile, they have a shortened life span due to multiple hematopoietic and physical aberrations. Their lungs are enlarged, due to infiltration of myeloid cells (macrophages and neutrophils). This is likely due to the expanded populations of granulocyte-monocyte progenitors in the bone marrow and spleen, as these cells are more responsive to sub-optimal levels of cytokines than their wild type counterparts. Extramedullary hematopoiesis results in

a 5-7 fold increase in spleen weight and cellularity, and the mice also suffer from severe osteoporosis, as a result of hyper-resorptive osteoclasts (Takeshita et al., 2002).

Interestingly, *SHIP-/-* mice share many characteristics with heterozygous PTEN mice (Di Cristofano et al., 1999). PTEN is also a phosphatase that functions to reduce PIP_3 levels, but its enzymatic activity targets the 3'-phosphate, thus producing $PI(4,5)P_2$. The similarity in mouse phenotypes suggests that elevated PIP_3 levels in *SHIP-/-* mice are responsible for many of the observed phenotypes. However, some *SHIP-/-* phenotypes are likely due to compensatory mechanisms initiated to counteract the damage incurred by SHIP loss. For example, as the SHIP knock-out mice age, immunoregulatory subsets such as T regulatory cells (T_{reg}) (Kashiwada et al., 2006) and myeloid suppressor cells (Paraiso et al., 2004) expand, likely in response to the inflammatory environment created by the elevated populations of hyper-responsive myeloid cells. T cell levels are also lower in *SHIP -/-* mice, and as the mice age they develop reduced $CD4^+/CD8^+$ double positive cells and increased activated T cells (Collazo et al., 2009). However, mice with conditional T cell deletion of SHIP do not display an expanded T_{reg} compartment (Tarasenko et al., 2007).

Since several of the phenotypes observed in *SHIP-/-* immune cells are due to cell extrinsic influences of ubiquitous SHIP loss, these may obscure cell autonomous effects. For example, *SHIP-/-* mice display B cell hyperactivity, but the total B cell population decreases over time, because the expanded myeloid cell population produce high levels of IL-6, an inhibitor of B cell development (Nakamura, 2004). Macrophages, classically viewed as phagocytic or antigen presenting components of the innate immune system, display functional plasticity when exposed to certain stimuli. In *SHIP-/-* mice, macrophages are skewed away from the classically activated phenotype, towards an alternatively activated M2 state (Rauh et al., 2005). Although this bias may be partly due to cell intrinsic effects of SHIP loss, polarization has primarily been attributed to the exogenous influence of basophil derived IL-4 (Kuroda et al., 2009). Thus ubiquitous knockouts, although tremendously helpful in increasing our knowledge of the *in vivo* effects of SHIP,

make it difficult to delineate the cell intrinsic effects of SHIP. The generation of mice in which deletion of SHIP has been restricted to targeted lineages has helped us resolve this problem. The construction of 'floxed' SHIP alleles permits inducible or tissue specific deletion and facilitates the identification of lineage specific function of SHIP.

1.2.5 Conditional SHIP knock-out mice

To examine the role of SHIP in the K/BxN serum transfer model of arthritis, I injected arthritogenic K/BxN serum into *SHIP-/-* mice and wild type (*SHIP+/+*) litter mates. For this initial experiment, we procured serum from a collaborator (Dr. David Lee, Harvard) and diluted it 1 part serum to 3 parts HBSS prior to administration (for the remainder of the disease courses, K/BxN serum was generated in-house and used at a dilution of 2 parts serum to 1 part HBSS). For the naive controls, HBSS was administered instead of K/BxN serum. Disease manifestation was assessed by evaluating several parameters over a ten day period; these parameters including changes in ankle thickness and the clinical index as described in the Materials and Methods. Additionally, at the termination of the disease course, both ankles were harvested and prepared for histology. Crosssections stained with hematoxylin and eosin were evaluated for levels of inflammatory infiltrate, cartilage degradation and bone erosion.

1.3 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory autoimmune disease, that affects approximately 1% of the global population (Firestein, 2003). The disease is characterized by painful swelling around the distal joints and causes progressive joint erosion and destruction of joint surfaces. Histological analysis of affected joints reveals leukocyte invasion of the joint, and proliferation of the synovial lining, accompanied by bone and cartilage erosion. Several mouse models of autoimmune arthritis have been developed in an effort to determine the molecular mechanisms underlying RA pathology. The K/BxN serum transfer model of arthritis is an autoantibody dependent model, in which pathogenic serum from the arthritic donor (K/BxN mouse) is transferred to a naive host (Korganow et al., 1999). Since the pathogenic autoantibodies are

generated in donor mice, the model bypasses the antigen priming step, allowing examination of the effector cell-mediated events leading to disease in the recipient.

1.3.1 K/BxN mice

K/BxN mice spontaneously develop arthritis at 4-5 weeks of age, and recapitulate several features of human rheumatoid arthritis such as symmetrical polyarthritis, pannus formation, inflammatory infiltrate, cartilage and bone erosion, and hyperproliferation of the synovium (Kouskoff et al., 1996). The strain was originally generated by crossing KRN mice, transgenic for a T cell receptor (TCR) that recognizes a bovine RNase epitope, with non-obese diabetic (NOD) mice. Unexpectedly, the KRN TCR in the context of the NOD derived MHC II molecule I-A^{g7}, also recognizes the ubiquitously expressed protein glucose-6-phosphate isomerase (GPI) (Figure 2). GPI reactive T cells aid K/BxN B cells to produce high titers of anti-GPI IgG (Maccioni, 2002), which binds to GPI that associates with the synovial lining of the joint, forming immune complexes (IC). These IC initiate an inflammatory cascade mediated by innate immune effector cells such as mast cells (Lee, 2002), neutrophils (Wipke & Allen, 2001) and macrophages (Solomon et al., 2005), all of which play an essential role in disease induction. Thus, administration of K/BxN serum containing anti-GPI immunoglobulin G (IgG) antibodies to a naive recipient permits examination of the lymphocyte-independent events post IC formation.

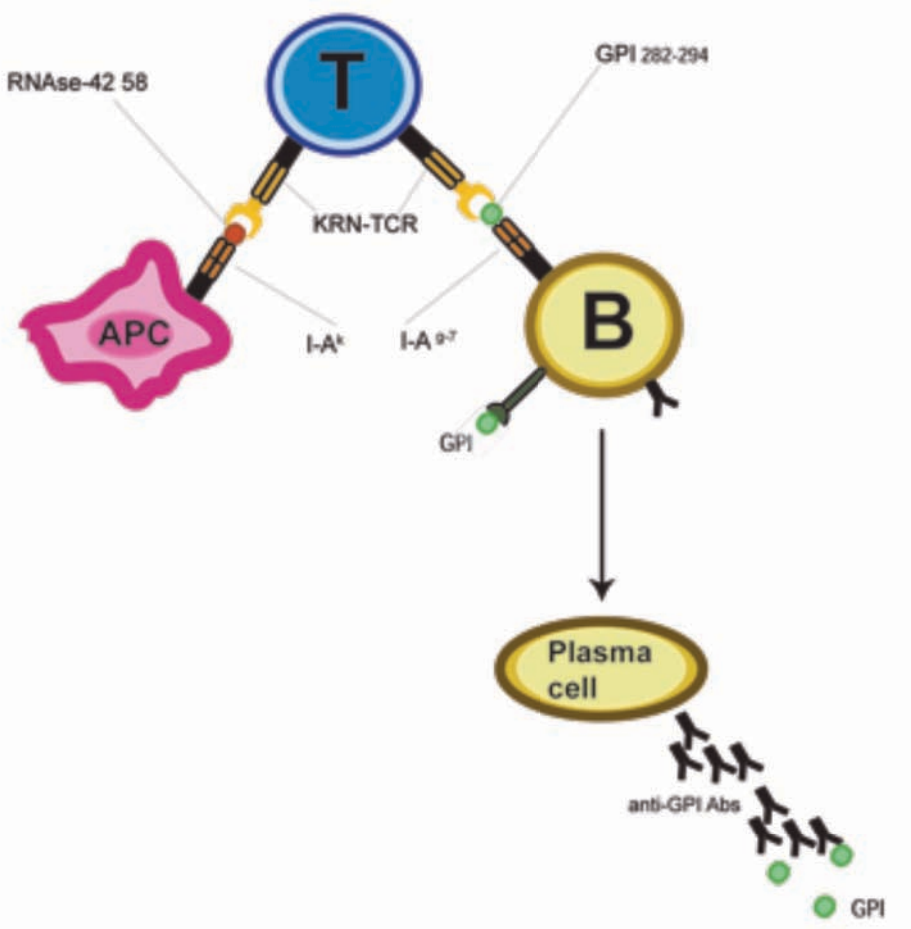


Figure 2. The specificity of the KRN TCR for GPI is responsible for autoimmunity in the transgenic K/BxN mice

K/BxN mice have a T cell receptor specific for both the RNase peptide 42-58, in the context of I-A^k (left side) as well as GPI, in the context of I-A ^{g7} (right side). B cells with I-A ^{g7} can act as APC and present GPI to T-cells, thus allowing the B cells to differentiate into plasma cells and produce anti-GPI autoantibodies. These auto antibodies will complex with the ubiquitously-expressed GPI, initiating disease.

1.3.2 The innate immune system is necessary for arthritis induction

Mast cells and neutrophils are amongst the earliest participants in K/BxN serum induced arthritis. Activation of these immune cell types via anti-GPI IC and soluble factors is necessary for the production of pro-inflammatory mediators. Histological studies have revealed that degranulated mast cells are localized in the synovial joint within two hours of serum transfer, long before any clinical symptoms are presented (Lee, 2002). Upon degranulation, mast cells release preformed granules containing soluble pro-inflammatory mediators such as IL-1β and TNF-α, both of which are important for disease progression, as they mediate chemotaxis, permeability and inflammation (Choe et al., 2003; Ji et al., 2002a). Neutrophils are also essential for the early stages of disease, infiltrating the synovial joint within 24 hours after serum transfer (Wipke & Allen, 2001). Like mast cells, neutrophils release pro inflammatory mediators, as well as degradative enzymes directed towards extracellular targets. Antibody-mediated depletion of neutrophils confers resistance to disease, while neutrophil depletion initiated after disease induction reverses the disease progression. This suggests that, in this context, neutrophils function as both disease initiators and amplifiers (Wipke & Allen, 2001).

The identification of cell lineages responsible for disease induction has inspired the elucidation of mechanisms underlying how arthrogenic IgGs and soluble factors interact with effector cells, and how these interactions translate into a cascade of pro-inflammatory signals (Figure 3). With respect to IC recognition, the activation of neutrophils and mast cells is dependent on certain Fcγ receptors (FcγR), which bind to the Fc fragment of IgG autoantibodies. The importance of these receptors was revealed by transferring K/BxN serum into a panel of *FcγR -/-* mice. *FcγR III-/-* mice developed less severe arthritis, while *FcγR I-/-* mice developed disease comparable to wild type mice (Ji et al., 2002b), implicating FcγRIII but not FcγRI, in disease progression. Further genetic studies involving FcγRVI deficient mice revealed the importance of this receptor for disease progression (Mancardi et al., 2011). Unlike other Fcγ receptors, FcγRIIB has an inhibitory function, and therefore loss of this receptor might be expected to exacerbate disease, as has been demonstrated in other arthritis disease models (Nakamura, et al., 2004; van den Berg, 2009). However, investigations into the effect of the FcγRIIB attenuation to

the K/BxN serum transfer model of arthritis have produced contradictory results. While one group found that loss of FcγRIIB in mice has no functional consequences (Ji et al., 2002b), another group observed that ablation of FcγRIIB accelerated the onset and severity of disease (Corr & Crain, 2002). It has since been suggested, however, that the genetic background of the mice influences disease manifestation, as FcγRIIB deficient mice of a mixed 129xC57B/6 background do not suffer from exacerbated disease (Pritchard et al., 2000).

In addition to IC engagement of Fcγ receptors, elements of the alternate complement cascade are also necessary for disease induction. Mice lacking components involved in the initial stages of the alternate complement pathway, such as complement factor B, C3, C5, or the C5a receptor, do not develop arthritis in the K/BxN serum transfer model (Ji et al., 2002b). The importance of C5 during disease was further illustrated through use of an anti-C5a antibody, the administration of which also conferred resistance to joint inflammation and destruction (Ji et al., 2002b). However, mice lacking C6, a component of the later stages of the alternate complement pathway, did develop disease. This demonstrates that the activity of the anti-GPI immune complexes is not dependent upon assembly of the membrane attack complex and facilitation of cell lysis, but rather the interaction of C5a with C5a receptors expressed on the surface of effector cells. C5a can act as a potent chemoattractant, inducing acute inflammation through activation of mast cells and neutrophils (Grant et al., 2002). At high concentrations, C5a can also stimulate the respiratory burst in neutrophils and promote vascular permeability by acting on endothelial cells (José et al., 1981). Although it is unclear exactly how the alternate pathway is activated within this context, it has been speculated that C3b binds to IgG deposited on the surface of cartilage, thus leading to the formation of enzymes that will cleave C3 and C5, thus producing C5a.

The initial activation of effector cells eventually culminates in joint damage and inflammation, typically attributed to the effects of several proinflammatory cytokines including TNF-α and IL-1. Clinical trials employing monoclonal antibodies against TNF-α and IL-1 have achieved beneficial results in a significant number of human

patients suffering from rheumatoid arthritis (Cohen et al., 2002; Lipsky et al., 2000). With respect to the K/BxN serum transfer model, the IL-1 receptor has been shown to be necessary, as evidenced by the resistance of *IL-1R-/-* mice to the effects of arthrogenic serum (Ji et al., 2002a). The role of TNF-α in this mouse model, however, is less clear, as blocking this inflammatory cytokine with a monoclonal antibody does not ameliorate disease (Ji et al., 2002a; Kyburz & Carson, 2000). Intriguingly, TNF-α receptor deficient mice administered arthrogenic serum display milder disease (Ji et al., 2002a), suggesting that the TNF-α receptor is interacting with unidentified ligands in the progression of K/BxN serum transfer arthritis.

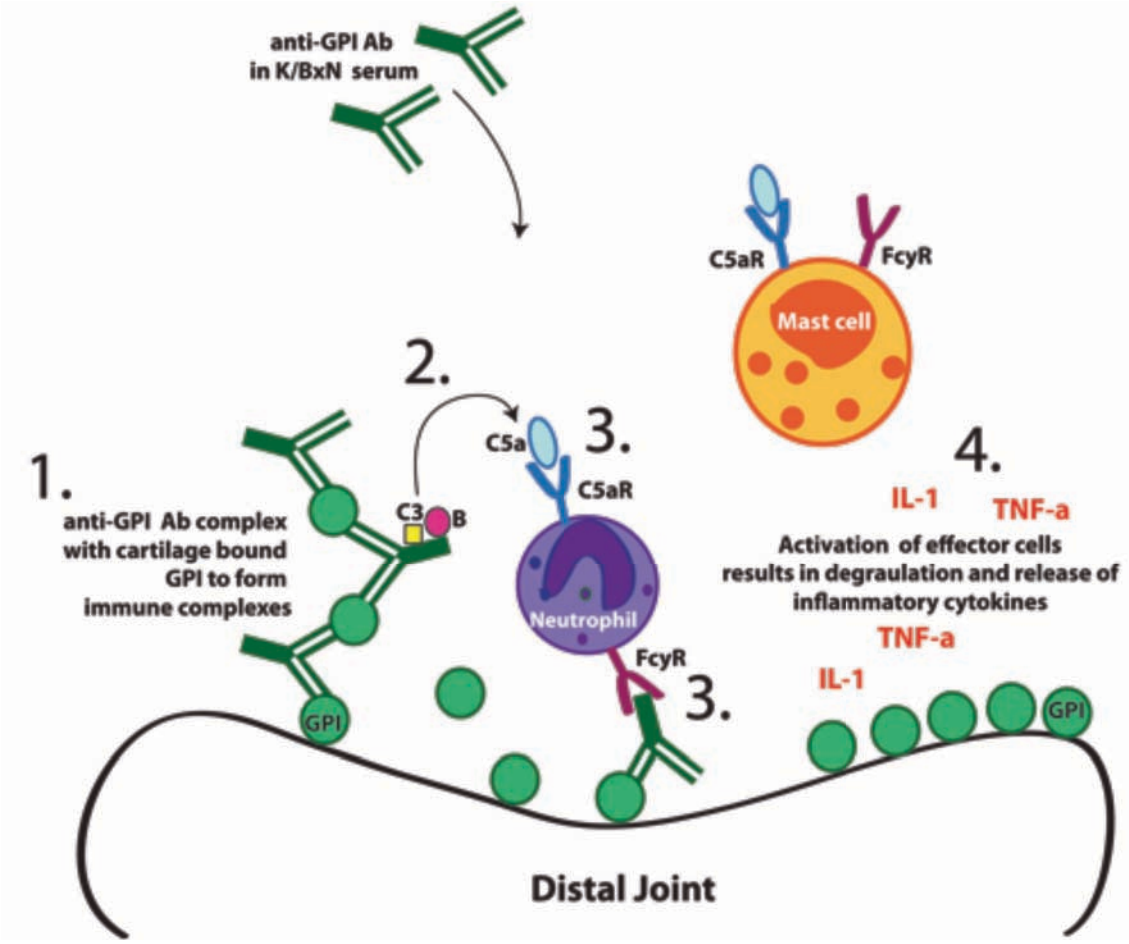


Figure 3. Immune complexes and C5a are involved in the initial stages of the K/BxN serum transfer model of rheumatoid arthritis

The K/BxN serum transfer model of rheumatoid arthritis occurs in a stepwise progression. Initially, immune complexes are formed when anti-GPI auto antibodies interact with GPI localized on the surface of distal joints (1). The interaction between the alternate complement pathway and GPI IC result in the release of C5a (2). The joint bound immune complexes or C5a then engage with receptors present on mast cells and neutrophils, resulting in their recruitment to the joint (3). There, they release various inflammatory chemokines, cytokines and enzymes such as IL-1 and TNF-α (4). Proliferation of joint synoviocytes and macrophages ensues, resulting in further release of IL-1, TNF-α and other factors which will act to erode the bone and cartilage.

1.3.3 SHIP and rheumatoid arthritis

Recently, decreased levels of SHIP have been observed in the synovial environment of human rheumatoid arthritis patients (Kurowska-Stolarska et al., 2011). In this study, the authors identified a microRNA-155 (miRNA)/SHIP pathway, which they postulate to be responsible for some of the exacerbated inflammatory response observed in the joints of rheumatoid arthritis patients. MiRNA are small ribonucleic acids which mediate mRNA expression of specific target genes; for example, miRNA-155 has been shown to post transcriptionally reduce SHIP (O'Connell et al., 2009). Kurowska-Stolarska and colleagues observed that miRNA-155 is up-regulated in the synovial membrane and synovial fluid of patients with rheumatoid arthritis. Furthermore, this increased miRNA-155 expression was associated with decreased expression of its target, SHIP, in macrophage and monocyte populations isolated from the human patients, as well as increased production of proinflammatory cytokines such as TNF-α.

1.3.4 Aim of the study

The aim of my study, in the most general sense, was to explore the role of SHIP within the context of an autoimmune inflammatory disease. In light of SHIP's role as a master regulator of immune cell signaling, and the implication of decreased SHIP levels in the pathology of human rheumatoid arthritis, these studies might provide insight into novel therapeutic applications. Since hematopoietic abnormalities, such as an expansion of the myeloid compartment, accumulate in the SHIP knock-out mice, I hypothesized that induction of the K/BxN serum transfer model of rheumatoid arthritis would result in exacerbated disease in the complete SHIP knock-out (*SHIP-/-*) mice.

Next, I wanted to explore the cell intrinsic effects of SHIP loss within our disease model. To this end, I employed conditional SHIP knock-out strains, including T cell, B cell, neutrophil/macrophage and neutrophil specific deleter strains. Ultimately, I elected to concentrate our studies on ablation of SHIP in neutrophils. Very little has been published on the role of SHIP in neutrophils, despite the importance of PI3K signaling to various biological functions of neutrophils. SHIP has been shown to

attenuate neutrophil effector functions in response to pathogen-associated agonists, however, other reports have noted that migration of *SHIP-/-* neutrophils is impaired. Furthermore, much of this work has been performed *in vitro*, thus the biological relevance of these phenotypes has not been confirmed. I employed both *in vivo* and *in vitro* techniques to assess the neutrophil intrinsic effect of SHIP ablation during the effector phase of rheumatoid arthritis (a stage in which neutrophils play a key role), and worked to elucidate a potential mechanism to explain the disease phenotype I observed.

2. Materials and Methods

2.1 Mice and K/BxN serum generation

All mice (with the exception of the *GEcreSHIP*$^{fl/fl}$) were housed in pathogen free mouse facilities at the The Biomedical Research Center, University of British Columbia. The *GEcreSHIP*$^{fl/fl}$ mice were maintained in double barrier facilities at the Calgary Health Sciences Center, University of Calgary. All procedures were conducted in accordance with guidelines set by the Canadian Council on Animal Care. Ubiquitous *SHIP -/-* were generated by crossing SHIP$^{fl/fl}$ to a ubiquitous cre expression strain (*pCxNLS-Cre*) (Andras Nagy, Universty of Toronto) to generate B/6 congenic *SHIP+/-* stain. These were back crossed once to parental SvImJ/129 to generate B/6:129 hybrid *SHIP+/-*. Mice were maintained on a B/6x129/Sv mixed background by intercross matings of *SHIP+/-* mice. Conditional knockout mice were generated by crossing SHIP$^{fl/fl}$ mice (Wang, 2002) (William G. Kerr, Moffitt Cancer Center) with either the *LysM-Cre* (Clausen, et al., 1999) (Jackson Labs,), *CD4-Cre* (Lee et al., 2001) (Jackson Labs), *CD19-Cre* (Rickert et al., 1995) (Jackson Labs) or *GE-cre* (Tkalcevic J *et al.*, 2000) (European Mutant Mouse Archive) transgenic lines. All conditional mice were back crossed for at least 10 generations and generated on a B/6 background, with the exception of the *CD19creSHIP*$^{fl/fl}$, which were maintained on a mixed B/6 x129/Sv background.

K/BxN serum transfer donors were generated by crossing KRN T-cell transgenic mice (provided by Christophe Benoist) (Kouskoff et al., 1996) with the NSG strain (Jackson Labs). Serum from 10-12 week old mice was obtained via cardiac puncture, pooled and stored at -20°C. In order to keep serum batches as consistent as possible, K/BxN mice were bred such that enough serum could be harvested to last several months.

2.2 K/BxN serum transfer mouse model of rheumatoid arthritis

Each mouse was administered intraperitoneal injections of 150 µl of serum diluted in HBSS, on day 0 and day 2. Forelimb and hindlimb clinical scores were noted daily for up to 10 days, and changes in ankle thickness were recorded with calipers (Mitutoyo Dial Thickness Gauge) every 24 or 48 hours. Clinical scores for each limb were

determined as 0= normal, 1= mild swelling/ redness, 2=obvious swelling of the ankle, 3= severe swelling for a total maximum score of 12 per mouse.

2.3 Statistical analysis

All data are presented as mean ± SEM. Comparisons were analyzed for statistical significance by two-tailed Student's t test, with $p < 0.05$ considered statistically significant.

2.4 Histology

Ankle joints were fixed in 10% buffered formalin for 24 hours and then transferred to 70% ethanol and stored at 4°C. Sample were then decalcified, paraffin-embedded, sectioned and stained with H&E or safranin-O by Wax-it Histology Services Inc. Samples were randomized for double blinded analysis and clinically scored based on the level of inflammatory infiltrate, or cartilage erosion and bone erosion observed in the cross section. A score of 0 (naive) to 4 (severe) was given for each parameter (please refer to Appendix A for a more detailed explanation of scoring). Pictures were taken at the 10x objective with a Zeiss Axioplan 2 microscope.

2.5 Analysis of cell populations in harvested tissues

Peripheral blood was collected by cardiac puncture or saphenous vein bleeds. RBC were lysed with MCRB RBC lysis buffer prior to flow cytometric analysis. For the ankle lavages, ankles were skinned and cut 0.5mm above the ankle joint and at the metatarsal region of the foot, just above the toes. Using a 30 gauge needle, the cellular contents of ankles were gently flushed with 3 injections of 100 µl volume HBSS/0.5% BSA and analyzed via flow cytometry.

2.6 Flow cytometry analysis

Samples were blocked with 10% rat or goat serum and anti-CD16/32 (AbLab, Vancouver, BC, clone 2.4G2) followed by staining with anti-Mac1 PE (BD Pharmingen, Mississauga, ON), anti-Gr1-APC (EBiosciences, San Diego, CA), anti-7/4-FITC (Abcam, Cambridge, UK), and anti-CD45-PerCP (BD Pharmingen, Mississauga, ON). For intracellular SHIP staining, cells were fixed with 2% PFA and permeabilized with

0.5% saponin (Sigma, St.Louis, MO) prior to staining with anti-SHIP (Santa Cruz, clone P1C1), followed by staining with anti-mouse IgG-Alexa Fluor 488 (EBiosciences, San Diego, CA). For analysis of FcγR levels, cells were directly incubated with anti-CD16/32 FITC (Ebiosciences, San Diego, CA, clone 93) in 10% goat serum in FACS buffer (PBS supplemented with 2mM EDTA and 0.05% sodium azide). Data were collected on a FACSCalibur cytometer (BD, Franklin Lanes, NJ) with CellQuest software and then analyzed with FloJo software(Treestar, Ashland, OR).

2.7 Isolation of BM PMN

To harvest BM, tibias and femurs were dissected from CO_2-euthanized mice and flushed with ice cold HBSS (calcium and magnesium free) supplemented with 0.5% BSA using a 25 5/8" long gauge needle. The resulting BM eluates were then gently pipetted and passed through a 70 micron nylon filter to remove cell clumps. BM cells were centrifuged at 1200 rpm for 5 minutes at 4°C. Each pellet was resuspended in 52% Percoll, and a discontinuous Percoll gradient was formed by layering 64% followed by 72% Percoll solutions beneath the resuspended BM. The gradients were centrifuged at 2100 rpm for 30 minutes at 4°C with a low brake setting (Eppendorf Centrifuge 5810, Hamburg, Germany). The cells visible at the interface between the 64% and 72% solution layers were carefully removed, resuspended in 10ml of HBSS/ 0.5% BSA and centrifuged at 1200 rpm for 5 minutes at 4°C with a low brake. The cell pellet was washed two more times with HBSS and left on ice. PMN viability was evaluated by trypan blue staining and cells were counted with a hemacytometer. % purity was determined morphologically by H&E staining of cytospins and by flow cytometry analysis of Gr-1 and 7/4 expression.

2.8 PMN stimulation

All cell stimulations were performed with freshly-isolated bone marrow PMN. Neutrophil activation by immobilized OVA anti-OVA immune complexes was performed as outlined previously (Tang et al., 1997). Briefly, 24-well plates were coated with poly-L-lysine (0.01%) followed by 2.5% glutaraldehyde to quench aldehyde groups. Plates were then coated with 1 mg/ml OVA (Sigma, St.Louis, MO) for 60 minutes, washed twice with PBS, and further incubated with 40 ug/ml rabbit-anti-OVA IgG (Sigma, St.Louis, MO) for one hour. The plates were again washed with PBS to removed any unbound antigen or

antibody, and PMN were plated at 2 x 10^6 cells/ml in HBSS containing calcium and magnesium, supplemented with 20 mmM HEPES (pH 7.4). For stimulations performed in suspension, PMN were resuspended in RPMI supplemented with 0.1% BSA, at a concentration of 10 x 10^6 cells/ml. 100 µL aliquots were then stimulated in the absence or presence of 100 nM C5a (Sigma, St.Louis, MO) or 100 nM LTB_4 (Santa Cruz Biotechnology, Santa Cruz, CA). PMN were stimulated with 100 nM C5a in the presence of fibrinogen (Ed Pryzdial, Center for Blood Research) coated 96 well plates (Maxisorp, NUNC, Rochester, NY) for 45 minutes.

2.9 Immunoblotting

PMN stimulations were terminated with the addition of 4x Laemmli sample buffer (9.2% SDS, 20% β-mercaptoethanol in glycerol). Whole cell lysates were boiled for 3 minutes at 95°C, sheared via repeated aspiration through a 1 ml insulin syringe, followed by centrifugation. Whole cell lysates (2 x 10^5 - 2 x 10^6 cells/lane) were fractionated by SDS-PAGE (10%) and transferred to PVDF membranes. Membranes were blocked with 5% BSA in PBS overnight at 4°C, washed three times with TBS-T (10x TBS, 100 mM Tris-HCl, 1.5 M sodium chloride, 200 mM potassium chloride, 0.05% Tween-20) and probed overnight with phospho-specific primary antibodies to detect phosphorylated SHIP, Akt (S473) and Erk1/2. Membranes were also probed for total SHIP and/or GAPDH as a loading control. Membranes were washed again with TBS-T and incubated with secondary antibodies directly conjugated to horseradish peroxidase for one hour at 20°C. Membrane were rinsed a final time with TBS-T and treated with treated with Western Lightning chemiluminescence reagent (Perkin Elmer, Boston, MA) for 60 seconds, and exposed to Kodak X-Omat Blue film prior to developing in a Kodak M35A X-Omat processor.

2.10 Measurement of MPO and Alkaline phosphatase release

MPO enzyme activity was measured using o-dianisidine dihydrochloride (Sigma, St.Louis, MO) as a substrate, as previously described (Xia & Zweier, 1997) with some modifications. In brief, 100 µl of cell free supernatant from stimulated PMN was added to 20 µl of 20 µM o-dianisidine dihydrochloride and 10 µl of 0.01% hydrogen peroxide (Fischer Scientific, Ottawa, ON) in one well of a 96 well plate, and incubated for 3 hours

at 37°C. Plates were then read with a spectrophotometer (Spectramax 150, Sunnyvale, CA) at 450nm. To determine total cell MPO activity, cell pellets were lysed with 0.02% CTAB (Sigma, St.Louis, MO) and assessed for MPO activity. The alkaline phosphatase assay was performed in a similar manner, except 50 µl of PMN supernatant was added to 50 µl of pNPP solution (Sigma, St.Louis, MO) (5 mg pNPP tablet dissolved in 50 ml of 0.1M diethanolamine buffer) in one well of a 96 well plate, and incubated for 3 hours at 37°C. Plates were then read with a spectrophotometer (Spectramax 150, Sunnyvale, CA) at 450 nm. To determine total cell alkaline phosphatase activity, cell pellets were lysed with 0.02% CTAB (Sigma, St.Louis, MO) and assessed for activity.

3. Results

3.1 Loss of SHIP exacerbates serum transfer induced arthritis

To examine the role of SHIP in the K/BxN serum transfer model of arthritis, I injected arthritogenic K/BxN serum into *SHIP-/-* mice and wild type (*SHIP+/+*) litter mates. For this initial experiment, we procured serum from a collaborator (Dr. David Lee, Harvard) and diluted it 1 part serum to 3 parts HBSS prior to administration (for the remainder of the disease courses, K/BxN serum was generated in-house and used at a dilution of 2 parts serum to 1 part HBSS). For the naive controls, HBSS was administered instead of K/BxN serum. Disease manifestation was assessed by evaluating several parameters over a ten day period; these parameters including changes in ankle thickness and the clinical index as described in the Materials and Methods. Additionally, at the termination of the disease course, both ankles were harvested and prepared for histology. Crosssections stained with hematoxylin and eosin were evaluated for levels of inflammatory infiltrate, cartilage degradation and bone erosion.

In comparison to the wild type equivalents, arthritic *SHIP-/-* mice displayed significantly altered values for several of these parameters. While ubiquitous loss of SHIP did not display significantly increased ankle thickness until day four (Figure 4), the clinical score of the hind limbs and forepaws showed signs of exacerbated disease two days after disease induction (Figure 4). Histological analysis of H&E stained synovial joint of *SHIP-/-* mice confirmed the presence of increased inflammatory infiltrate in comparison to wild type mice (Figure 5). Bone and cartilage erosion were both visible in the histological cross sections, thus confirming joint damage, but significant differences in cartilage erosion and bone erosion between the genotypes were not discernible.

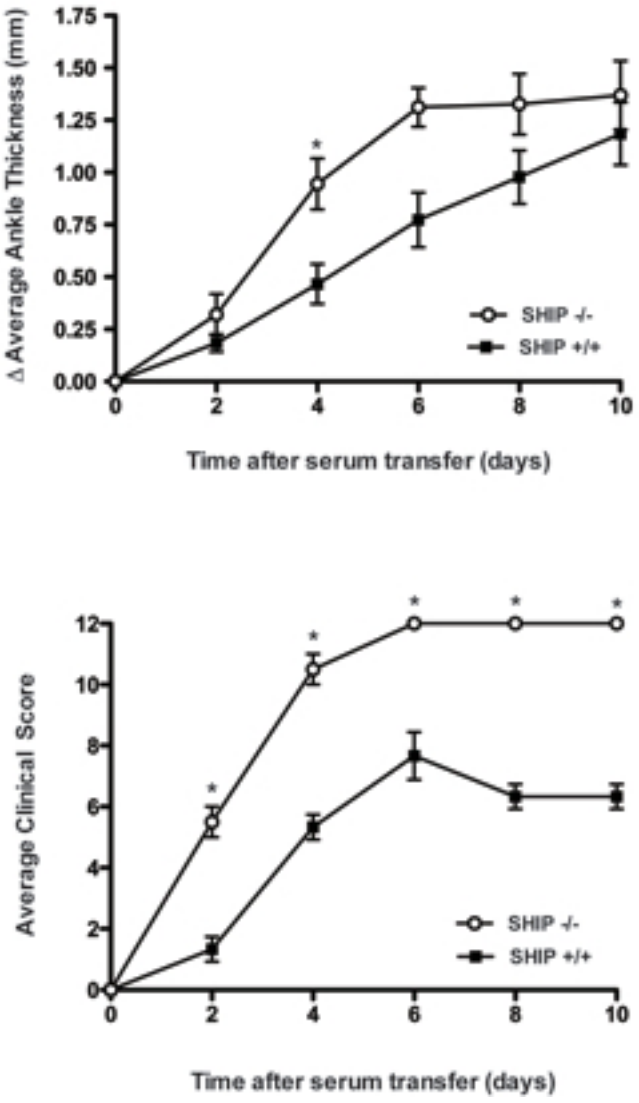


Figure 4. Loss of SHIP exacerbates serum transfer induced arthritis

Arthritis was induced in 6-8 week old male *SHIP-/-* or *SHIP+/+* mice (6 mice per genotype) via administration of diluted K/BxN serum on day 0 and day 2, and symptoms were compared between genotypes over a 10 day course. Average change in ankle thickness was measure with microcalipers, every 48 hours (top panel). Clinical disease scores were determined by assessing the severity of disease in each ankle and paw (4 limbs total) on a scale from 0-3, the sum of which is the clinical score (bottom panel). Data shown are mean ± SEM. *p<0.05 compared to *SHIP+/+* (wild type).

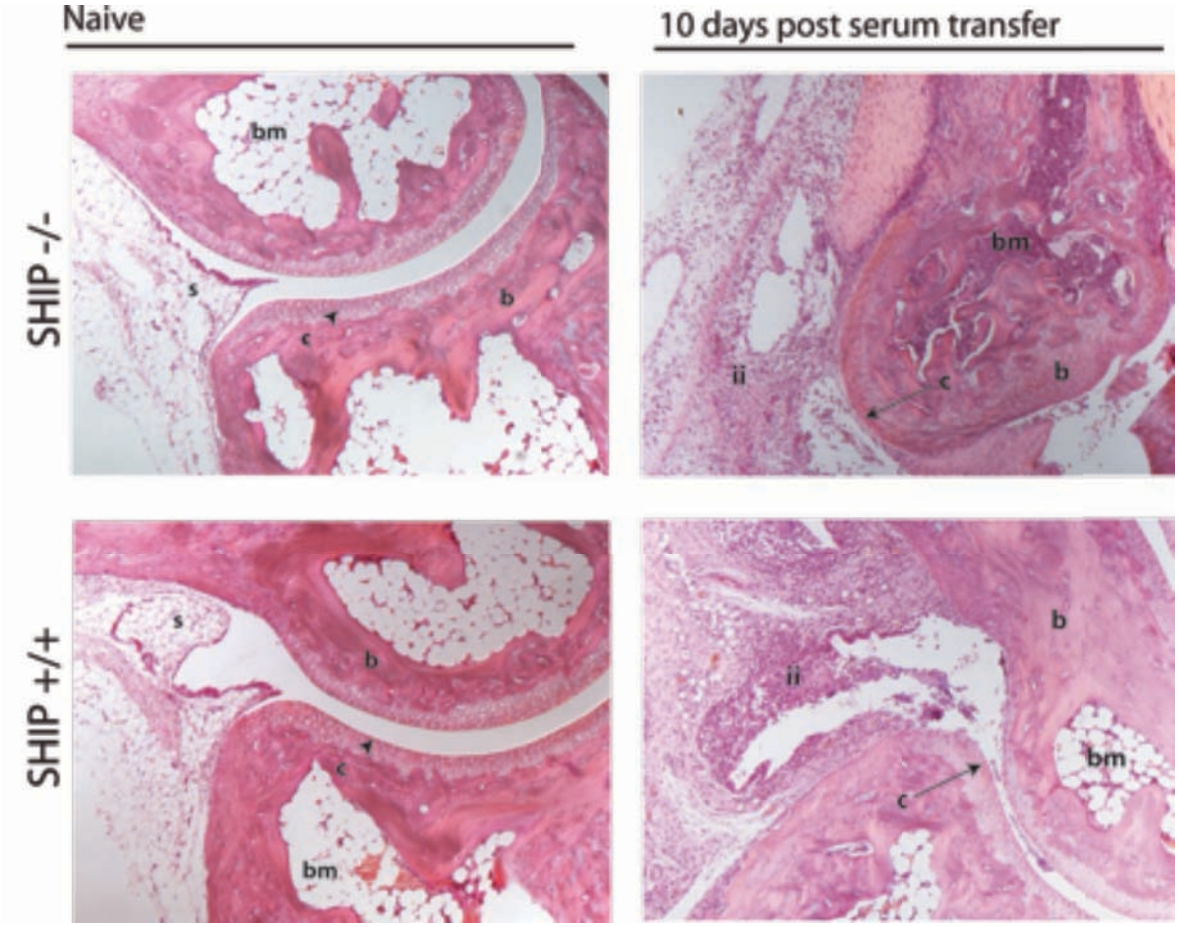


Figure 5. K/BxN serum transfer arthritis results in joint damage

Histological sections for H&E stained ankle joints from *SHIP-/-* and wild type mice. Ankle sections were taken 10 days after disease initiation (original magnification 10x). b, bone; bm, bone marrow; c, cartilage; ii, inflammatory infiltrate; s, synovium. Sections are representative of both ankles from 6 mice/genotype, for a total of 12 samples.

3.2 50% reduction in SHIP expression increases onset of arthritis and ankle swelling

SHIP heterozygous mice are considered to be phenotypically comparable to wild type controls. Unlike their wild-type counterparts, bone marrow progenitors from *SHIP+/-* mice are not hyper-responsive to cytokines such as GM-CSF, M-CSF and IL-3 (Helgason et al., 1998). However, significantly elevated populations of circulating polymorphonuclear cells (PMN) have been observed in mature *SHIP+/-* mice (Helgason et al.,1998). Assessment of bone marrow and the peripheral blood of heterozygous mice, confirmed that SHIP protein expression was indeed 50% reduced in PMNs of these tissues (Fig 6).

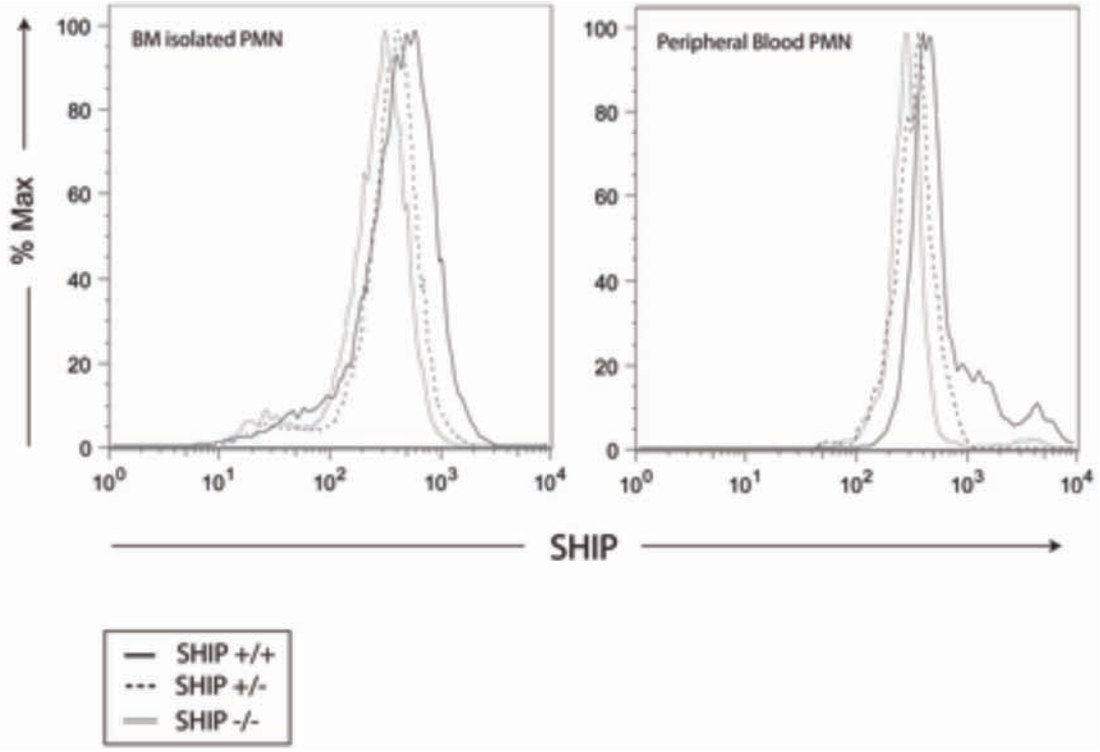


Figure 6. SHIP+/- PMN express 50% less protein than wild type PMN

SHIP+/+, +/- and *-/-* polymorphonuclear cells (PMN) were isolated from bone marrow via a discontinuous Percoll gradient. Blood PMN from saphenous vein bleeds were were gated ($CD45^+Gr\text{-}1^+7/4^+$) and intracellular expression of SHIP was determined by flow cytometry. Data are representative of 2 independent experiments (n=3 per genotype).

We then induced arthritis in *SHIP +/-* mice, concomitantly with *SHIP +/+* (wild type control) and *SHIP-/-* mice. Disease severity was so great in the ubiquitous knock-outs that they reached their humane end point and were sacrificed two days after disease induction (Figure 7). *SHIP+/-* mice developed a more severe disease phenotype than wild type mice, yet less severe than *SHIP-/-* mice, as evidenced by changes in ankle thickness and clinical scoring (Figure 7). Histological analysis after 2 days of disease further emphasized the significantly earlier onset of disease in *SHIP-/-* mice. Morphological assessment of inflammatory infiltrate and cartilage erosion releaved both were significantly higher in the ubiquitous knockouts, whereas wild type litter mates displayed very slight increases in scores for these parameters (Figure 8 A,B).

The exacerbated disease observed in the arthritic *SHIP-/-* mice likely reflects the elevated granulocyte-monocyte populations in the circulation of mature *SHIP-/-* mice which could result in an enhanced response to the anti-GPI immune complexes. Ubiquitous loss of SHIP results in an expansion of the myeloid compartment, which includes neutrophils and macrophages, and likely contribute to the observed disease phenotype. Since loss of SHIP has been observed to decrease the threshold for degranulation, hyper-responsive *SHIP-/-* mast cells likely also contribute to the exacerbated joint damage. While multiple factors could contribute to the disease phenotype, any attempt to discern the *in vivo* cell intrinsic effects of SHIP during the effector stages of autoimmune arthritis is likely to be obscured by the plethora of additional defects present in the ubiquitous knock-out mouse.

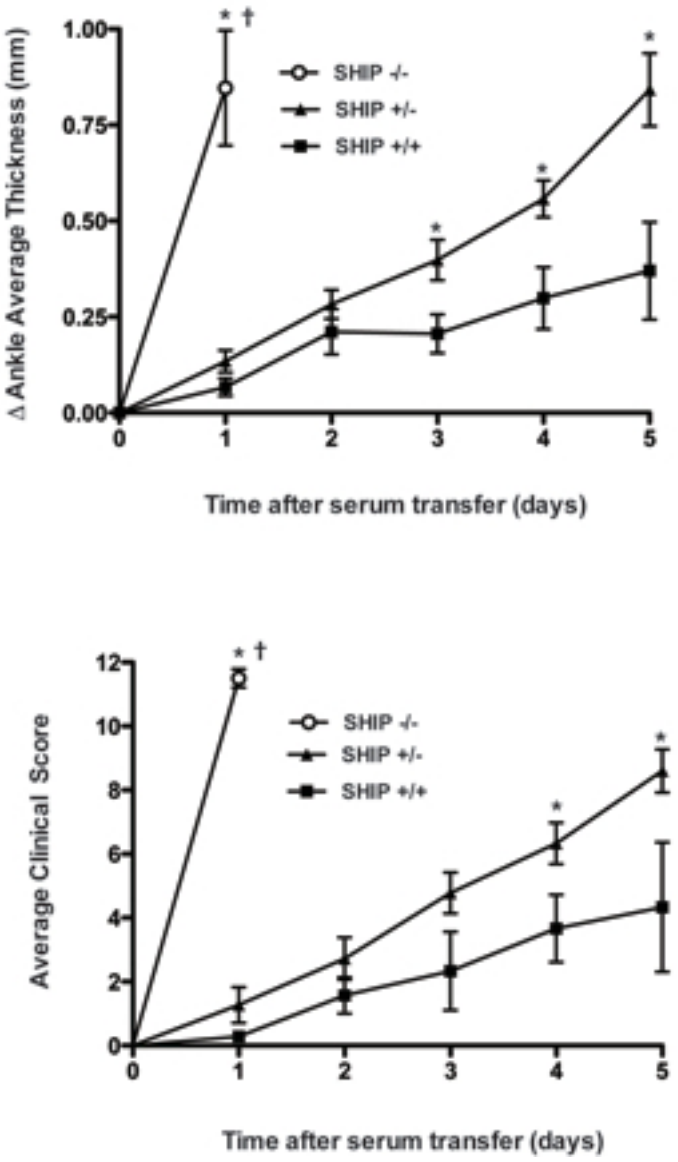


Figure 7. SHIP dampens K/BxN serum induced autoimmune arthritis

SHIP +/+, *+/-* and *-/-* mice (n=6 per genotype) were administered K/BxN serum i.p on day 0 and day 2. Changes in ankle thickness (upper panel) were measured at daily intervals with micro-calipers. Clinical disease scores were assessed (see Materials and Methods). †, *SHIP -/-* mice were euthanized on day 2, due to severe morbidity. n= 6 (*SHIP +/+,+/-*) n=3 (*SHIP -/-*). *= significantly different than *SHIP +/+* (wild type) ($p<0.05$). Data shown are mean ± SEM.

A.

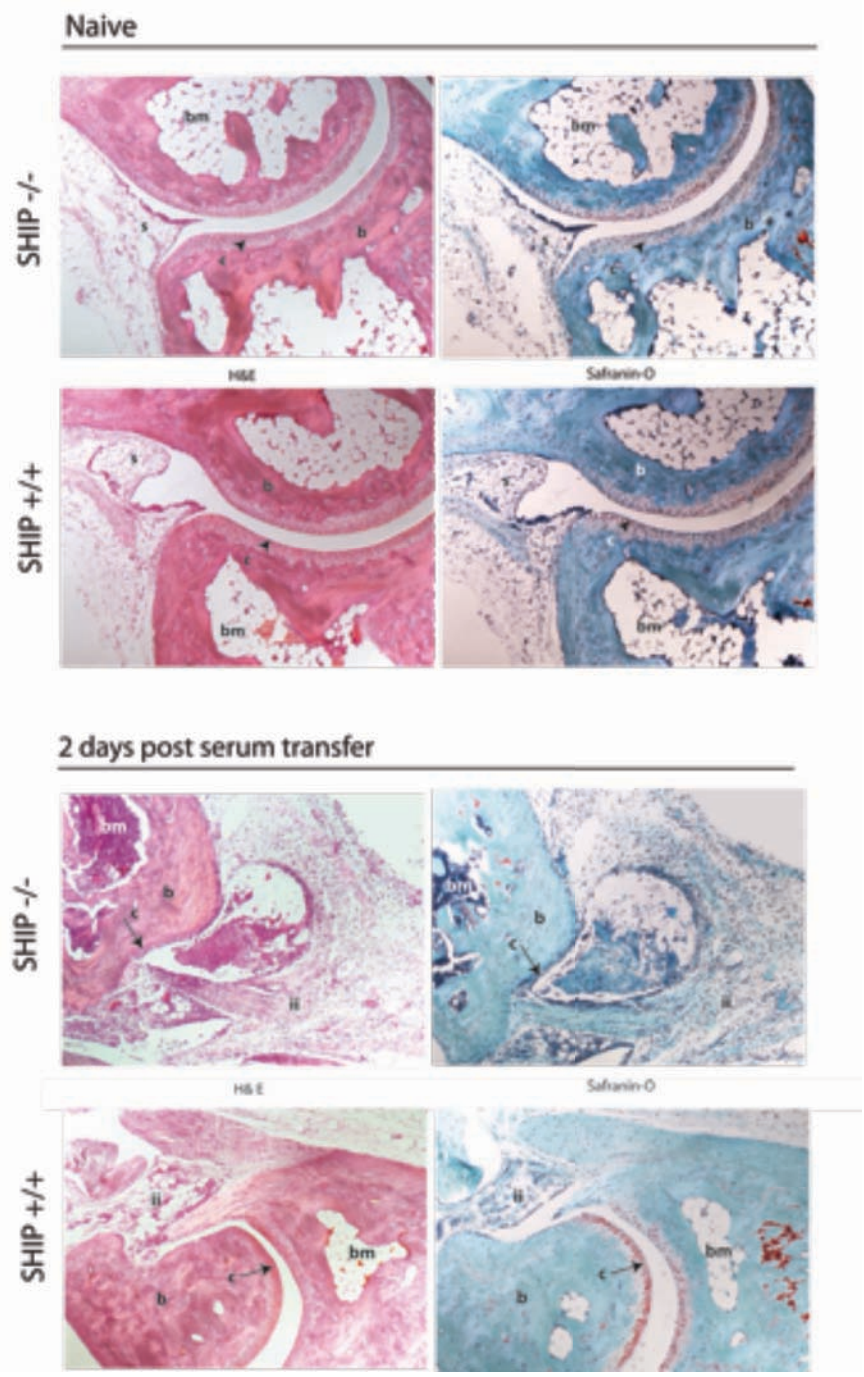


Figure 8. SHIP limits K/BxN serum induced autoimmune arthritis

A, Histological sections of ankle joints from *SHIP-/-* and wild type mice, stained with H&E or safranin-O. Ankle sections were taken 2 days after disease initiation (original magnification 10x). b, bone; bm, bone marrow; c, cartilage; ii, inflammatory infiltrate; s, synovium. n= 6 (*SHIP+/+*) n=3 (*SHIP-/-*).

B.

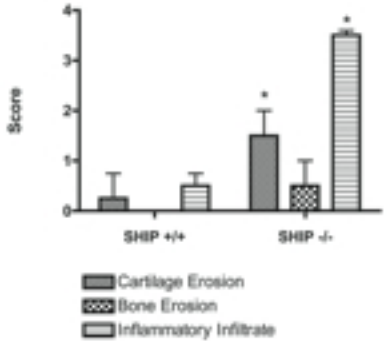


Figure 8. (*continued*) SHIP limits K/BxN serum induced autoimmune arthritis

B, Histological scores were based on evaluation of several parameters on a scale of 0 (no disease) to 4 (severe); inflammatory infiltrate, cartilage erosion and bone erosion. n= 6 (*SHIP+/+*) n=3 (*SHIP-/-*). *= significantly different than *SHIP+/+* (wild type) ($p<0.05$). Data shown are mean ± SEM.

3.3 Development of arthritis is not altered in mice with T or B cell specific deletion of SHIP

The K/BxN serum transfer model bypasses the need to develop endogenous autoantibodies and therefore is thought to be lymphocyte independent, so we hypothesized that loss of SHIP in T or B cell lineages would not affect disease. To test this, we induced disease in mice with T-cell specific (CD4cre)(Lee et al., 2001) and B-cell specific (CD19cre)(Sawada et al., 1994) deletion of SHIP. Changes in ankle thickness and clinical scores were not significantly different between knock-outs and wild type mice (Figure 9), thus confirming our hypothesis.

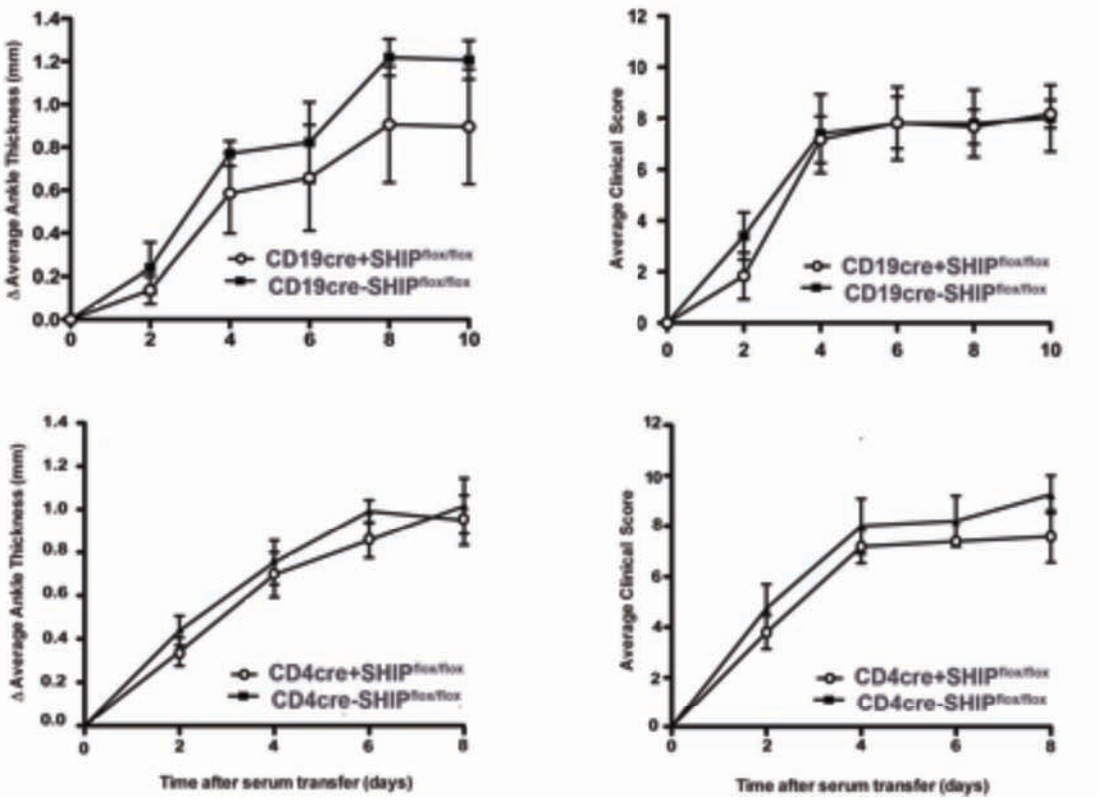


Figure 9. Conditional deletion of SHIP in T or B cells does not affect disease progression or outcome

Following induction of arthritis, changes in ankle thickness and clinical scores were evaluated every 48 hours, in *CD4cre-SHIP*$^{fl/fl}$ (wild type), *CD4cre+SHIP*$^{fl/fl}$ (*SHIP-/-*) (n=5/genotype) and *CD19cre-SHIP*$^{fl/fl}$ (wild type), *CD19cre+SHIP*$^{fl/fl}$ (*SHIP-/-*)(n=6/genotype). Data shown are mean ± SEM. *= significantly different than wild type controls ($p<0.05$).

3.4 Characterization of macrophages isolated from LysMcreSHIP mice

Next, we sought to explore how the loss of SHIP in the granulocyte/monocyte lineage cells would affect disease outcome. Therefore we generated a conditional SHIP knock out mouse, in which ablation of SHIP was restricted to macrophages and neutrophils. To this end, we employed the LysMcre deleter stain, which was generated by 'knocking in' cre recombinase to the endogenous lysozymeM loci (Clausen, et al., 1999). LysMcre deletes loxP flanked alleles in granulocyte and monocyte precursors with the ability to differentiate into macrophages or neutrophils; however, deletion has also been reported in a small percentage of dendritic cells (Clausen et al., 1999; Jakubzick et al., 2008).

In mice with ubiquitous loss of SHIP, resident peritoneal, alveolar and intestinal macrophages are skewed towards an alternately activated M2 phenotype. This M2 polarization has predominantly been attributed to the production of high levels of IL-4, by SHIP-/- basophils (Kuroda et al., 2009). However, a macrophage intrinsic contribution to an M2 phenotype has been hypothesized (Sly et al., 2007). We observed that peritoneal macrophages were indeed M2 polarized, in comparison to their wild-type litter mates. This was evidenced by a low IL-12/IL-10 ratio in response to LPS stimulation (Figure 10 D) and increased expression of the M2 marker Arg-1 (Figure 10 A, B, C). Interestingly, the expression of another M2 marker, Ym-1, which is high in macrophages from ubiquitous SHIP knock-out mice, was not altered in LysMcre+SHIP$^{fl/fl}$ macrophages (Figure 10 C), suggesting that other SHIP deficient lineages also contribute to the complete M2 polarization observed in the ubiquitous SHIP KO mice. Nevertheless, our results suggest that SHIP plays a macrophage intrinsic role in attenuating M2 polarization.

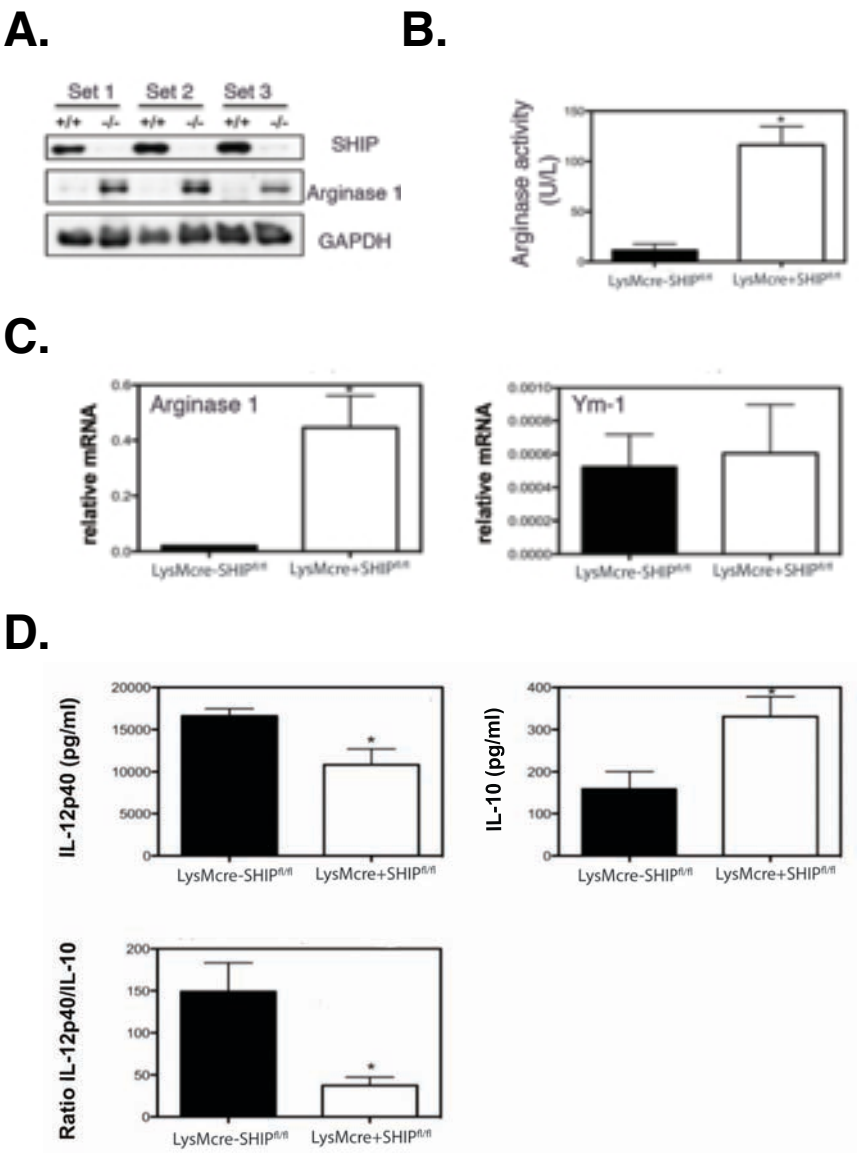


Figure 10. Peritoneal macrophages isolated from *LysMcre+SHIP$^{fl/fl}$* mice are M2 skewed

A, Peritoneal macrophages were isolated from *LysMcre-SHIP$^{fl/fl}$* (wild type) and *LysMcre+SHIP$^{fl/fl}$* mice. Western blot analysis of whole cells lysates showing expression of SHIP, arginase and GAPDH (loading control) of peritoneal macrophages harvested from separate mice (n=3 per genotype).
B, Arginase activity (n=3 per genotype).
C, Expression of M2-markers Arginase 1 and Ym1 (n=3 per genotype).
D, Peritoneal macrophages were stimulated with 100ng/ml LPS for 24 hours. Supernatants were harvested and analyzed for production of IL-12p40 and IL-10 by ELISA. A higher IL-12p40/IL-10 ratio is indicative of an M1 polarized phenotype, a lower ratio indicates M2-polarization. Error bars are the mean ± SEM (n=3). *= significantly different than wild type controls ($p<0.05$).

3.5 Loss of SHIP in the granulocyte-monocyte lineages results in exacerbated arthritis

Following administration of K/BxN arthrogenic serum, mice with conditional loss of SHIP in macrophages and neutrophils (LysMcre+SHIP$^{fl/fl}$) developed more severe arthritis than their wild type littermates (LysMcre-SHIP$^{fl/fl}$). Over a 10 day disease course, the change in ankle thickness and clinical score were significantly greater in SHIP conditional knock-out mice at day 2 and day 4 (Figure 11). Histological analysis of ankle sections 10 days post disease initiation revealed increased inflammatory infiltrate in the ankles of SHIP knock-out mice (Figure 12 A, B). Ankle lavages were also performed on day 10, and revealed a significantly greater percentage of neutrophils in the joint of the LysMcre+SHIP$^{fl/fl}$ mice.

The earlier appearance of increased ankle thickness and edema in the hind and front paws led us to refocus our analyses to the early phase of the disease. Additionally, in light of the disease phenotype observed in the heterozygous SHIP mice, we explored whether heterozygous SHIP expression in macrophages and neutrophils (LysMcre +SHIP$^{fl/+}$) would exacerbate disease. Interestingly, we observed that heterozygous SHIP expression was sufficient to increase disease severity, although not as significantly as total loss of SHIP (Figure 13). Histological analysis of ankle joints 4 days after serum transfer, however, only revealed a significant increases in inflammatory infiltrate of mice with a complete loss of SHIP (Figure 14 A,B,C). Ankle lavages of LysMcre+SHIP$^{fl/fl}$ mice at 4 days following disease induction indicated that the majority of hematopoietic cells in the ankle joints of arthritic mice were neutrophils (Figure 14 D).

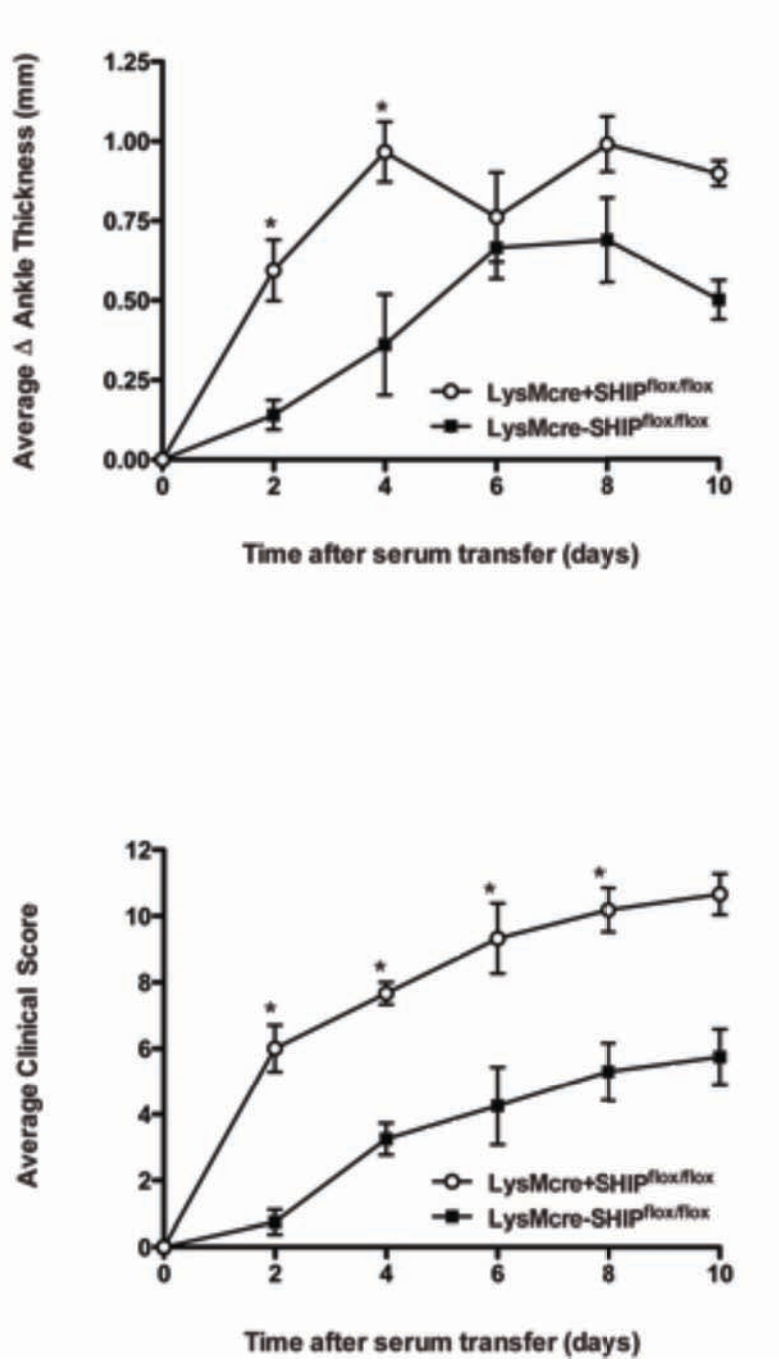


Figure 11. Neutrophil/macrophage specific (*LysMcre*) deletion of SHIP exacerbates K/BxN serum induced arthritis

Changes in ankle thickness (upper panel) were measured at daily intervals with micro-calipers. Clinical disease scores were assessed by determining the severity of disease in each ankle and paw (4 limbs total) on a scale of 0 to 3. The sum of of these scores constituted the average clinical score (lower panel). n=6/ genotype. *= significantly different than *LysMcre-SHIP*$^{fl/fl}$ (wild type) ($p<0.05$). Data shown are mean ± SEM.

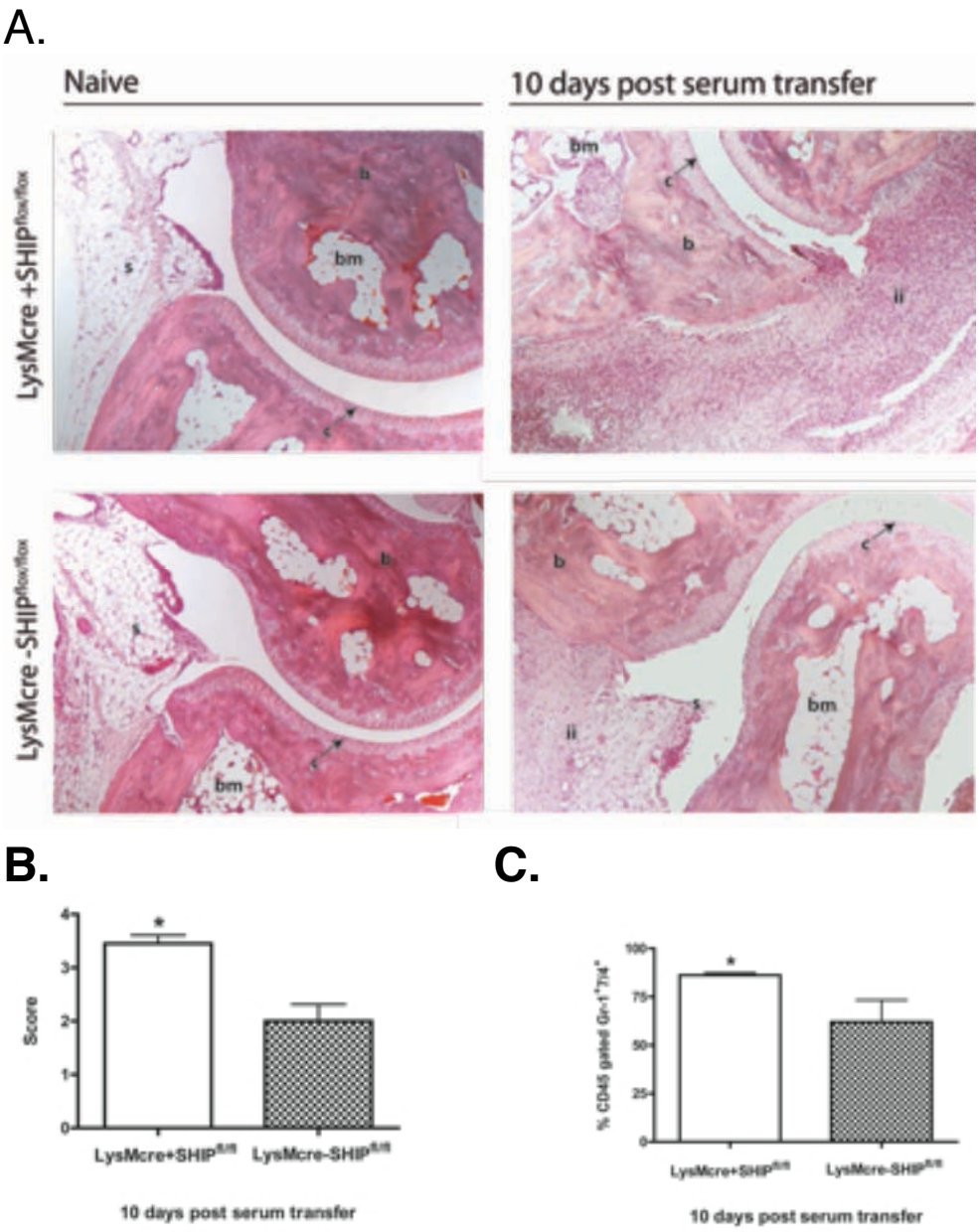


Figure 12. *LysMcre+SHIP$^{fl/fl}$* mice exhibit more severe joint damage 10 days following disease induction

A, Histological sections for H&E stained ankle joints from *SHIP-/-* and wild type mice harvested 10 days after disease initiation (original magnification 10x). b, bone; bm, bone marrow; c, cartilage; ii, inflammatory infiltrate; s, synovium.
B, Histological scoring was attributed through evaluation of inflammatory infiltrate in each of the sections.
C, Ankle lavages were performed 10 days post serum transfer. Cells were stained for CD45/Gr-1/7/4 to identify neutrophils. *= significantly different than *LysMcre-SHIP$^{fl/fl}$* (wild type)($p<0.05$). Data shown are mean ± SEM. (n=6 per genotype.

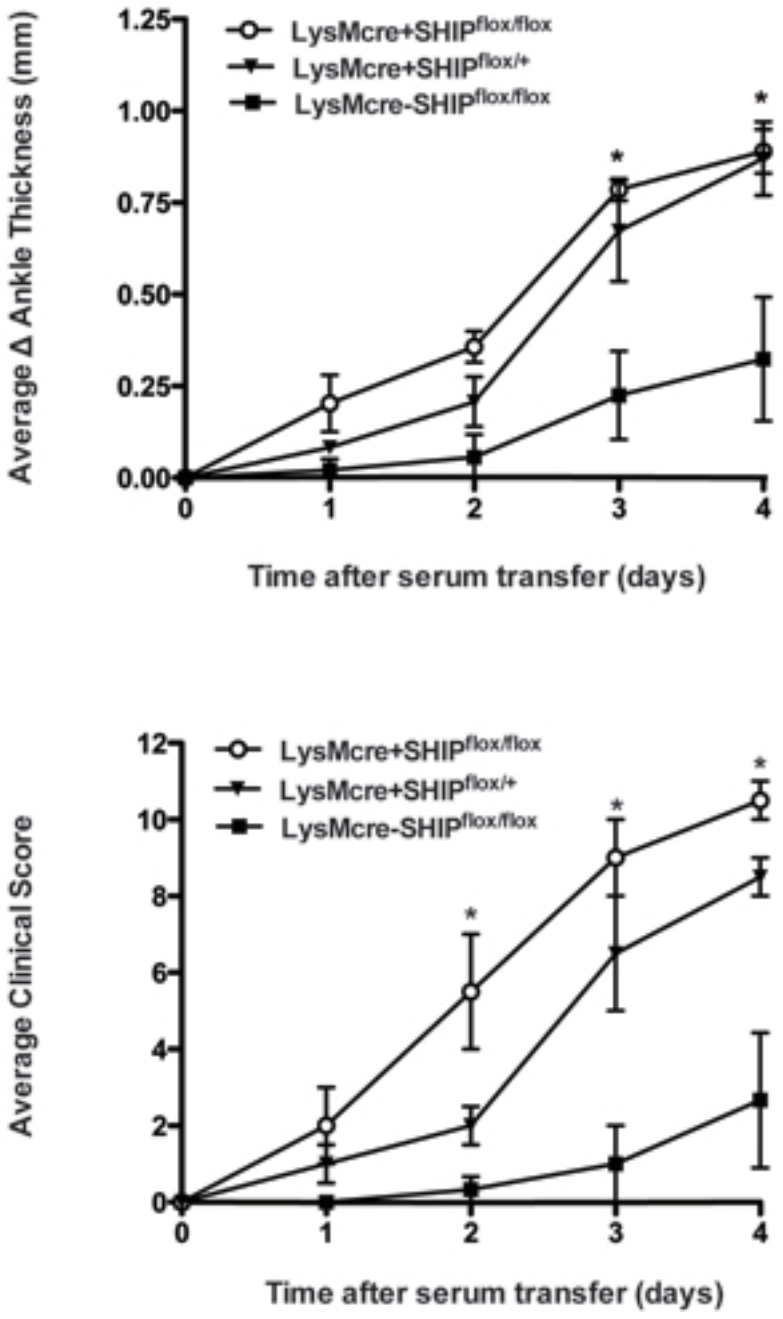


Figure 13. PMN and macrophage specific deletion of SHIP exacerbates K/BxN arthritis

LysMcre-SHIP$^{fl/fl}$ (n=4), *Lyscre+SHIP*$^{fl/+}$ (n=3) and *LysMcre+SHIP*$^{fl/fl}$ (n=5) mice were administered K/BxN serum i.p. on days 0 & 2. Clinical disease scores (bottom panel) and change in ankle thickness (top panel) measured by micro calipers. *= significantly different than wild type *LysMcre-SHIP*$^{fl/fl}$ (wild type) ($p<0.05$). Data shown are mean ± SEM.

A.

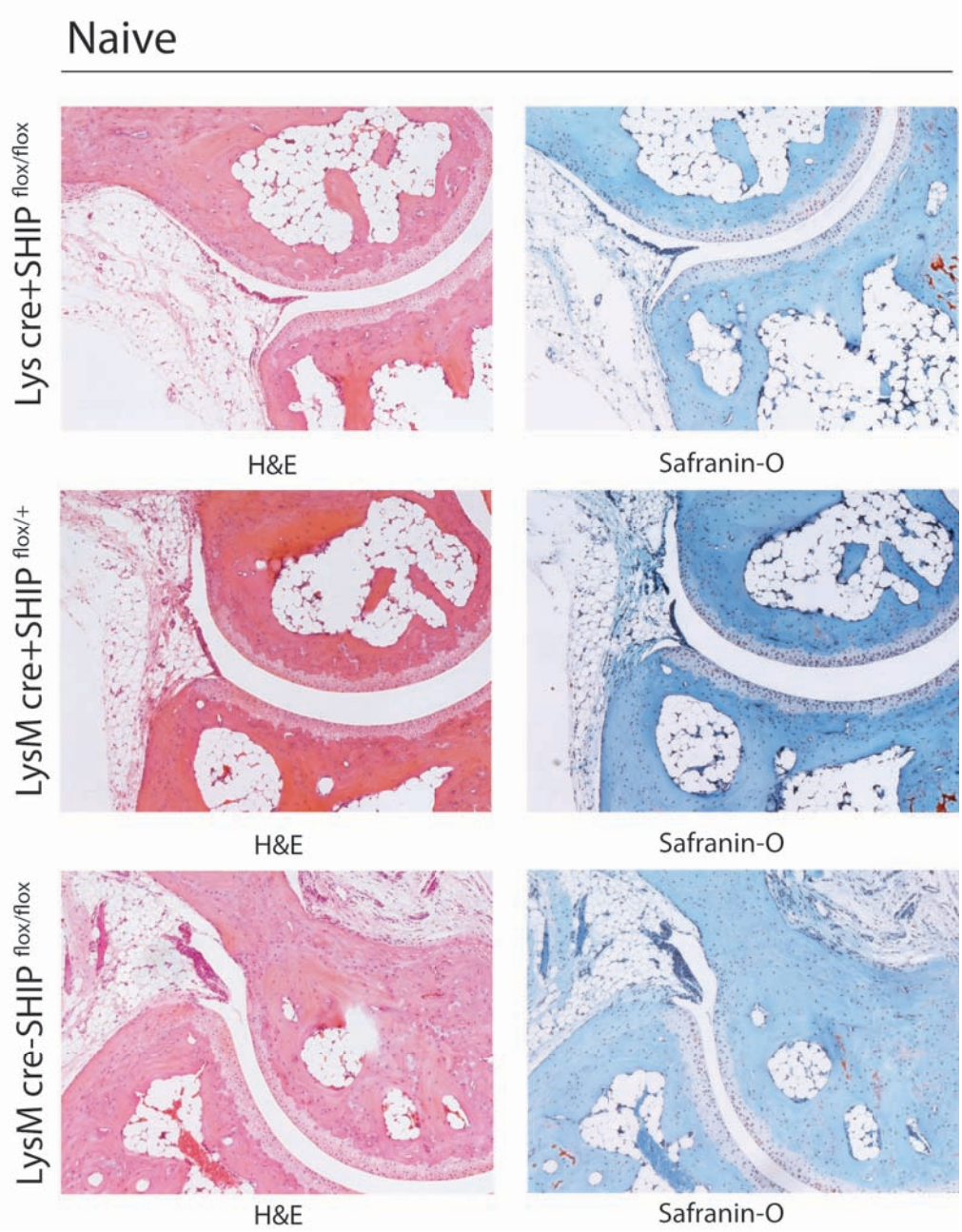


Figure 14. Analysis of the joint 5 days post serum transfer confirms an aggravated disease phenotype in *LysMcre+SHIP$^{fl/fl}$* mice

A, Histological sections of naive H&E and safranin-O stained joints from each genotype. n=4 per genotype.

B.

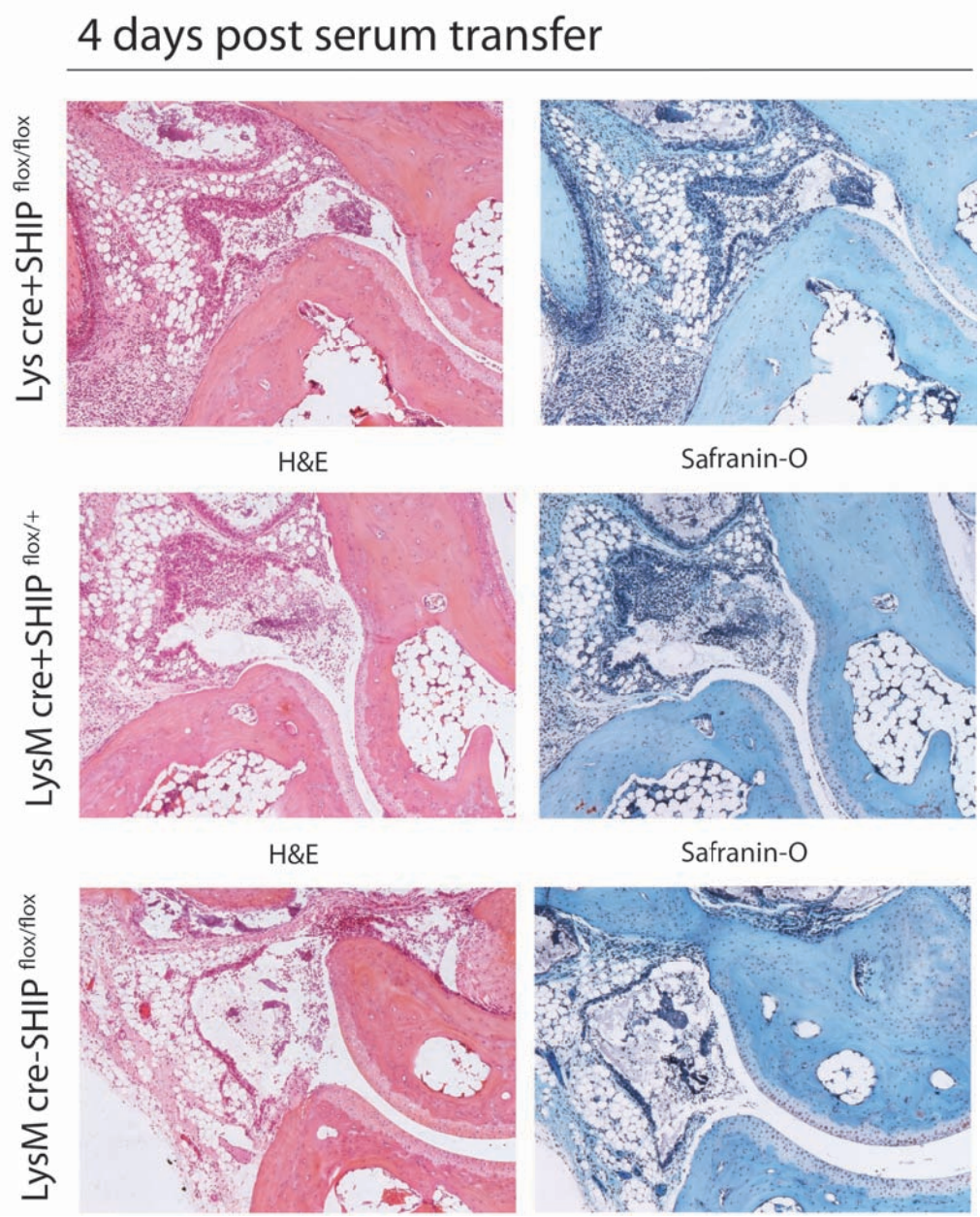
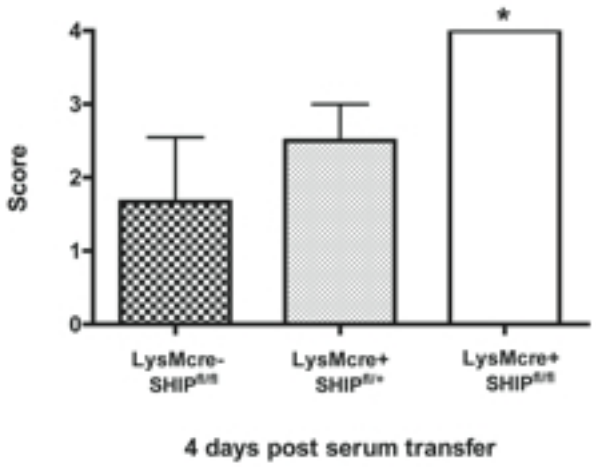


Figure 14. (*continued*) Analysis of the joint 5 days post serum transfer confirms an aggravated disease phenotype in *LysMcre+SHIP*$^{fl/fl}$ mice

B, Histological sections of joints from K/BxN serum injected *LysMcre-SHIP*$^{fl/fl}$ and *LysMcre+SHIP*$^{fl/fl}$ harvested 4 days post serum transfer and stained H&E and Safranin-O stained. n=4 per genotype.

C.



D.

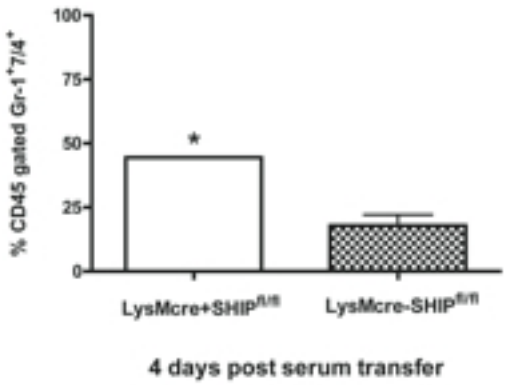


Figure 14. (*continued*) Aggravated disease phenotype in *LysMcre +SHIP$^{fl/fl}$* mice 5 days post serum transfer

C, Histological scoring of inflammatory infiltrate 4 days post serum transfer **D**, Ankle lavages were performed 4 days following disease induction, and the number neutrophils present was assessed, as defined as % of hematopoietic cells (CD45$^+$ gated) which were Gr-1$^+$ and 7/4$^+$.Data are mean ± SEM. *Significantly different than *LysMcre-SHIP$^{fl/fl}$* (wild type)($p<0.05$). n=4 per genotype.

3.6 Conditional deletion of SHIP in neutrophils results in earlier disease onset

I then explored the neutrophil intrinsic role of SHIP during the effector phase of autoimmune arthritis. Our decision to focus on neutrophils was based on several factors. First, although the PI3K pathway is integral to numerous neutrophil functions (reviewed in (Hawkins et al., 2010)), very little is known about the role of SHIP in these activities. Secondly, while the effector phase is mediated by various cell types, neutrophils have systematically been associated with the development of disease in various arthritis models and experimental approaches (Kim et al., 2006; Lawlor et al., 2004; Nandakumar & Svensson, 2003; Tanaka et al., 2006; Wipke & Allen, 2001). Finally, I had access to a neutrophil specific conditional knock out strain, *GE-cre*, which had already been crossed to mice with floxed SHIP alleles (*SHIP$^{fl/fl}$)* by our collaborators. Cre expression in the *GEcre* strain is driven by the neutrophil elastase (*Elane*) promoter, *GEcreSHIP$^{fl/fl}$* mice are reported to be phenotypically normal and viable (Dr. Paul Kubes and Dr. Bjorn Petri, personal communication).

I observed that neutrophil restricted deletion of SHIP in mice (*GEcre+SHIP$^{fl/fl}$*) was sufficient to significantly increase clinical symptoms and ankle thickness 48 hours after disease induction (Figure 15). 72 hours post serum injection, ankle swelling was also visible in the region of the ankle joint of the wild type mice (*GEcre-SHIP$^{fl/fl}$*) (Figure 16A). In addition to pronounced swelling and acute inflammation (demarcated by enhanced redness) in this region, the conditional knockouts also presented with visible edema on the dorsum of the hind paw (Figure 16A). Histological analysis of the synovial joint of *GEcre+SHIP$^{fl/fl}$* mice confirmed the presence of inflammatory infiltrates in the synovium 72 hours post serum transfer (Figure 16B). Ankle lavages at 72 hours confirmed a greater percentage of the hematopoietic cells in the joints of *GEcre+SHIP$^{fl/fl}$* mice were neutrophils. Initial infiltration of circulating neutrophils into the synovial joint is reported to occur early during disease progression (within 24-48 hours) after serum transfer (Wipke & Allen, 2001). Thus, our initial studies focused on events occurring during the earliest stages of diseases. Cartilage and bone damage are typically indiscernible before one week of disease, therefore I only evaluated inflammatory infiltrate.

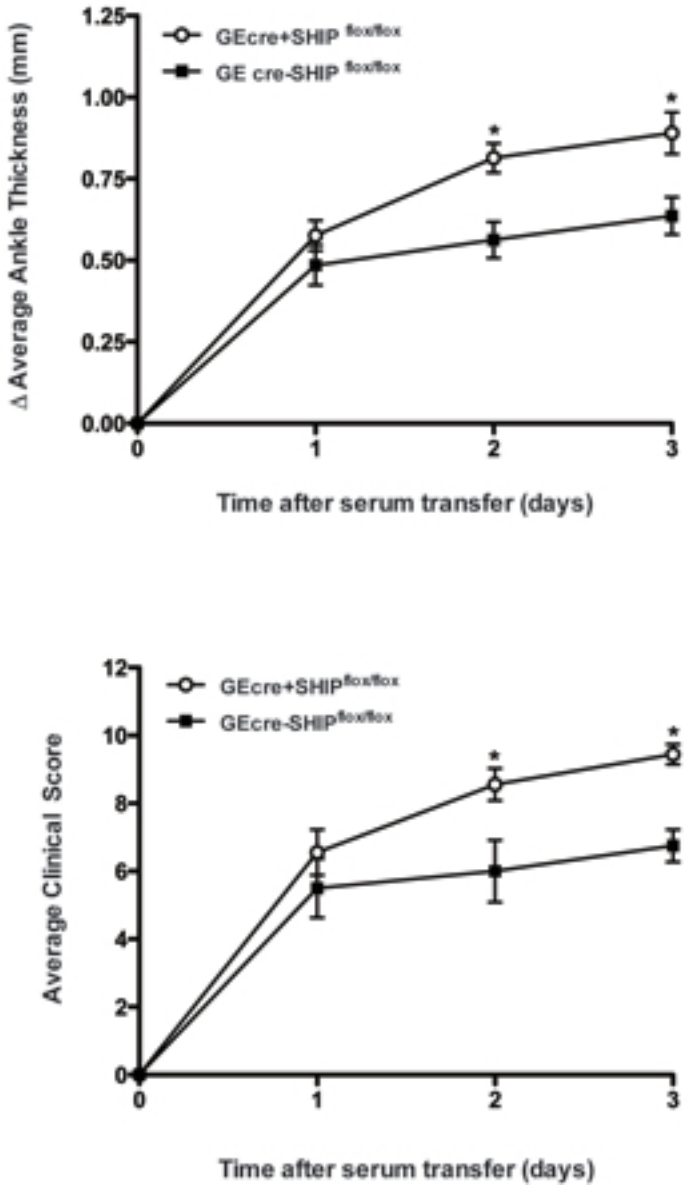


Figure 15. Neutrophil specific deletion (*GEcre*) is sufficient to exacerbate K/BxN serum induced arthritis

GEcre+SHIP$^{fl/fl}$ (n=9) and *GEcre-SHIP*$^{fl/fl}$ (n=4) mice were administered K/BxN serum i.p on day 0. Average change in ankle thickness was measured with micro calipers, every 24 hours (top panel). Clinical disease scores were determined by assessing the severity of disease in each ankle and paw (as outlined in materials and methods). Data are mean ± SEM. *= denotes significantly different than *GEcre-SHIP*$^{fl/fl}$ (wild type) ($p<0.05$).

A.

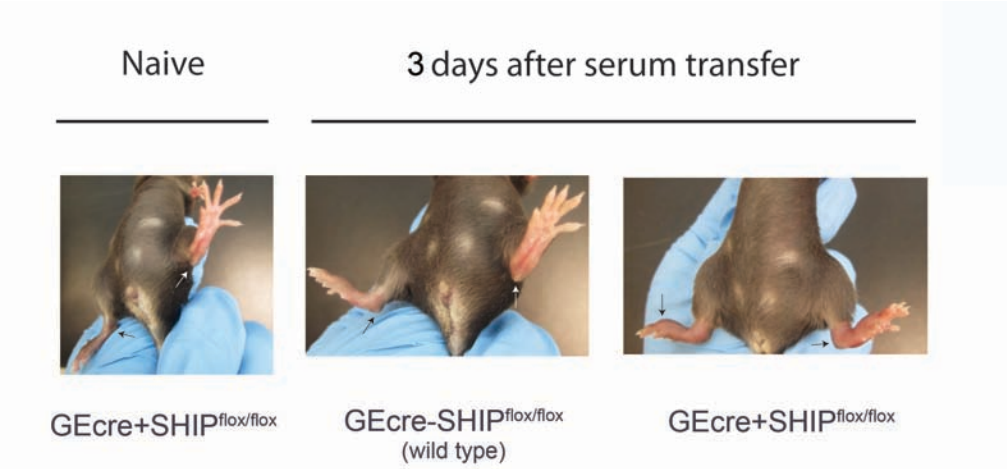


Figure 16. Analysis of the joints of *GEcre* mice exhibit more severe pathology 3 days post disease induction

A, Photographs of hind limbs of mice 3 days post serum transfer. Arrows indicate regions prone to swelling. Pictures are representative of 5-9 mice/ genotype.

B.

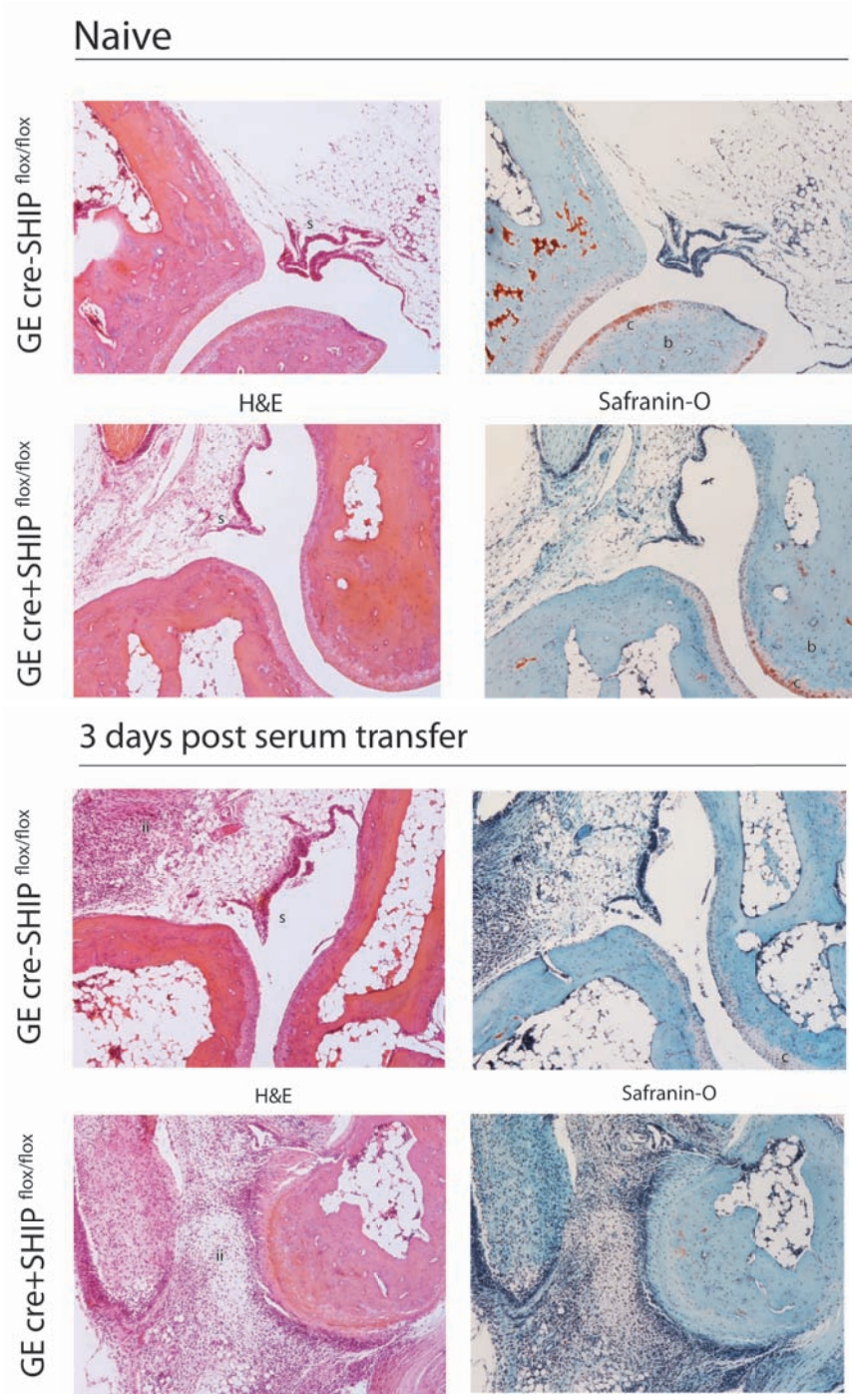
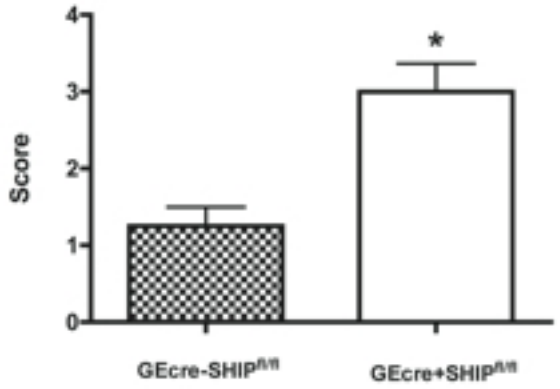


Figure 16 *(continued)*. Analysis of the joints of *GEcre* mice exhibit more severe pathology 3 days post disease induction

B, Ankles were harvested 3 days after disease induction and prepared for H&E and safranin-O staining. 10x magnification. ii=inflammatory infiltrate, s= synovium, c=cartilage. Pictures are representative of 5-9 mice/genotype.

C.



D.

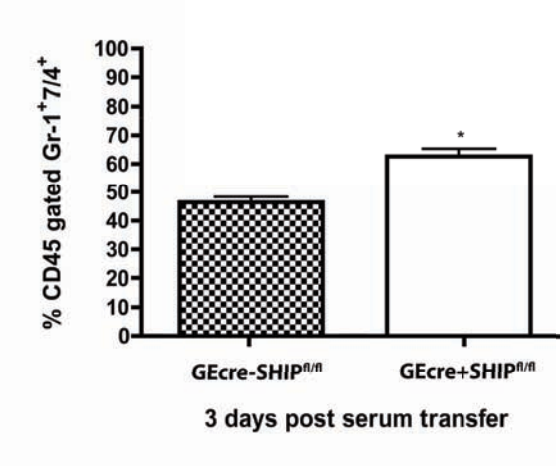


Figure 16 *(continued)*. Analysis of the joints of *GEcre* mice exhibit more severe pathology 3 days post disease induction

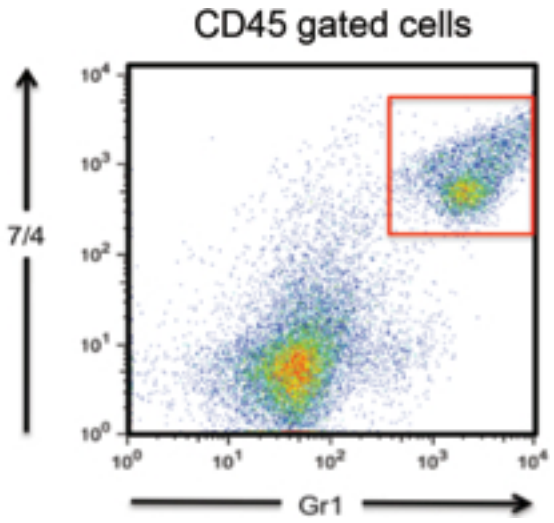
C, Histological samples were blind scored, based on degree of inflammatory infiltration on a scale of 0 (naive) to 3.
D, Ankle lavages were performed on the left ankle, and the number neutrophils present was assessed, as defined as % of hematopoietic cells ($CD45^+$ gated) which were $Gr\text{-}1^+$ and $7/4^+$. Data are mean ± SEM.
*Significantly different than *GEcre-SHIP$^{fl/fl}$* (wild type)($p<0.05$).

3.7 Neutrophil specific deletion of SHIP does not affect circulating PMN levels

Loss of SHIP in neutrophils alone was sufficient to induce exacerbated arthritis. This indicated that ablation of SHIP resulted in neutrophil intrinsic defects that were responsible for the disease phenotype. Since increased levels of neutrophils have been observed in the peripheral blood of *SHIP+/-* and *SHIP-/-* mice (Helgason et al., 1998), I hypothesized that neutrophils might constitute a larger pool of circulating cells in *GEcre +SHIP$^{fl/fl}$* than in wild type mice, which would lead to increased effector cells infiltrating the synovial joint during disease, and increased inflammatory infiltrate and ankle swelling. However, naive *GEcre+SHIP$^{fl/fl}$* mice did not display significantly different levels of circulating neutrophils than wild type controls (Figure 17).

We also explored whether SHIP loss affected bone marrow PMN numbers, and expression of surface markers. Employing a discontinuous Percoll gradient, our isolation protocol yielded similar numbers of neutrophils across all genotypes during each experiment, indicating that loss of SHIP did not impart a quantitative change in neutrophil numbers. Surface expression levels of hematopoietic marker CD45, or neutrophil markers Gr-1, and 7/4 was not altered by loss of SHIP in unstimulated PMN (data not shown).

A.



B.

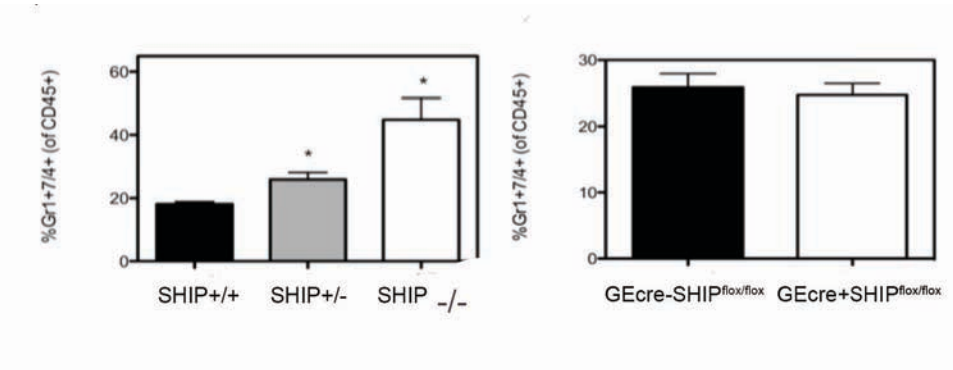


Figure 17. Elevated circulating neutrophil levels in *SHIP-/-* and *SHIP+/-* mice

A, Levels of circulating neutrophils were assessed by flow cytometry. Gating scheme for neutrophils were based on gating for CD45+ positive Gr-1$^+$/7/4$^+$ cells.
B, Peripheral blood from 10-12 week old *SHIP+/+, +/-* and *-/-* mice (n=6) and 14 week old *GEcreSHIP$^{fl/fl}$* mice (n= 10 (cre+), 5 (cre-)) was assessed for neutrophils. Data are mean ± SEM. *= significantly different than *GEcre-SHIP$^{fl/fl}$* mice (wild type)($p<0.05$).

3.8 Loss of SHIP affects pathways downstream of FcγR activation

During the early stages of rheumatoid arthritis, neutrophils are activated by IgG immune complexes (IC) deposited on the surface of the distal joints. These IC stimulate murine neutrophils via low-affinity activating Fc receptors FcγRIIIA and FcγRIV, (Jakus et al., 2008) thus contributing to the earliest stages of the inflammatory cascade. While different immune cell types express different members of the FcγR family, both FcγRIIIA and FcγRIV are expressed on murine neutrophils. To explore whether SHIP plays a neutrophil intrinsic role in regulating FcγR signaling, we first determined if cell surface expression of this family of receptors is altered. Basal levels of FcγRIII and II were not affected by loss of SHIP, as evidenced by cell surface staining of unstimulated BM isolated neutrophils with a common antibody against FcγRIII and FcγRII in (Figure 19). Unfortunately, an antibody against FcγRVI is not commercially available, so we could not assess the basal level of FcγRVI in *SHIP-/-* PMN.

To determine how activation of FcγR might be affected by loss of SHIP, we utilized an *in vitro* assay to mimic the interaction of isolated neutrophils with immobile OVA anti-OVA immune complexes (IC). *SHIP-/-* or wild type PMN were incubated with immobilized IC for various periods of time. Phosphorylation of Erk1/2 and Akt were then evaluated and found to be increased in the *SHIP-/-* PMN (Figure 20).

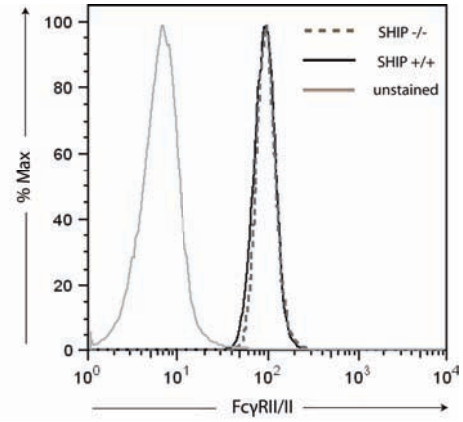


Figure 18. Equal levels of FcγRIII/II in SHIP-/- and WT naive mice

Expression of FcγRIII/II (CD16/32) in unstimulated bone marrow PMN was assessed via flow cytometry for *SHIP-/-* and wild type genotypes. The figure is representative of three independent experiments with similar results.

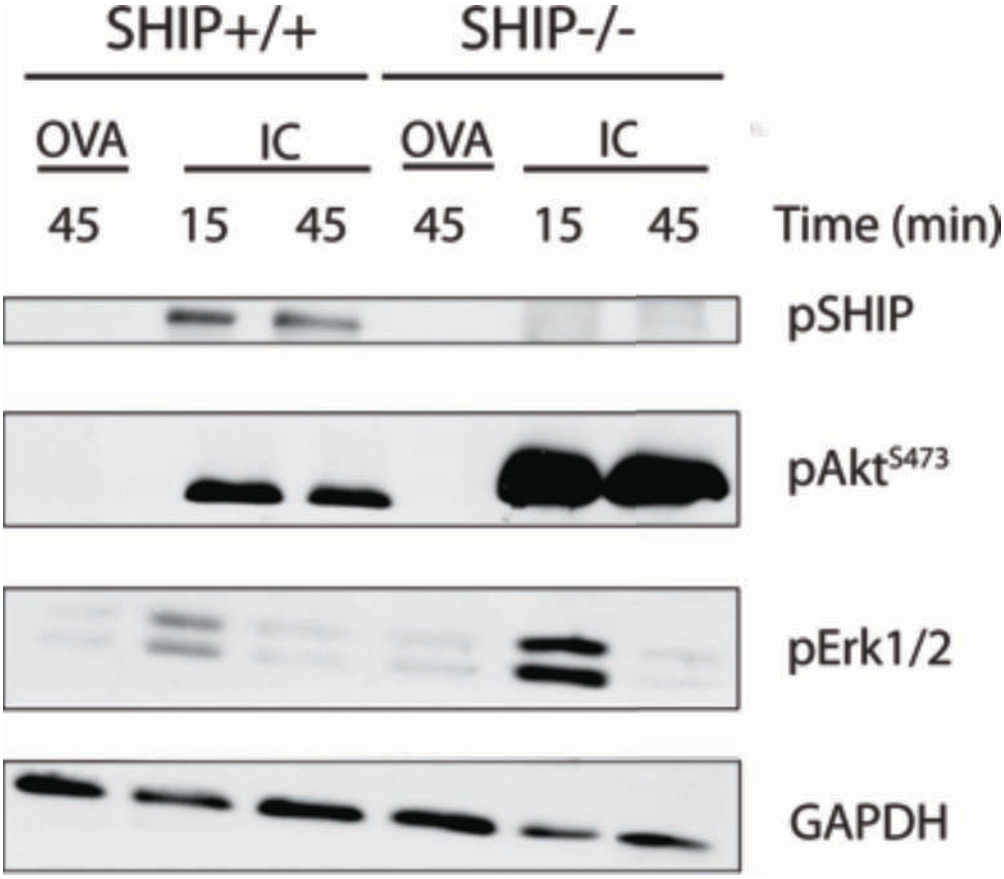


Figure 19. Loss of SHIP enhances activation of Akt and Erk following immune complex stimulation of BM isolated PMN

2 x 10^6 bone marrow isolated PMN from *SHIP-/-* or *SHIP+/+* mice (2 mice per genotype) were resuspended in HBSS (with calcium and magnesium) and plated on immobilized OVA anti-OVA immune complexes for 15 or 45 minutes, or immobilized OVA for 45 minutes (negative control) at 37°C. Non adherent cells were removed and adherent cells were lysed with 1x Laemeli sample buffer. Whole cell lysates (1 x 10^6 cells/lane) were fractionated by SDS-PAGE (10%) and transferred to PVDF membrane. Membranes were probed with phospho-specific antibodies to detect phosphorylated SHIP, Akt (S473) and Erk1/2. Membranes were also probed for GAPDH as a loading control.

3.9 Loss of SHIP affects signaling pathways downstream of C5a and LTB_4

While the interaction between IC and neutrophils plays an important role in the earliest stages of arthritis induction, several soluble factors are also critical to this process, such as the chemoattractants C5a and LTB_4. The importance of C5a interacting with C5a receptor present on the surface of neutrophils has been well documented (Grant et al., 2002, illustrated in Figure 3). Recently, accumulation of LTB_4 and surface expression of the LTB_4 receptor on neutrophils has also been shown to be necessary for the production of the pro-inflammatory cytokine IL-1, and its delivery to the joint (Chou et al., 2010; Kim et al., 2006). Both LTB_4 and C5a bind to G protein coupled receptors (GPCR), and since ligation of GPCR has been known to recruit SHIP to the plasma membrane, we hypothesized that loss of SHIP would affect signaling pathways downstream of PMN stimulation with C5a and LTB_4. We confirmed that SHIP was activated in wild type *SHIP+/+* PMN as evidenced by phosphorylation of *SHIP +/+*, but not in *SHIP-/-* PMN (Figure 21). We observed phosphorylation of Erk1/2 and Akt after one minute in both genotypes, but the level of phosphorylation after 15 minutes was greater in *SHIP-/-* PMN.

Based on the altered signaling we observed in *SHIP-/-* neutrophils, we explored the effect of PMN stimulation upon biological functions associated with neutrophil activation. We elected assessed neutrophil degranulation by observing the release of secondary granules (also known as specific granules) myleoperoxidase (MPO) and alkaline phosphatase following stimulation with C5a. We found that loss of SHIP resulted in significantly greater release of these secondary granules (Figure 22) which, in turn, is a potential explanation for the exacerbated disease severity we observed in the *GEcre+SHIP$^{fl/fl}$* mice.

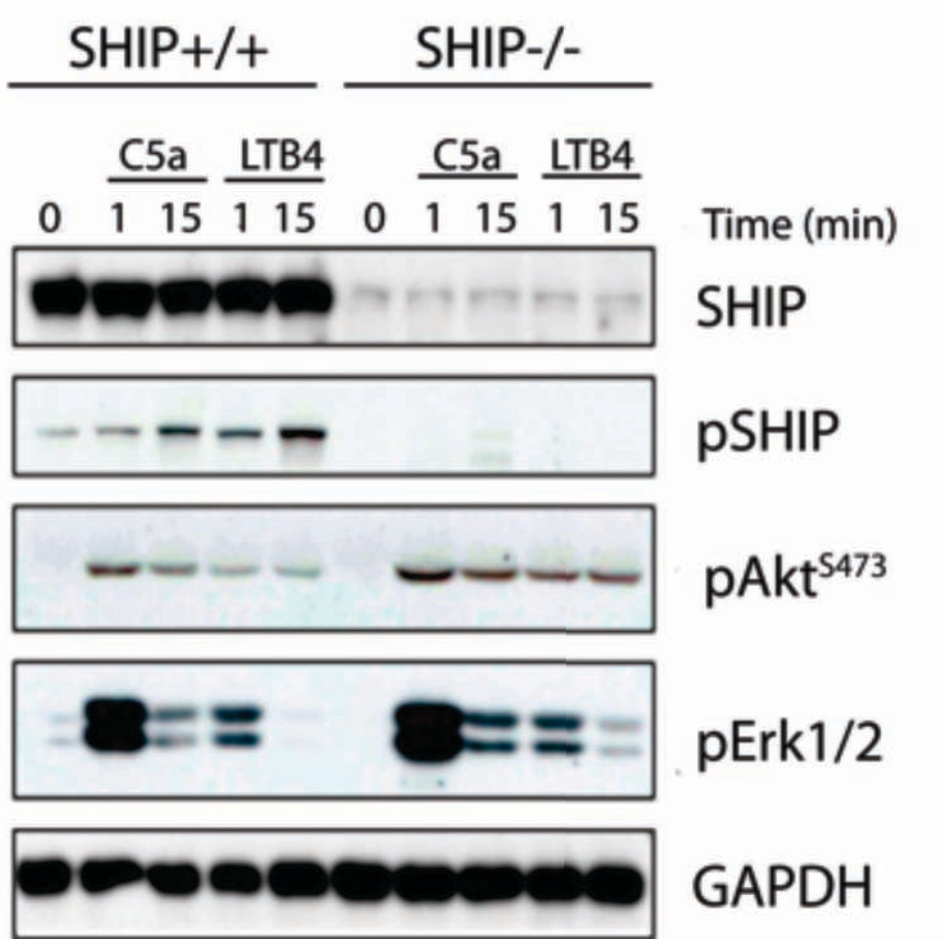
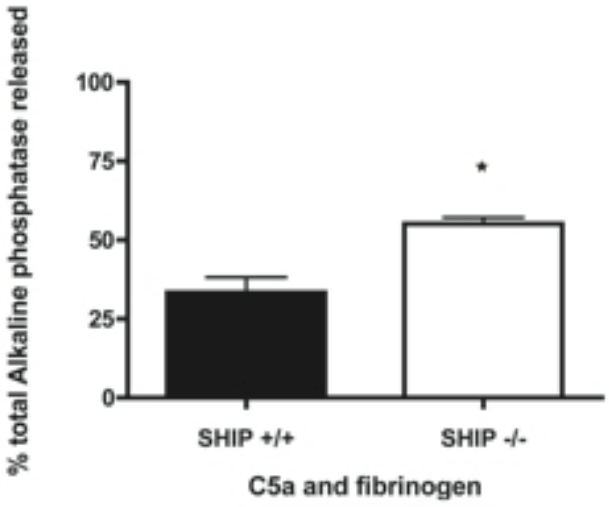


Figure 20. Akt activation is enhanced in *SHIP-/-* PMNs stimulated with C5a or LTB_4

Freshly isolated BM PMN from *SHIP+/+* and *SHIP-/-* mice (3 mice per genotype) were resuspended in RPMI+0.1% BSA at a concentration of $10x10^6$ cells/ml. Aliquots (100 uL) were stimulated in the presence or absence of 100 nM C5a or 100 nM LTB_4 for the indicated times (0, 1 or 10 min). Stimulations were stopped by the addition of 4 times Laemmli sample buffer. Whole cell lysates ($2.5x10^5$ cells/lane) were fractionated by SDS-PAGE (10%) and transferred to PVDF membrane. Membranes were probed with phospho-specific antibodies to detect phosphorylated SHIP, Akt (S473) and Erk1/2. Membranes were also probed for total SHIP and GAPDH as a loading control.

A.



B.

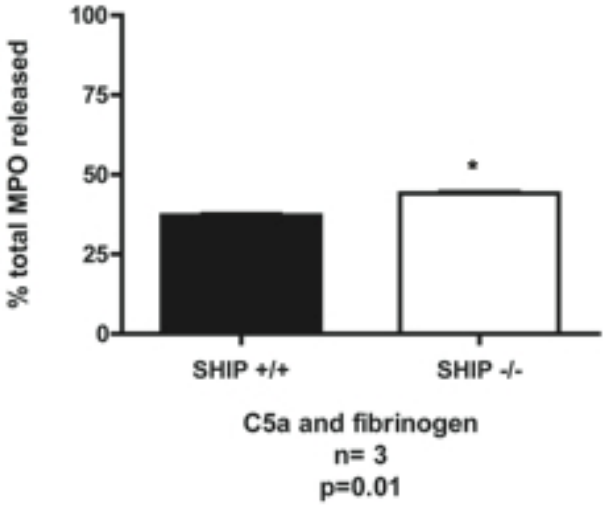


Figure 21. Release of enzymes following PMN stimulation is increased in *SHIP-/-* PMN

A, Freshly isolated BM PMN were plated on surfaces coated with fibrinogen, and incubated with 100nM C5a for 45 minutes. Alkaline phosphatase activity was then assessed by determining the percentage of enzyme in the supernatant versus the sum of enzyme released and the enzyme retained in the cell, following PMN stimulation.
B, MPO activity was also evaluated following PMN stimulation. Data are mean ± SEM. *Significantly different than *SHIP+/+* mice (wild type)($p<0.05$). n=3 per genotype.

4. Discussion and Conclusion

4.1 Disease manifestation in the ubiquitous SHIP mice and LysMcre

The aim of this thesis was to explore the role of SHIP in the effector phase of autoimmune arthritis. To this end, we utilized a genetic approach, employing several conditional SHIP knock-out lines in combination with the K/BxN serum transfer model, in order to parse the cell type specific requirement for SHIP in the development of autoimmune inflammatory disease.

While the exacerbated disease phenotype observed in the homozygous SHIP mice was predictable, we also observed that a 50% loss of SHIP expression was sufficient to increase disease severity. Biologically relevant *SHIP+/-* phenotypes, both i*n vivo* or *in vitro*, have been rarely reported. *SHIP+/-* mice challenged with T cell independent antigens were shown to exhibit intermediary enhanced responsiveness, as evidenced by elevated serum IgG levels (Helgason et al., 2000), while the ability of *SHIP +/-* mast cells to release total granule contents following stimulation, is higher than wild type controls (Huber et al., 1998). Thus, the disease manifestation we observed in arthritic heterozygous SHIP mice was unexpected. While I did not explore this finding further, we hypothesize that this may be due to increased circulating PMN levels. Interestingly, we initially hypothesized that the exacerbated phenotype observed in the neutrophil specific SHIP knock out mice might also be due to increased circulating neutrophil frequency. However, we observed that there was no significant difference between in the neutrophil population within the peripheral blood of mice with neutrophil restricted ablation of SHIP versus wild type controls. This suggests that modulation of neutrophil populations, perhaps via apoptosis or survival, is mediated by neutrophil extrinsic effects in *SHIP -/-* and *SHIP+/-* mice. To determine, *in vivo*, the role of SHIP in PMN, the half life of circulating *SHIP-/-* PMN could be elucidated with BrdU 'pulse chase' experiments. In this method, mice are administered BrdU to label cycling cells. Analysis of cycling mature $BrdU^{bright}Gr1+$ and $BrdU^{dim}$ Gr1+ immature granulocytes via flow cytometry can be used to measure the size of these populations in the bone marrow. Furthermore, analysis of the kinetics of BrdU+ PMN decay could be used to determine the life span of circulating neutrophils. By employing these techniques in ubiquitous, *LysMcre* and *GEcre* SHIP knockouts, we can, in the future, determine which aspects of

PMN homeostasis are regulated by SHIP and if the mechanisms are extrinsic or cell intrinsic.

We also found that granulocyte/monocyte restricted loss of SHIP promoted an M2 biased macrophage phenotype. Part of our initial aim was to explore how the loss of SHIP in macrophages and an M2 skewed macrophage phenotype might contribute to the disease phenotype observed in the *LysMcre+SHIP$^{fl/fl}$* mice. However, exploration of SHIP's role in macrophages during autoimmune inflammatory disease was beyond the scope of this thesis. Several more preliminary experiments would need to be conducted to determine if loss of SHIP in macrophages is sufficient to exacerbate disease, and since a macrophage specific cre strain has yet to be developed, designing an experiment to test the cell intrinsic effects of SHIP loss in this cell lineage is challenging. As mentioned previously, *LysM-Cre* also deletes in neutrophils. Furthermore, it should be noted that osteoclasts are derived from the monocyte lineage and are instrumental to the modulation of bone and tissue remodeling. While not widely used for the examination of conditional alleles in osteoclasts, it would be pertinent to examine SHIP expression levels of osteoclasts in *LysM-Cre* mice, as osteoclasts are derived from the monocyte lineage. Furthermore, *SHIP-/-* osteoclasts are hyper resorptive (Takeshita et al., 2002), and may contribute to the disease pathology.

In lieu of a genetic approach, some studies have employed macrophage depletion followed by cellular reconstitution to show their significance in disease. Local administrations of chlodronate liposomes have been shown to effectively deplete macrophages from wild type mice (Van Rooijen & Sanders, 1996). The host mice can then be reconstituted with macrophages from genetically manipulated donors. This method has been widely utilized (Solomon et al., 2005), however, it must be noted that this approach is highly toxic and requires a large number of animals, as we found that administration of chlodronate liposomes can result in the mortality of up to 2/3 of the experimental population (data not shown).

4.2 Loss of SHIP affects pathways downstream of FcR and GPCR engagement in neutrophils

Our preliminary data indicates that loss of SHIP in neutrophils increased in the phosphorylation of key signaling proteins in response to Fcγ receptor ligation with

immobile IC. While SHIP mediated inhibition of Fcγ receptor signaling has been described in macrophages, this is, to my knowledge, the first report in neutrophils. Increased phosphorylation of Akt and Erk1/2 following IC stimulation observed in *SHIP-/-* neutrophils supports our hypothesis that loss of SHIP in neutrophils confers a hyper-responsive phenotype, yet this remains to be confirmed experimentally. I propose several assays to examine how the loss of SHIP affects PMN effector functions post IC stimulation. Activation of neutrophils can result in the release of various preformed granules into the extracellular environment. These granules contain cytotoxic molecules such as enzymes (MPO and gelatinase) and anti-microbial peptides, and there are numerous assays to quantify their release from neutrophils upon activation. In addition, evaluating neutrophil activation via quantification of super oxide production following neutrophil stimulation could be assessed via the real time cytochrome c reduction test. It would also be interesting to explore if SHIP attenuation in neutrophils affects release of pro-inflammatory factors such as TNF-α and IL-1β, since cytokines are essential to cultivating and maintaining the inflammatory cascade during arthritis. To this end, an enzyme linked immunosorbent assay (ELISA) could be employed to quantify the amount of cytokines released by isolated PMN cultured for a short duration.

We did not continue to explore the molecular mechanisms underlying SHIP mediated attenuation of IC stimulation in neutrophils, and many questions remain. However, based on research conducted in other primary cell types, we hypothesize that recruitment of SHIP to the inhibitory receptor FcγRIIB plays a role in the observed phenotype. Upon activation, SHIP is normally recruited, via it's SH2 domain, to the immunoreceptor tyrosine inhibitor motifs (ITIM) on the cytoplasmic tail of the FcγRIIB receptor. This recruitment to the plasma membrane allows SHIP to dephosphorylate PIP_3, thereby reducing PI3K driven activities. Thus, since PMN express the inhibitory FcγRIIB receptor, the hyper-responsiveness of IC stimulated *SHIP-/-* PMN is likely due to inability of activated FcγRIIB to recruit SHIP, resulting in an inability to attenuate PI3K mediated pathways downstream of FcγRIIIA and FcγRIV ligation. As mentioned in the introduction of this thesis, a similar ITIM dependent mechanism has been identified in B cells and mast cells.

From the standpoint of clinical immunologists, FcγRIIB receptor mutations have been of great interest due to their potential in influencing the clinical outcome of autoimmune diseases. This outlook has been encouraged by observations in various antibody and

IC mediated disease models (for a review see (Smith & Clatworthy, 2010)). Furthermore, the contribution of the FcγRIIB to immune system homeostasis has been revealed by the enhanced responses of *FcγRIIB -/-* mice to antibody or IC mediated reactions (Takai et al., 1996). While a direct link between FcγRIIB polymorphisms and rheumatoid arthritis has yet to be established in humans, FcγRIIB mutations in the synovial macrophages of patients suffering from rheumatoid arthritis have been observed, and speculated to contribute to elevated TNF-α and MMP production (Blom et al., 2003).

It is also important to mention, however, that interactions between SHIP and immunoreceptor tyrosine activation motifs (ITAM) of certain receptors have also been reported. Previous studies have demonstrated that SHIP can interact with phosphorylated ITAM bearing peptides derived from the γ subunit of Fc receptors *in vitro* (Maresco et al., 1999). One functional consequences of this interaction was later demonstrated, as studies employing macrophages revealed that SHIP negatively regulates FcγR mediated phagocytosis via the ITAM on IgG receptors, in an ITIM independent mechanism (Nakamura, 2002). In this study, the authors demonstrated that SHIP was efficiently phosphorylated in *FcγRIIB-/-* macrophages, and that clustering of a receptor chimera containing ITAMs without the co-engagement of FcγRIIB is sufficient for phosphorylation of SHIP. They also utilized a macrophage cell line expressing an ITAM containing human-restricted Fcγ receptor (FcγRIIa), transfected with a dominant negative form of SHIP to inhibit endogenous SHIP. Phagocytosis was induced via red blood cells coated with antibody that allowed for selective ligation to FcγRIIa but not FcγRIIB. Cells transfected with the dominant negative mutants displayed significantly increased phagocytosis. It remains to be determined how and if receptors bearing intracellular ITIM and ITAM play a role in SHIP mediated attenuation of neutrophil function; it is also entirely plausible that SHIP employs both types of tyrosine motifs to exert its function.

I also observed an increased in phosphorylation of Akt following neutrophil stimulation of the GPCR via C5a or LTB_4. These soluble factors were selected because they are indispensable for neutrophil mediated effects during the early phases of the K/BxN serum transfer arthritis. Given the importance of the neutrophil LTB_4 receptor

expression to IL-1β production and delivery to the joint, it will now be important to quantify the IL-1β levels in the arthritic joints of mice with neutrophils restricted loss of SHIP (*GEcre+SHIP*$^{fl/fl}$ mice). I hypothesize that IL-1β and possibly other cytokine levels during the early stages of disease will be significantly higher in the knock-outs, due to the hyper responsiveness of *SHIP-/-* neutrophils.

4.3 SHIP likely regulates several aspects of neutrophil biology

While I elected to focus on how SHIP affects responses downstream of receptors ligated during disease, it is important to acknowledge that SHIP likely regulates multiple stages of PMN activity. PI3K activity is key to numerous stages of neutrophil function and development, and as a negative regulator of the PI3K signaling, it is possible that ablation of SHIP could also affect these processes (Figure 22). Furthermore, I cannot rule out that these known or potential neutrophil intrinsic effects of SHIP loss may contribute to the early onset and disease manifestation observed in the *GEcre+SHIP*$^{fl/fl}$ mice.

Addressing the importance of PI3K in all aspects of neutrophil biology merits an entire thesis (for an excellent review refer to (Hawkins et al., 2010)), thus future studies will selectively address processes which may influence by the neutrophil intrinsic role of SHIP and the extent to which they may contribute the observed disease phenotype; chemotaxis, trans-endothelial migration, survival and apoptosis.

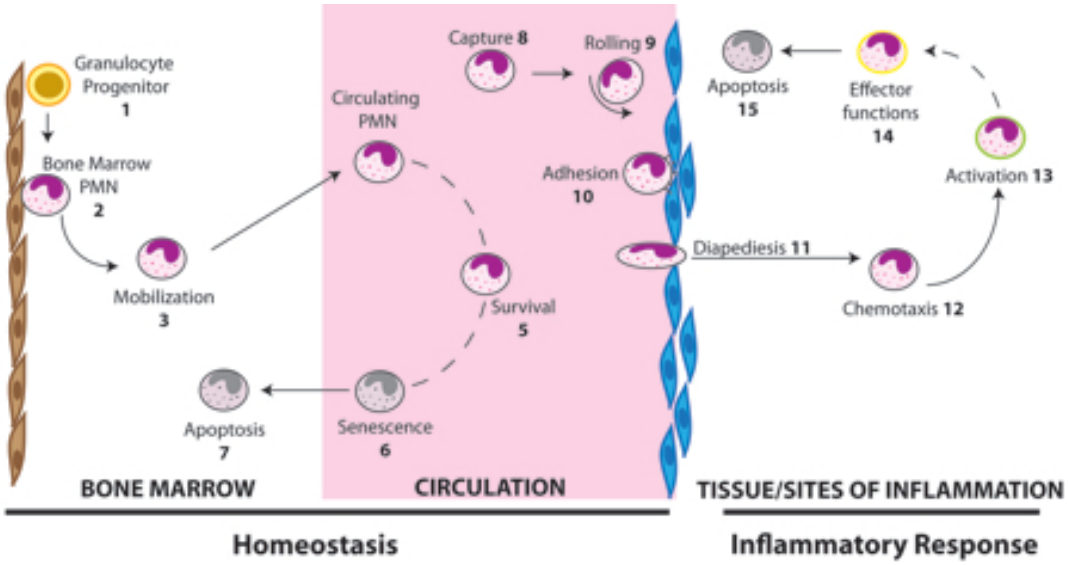


Figure 22. Life cycle of the polymorphonuclear leukocyte (PMN)

PMN generated from granulocyte progenitor in the bone marrow (BM) (**1**) are retained via adhesion to the bone marrow stroma (**2**). Mobilizing agents can induce the release of BM PMN into the circulation (**3**). Although circulating PMN have a short lifespan, apoptosis may be delayed by survival factors such as G-CSF (**5**). Senescent PMN can be sequestered in the bone marrow and removed from the circulation via bone marrow macrophages (**6,7**). When circulating PMN encounter inflamed tissue, they can become captured by microvasculature (**8,9**). Once adhered to endothelia (**10**), PMN transmigrate into the inflamed tissue (**11**) and migrate along the chemotactic gradient to the site of infection (**12**). Activated PMN can delay apoptosis (**13**) and release pro-inflammatory cytokines, chemokines, reactive oxygen species and granules containing proteases (**14**) to perpetuate an inflammatory cascade. Apoptosis of PMN is important for the resolution of inflammation (**12**).

4.3.1 Chemotaxis

SHIP-/- neutrophils display normal directional, but delayed migration in response to the chemoattractants fmlp and C5a (Nishio et al., 2007), due to delayed cellular polarization. Given the necessity of C5a and C5a receptor expression on neutrophils to K/BxN serum transfer arthritis (Monach et al., 2010), this finding appears contrary to the significantly higher percentage of neutrophils we observed in the ankle lavages of *GEcre+SHIP*$^{fl/fl}$ mice. However, the extent to which PI3K plays a role in C5a- and fmlp-mediated neutrophil chemotaxis has been suggested to be insignificant *in vivo* (Heit et al., 2008a), and limited to modulating the initial response to certain chemoattractants. This idea is substantiated by the ability of PI3K isoforms (p10δ and p10γ) to significantly accelerate initial chemotactic responses to fmlp, but not for prolonged periods of time (Heit et al., 2008b). Thus while modulation of PI3K can amplify or delay early responses to certain chemoattractants, the biological ramifications of this enhancement are very minor.

4.3.2 Transendothelial migration

Another important role of neutrophils during inflammation is transendothelial migration. Proinflammatory agents released by pathogens or activated cells can remodel the endothelial lining of local capillaries, resulting in the increased expression of adhesive molecules. This event, in conjunction with the influences of chemoattractants, recruits circulating neutrophils, which will then adhere to the post capillary venules. To test neutrophil-endothelial interactions *in vivo*, interactions can be visualized in the cremaster muscle of a male mouse which has been superfused with a neutrophil chemoattractant. Interestingly, neutrophils in the cremaster of a MIP-2 superfused *GEcre+SHIP*$^{fl/fl}$ mouse, demonstrated decreased rolling velocity and adhesion (Dr. Paul Kubes, University of Calgary, personal communication). This suggests that SHIP may play a role in facilitating neutrophil rolling and adhesion to the vascular endothelium. Furthermore, adhesion to ICAM-1 mediated by the β_2 integrin LFA-1 is enhanced in a cell line which over expresses SHIP (Rey-Ladino et al., 1999), thus further supporting the idea that SHIP can modulate cell adhesion. In the K/BxN model of rheumatoid arthritis, neutrophil rolling and adhesion are important events during disease progression. Since we observed exacerbated

disease in the *GEcre+SHIP$^{fl/fl}$* mice, we speculate that the decreased rolling and adhesion of *SHIP-/-* PMN to vascular endothelia is not a dominating phenotype, as we would expect the *GEcre+SHIP$^{fl/fl}$* mice to be resistant to develop mild disease if this were the case. In the future, it may be interesting to explore how attenuation of SHIP affects leukocyte adhesion in the presence of other factors, such as LTB_4 and C5a.

4.3.3 Survival and apoptosis

Once released from the bone marrow, circulating neutrophils can be 'primed' by various stimuli they encounter, thus modulating their responsiveness. During rheumatoid arthritis, the volume of fluid increases in the synovial cavity of the joint and becomes a source of pro-inflammatory mediators such as GM-CSF, IFNγ, IL-1β (Beaulieu, 1994; McInnes & Schett, 2007). Furthermore, priming of isolated PMN with GM-CSF has been shown to increase their sensitivity to immune complexes *in vitro* (Fossati, 2002). While *SHIP-/-* hematopoietic progenitors are hyper-responsive to suboptimal levels of several factors such as GM-CSF (Helgason et al., 1998), responsiveness of *SHIP-/-* PMN to cytokines has yet to be evaluated. Exposure to the cytokine milieu in the circulation might also influence neutrophil survival. The role SHIP plays in neutrophil viability has not been assessed, although *SHIP-/-* PMN demonstrate decreased sensitivity to various apoptotic stimuli (Gardai et al., 2002; Liu et al., 1999) suggesting that SHIP has a cell intrinsic role in regulating neutrophil survival. SHIP has also been implicated in mediating neutrophil apoptosis within the context of inflammatory environments (Gardai et al., 2002). It would be interesting to explore if ablation of SHIP confers increased sensitivity to sub-optimal levels of GM-CSF and survival factors such as G-CSF. Furthermore, there are several other factors known to enhance PMN survival, including β2 integrin receptor ligation, C5a, leukotriene B_4 (LTB_4), cross-linking of FcγRs, and the sensitivity of *SHIP-/-* PMN to each of these agents could also be evaluated.

Pinpointing the receptors and responses most dependent on SHIP influences on PI3K *in vivo* is complicated by the fact that neutrophil function is guided by a large number of interdependent stimuli and events. In this thesis we performed a preliminary assessment of exploring SHIP function in mediating neutrophil effector

functions in the murine system. One promising avenue to explore, in addition to the experiments previously suggested, would be to extend this project to the human system. Neutrophils can also be readily isolated from peripheral blood, and the majority of current *in vitro* experiments concerning neutrophils utilize human samples. Furthermore, a pharmacological SHIP activator (Aquinox Pharmaceuticals) and inhibitor (Kerr, 2010) have been developed, allowing for the generation of knowledge more relevant to therapeutic applications. Thus, these studies are now timely and feasible.

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Appendices

Appendix A. Parameters and examples of histological evaluation

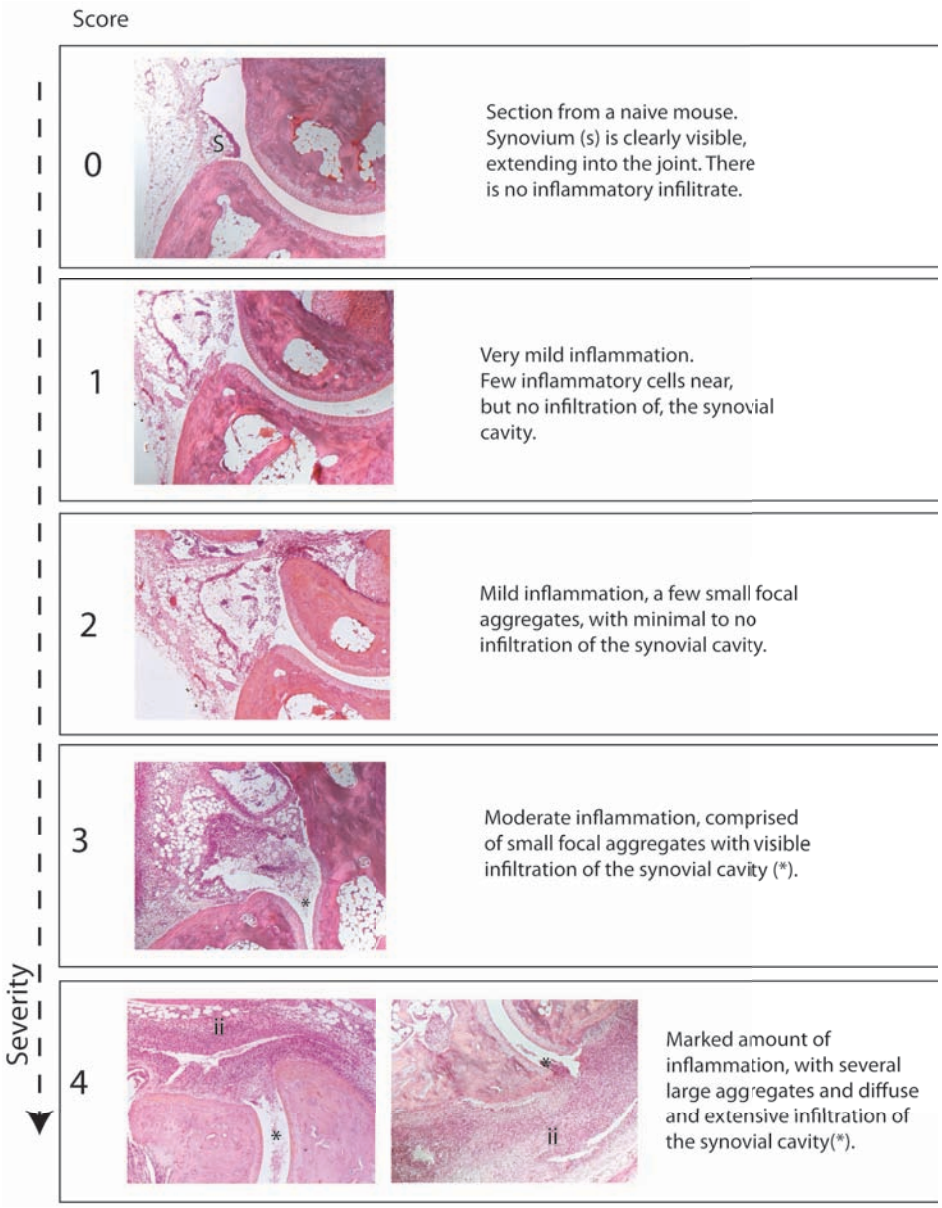


Figure 23. Histological scoring of inflammatory infiltrate

Following termination of the disease course, hind paws were fixed in 10% neutral buffered formalin, decalcified, and cut into 5 µm sections. Specimens were then stained with hematoxylin and eosin for general evaluation of joint structure. Ankle Inflammatory infiltrate present in the joint was assessed on a scale of 0 to 4. To determine the extent of disease severity, this histological scoring method was used in conjunction with other read-outs, such as the clinical scoring and changes in ankle thickness.

Appendix B. The role of Tie2 in mast cell function

Introduction

Mast cells have been implicated in numerous physiological and pathological processes. Once thought to function primarily as pro-inflammatory cells in allergic reactions, mast cells are now known to participate in host defense during both acquired and innate immune responses, and in a wide spectrum of inflammatory diseases (Benoist, 2002; Puxeddu, et al., 2003). Furthermore, mast cells have been recognized as the source of a variety of multi-functional cytokines, introducing potential roles for mast cells in angiogenesis, fibrosis, and wound healing (Martin & Leibovich, 2005; Noli & Miolo, 2010). Given their biological and medical relevance, an interest in the mechanisms underlying mast cell regulation and development has been renewed. In an effort to identify novel genes that play a role in the regulation and function of mast cells, our lab utilized microarray to survey their global gene expression. We found that Tie2 mRNA was expressed by bone marrow derived mast cells (BMMC). To our knowledge, this is the first report of Tie2 protein expression on mast cells. This was of special interest as the expression of Tie2 had been generally thought to be restricted to the endothelium of growing blood vessels, and hematopoietic stem cells (HSC) (Arai, et al., 2004; Kukk, et al., 1997). Tie2 is a receptor tyrosine kinase (Figure 24), and upon binding to its ligand, Angiopoietin-1 (Ang-1), it can initiate signal transduction pathways, including the MAPK/ERK, PI3K, and Akt pathways (DeBusk, et al., 2004; Jones, 2000). Through these pathways, Tie2 activation promotes quiescence and niche adhesion in HSC, and regulates the survival, apoptosis, adhesion, and migration of endothelial cells. We hypothesize that, similar to its functions in endothelial cells and HSC, activating Tie2 via Ang-1 regulates mast cell development and function.

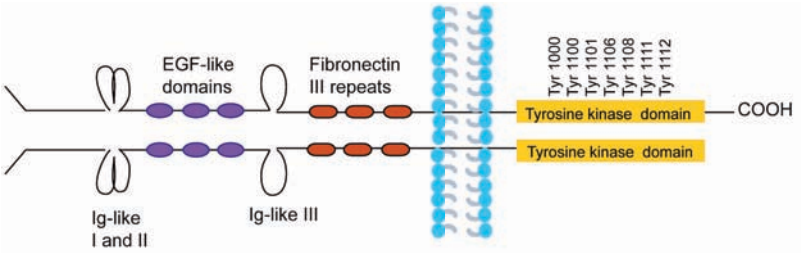


Figure 24. Structure of Tie2

The extracellular-domain of Tie2 contains an immunoglobulin-like loop, 3 EGF-like domains, another If-like loop, and three fibronectin type III repeats. The cytoplasmic regions contain tyrosine kinase domains including a number of sites of phosphorylation and protein interaction.

Methods and Materials

Cell culture and derivation

BMMC were derived from femurs and tibias from mature B/6 mice. Mast cells were maintained in IMDM supplemented with 10% FBS, 150µM monothioglycerol (MTG, Sigma, St.Louis, MO), 100U/ml penicillin, 100µg/ml streptomycin (Sigma, St.Louis, MO) and 30ng/ml recombinant mouse IL-3 (Stem Cell Technologies Inc, Vancouver, BC). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere, selected for non-adherence, and considered to be BMMC after six weeks in culture, as verified by Wright-Giemsa staining or flow cytometry. ESMC were derived as outlined previously (Tsai et al., 2000). *Tie2-/-* , *Tie2 +/-*, R1 (wild type) ES cells were provided by Dr. Mira Puri (University of Toronto). Growth factors and cytokines required were all purchased from Stem Cell Technologies Inc (Vancouver, BC).

Cell stimulations and Immunoblotting

BMMC were starved of IL-3 for 16 hours and then stimulated with a vehicle control or Ang-1 (100ng/ml, Alexis Biochemicals, USA). Whole cells lysates were prepared in Laemmli sample buffer, boiled and loaded onto a 10% SDS-PAGE gel for fractionation. Proteins were then transferred to a PVDF membrane, blocked with 3% BSA, and probed with antibodies to detect Grb2 (loading control), phospho-Erk1/2, and phospho-Akt (S473). Membranes were washed again and incubated with secondary antibodies directly conjugated to horseradish peroxidase for one hour at room temperature. Membrane were rinsed with TBS-T and treated with treated with Western Lightning chemiluminescence reagent (Perkin Elmer, Boston, MA) for 60 seconds, and exposed to Kodak X-Omat Blue film.

Flow cytometry

Cells were blocked with 10µg/ml anti-FcγRIII/II antibody (clone 2.4G2, AbLab, Vancouver, BC) with 10% goat serum in FACS buffer, for 45 minutes at 4°C. Cells were incubated with antibodies directly conjugated to fluorophores such as anti-c-kit (Ebiosciences, San Diego, CA), anti-Tie2 (clone 33, Calbiochem, San Diego, CA), or anti-FcεRI (Ebiosciences, San Diego, CA). Cells were washed and resuspended with FACS buffer, then processed with a FACS Calibur (BD, Franklin Lanes, NJ), and analyzed with FloJo software (Treestar, Ashland, OR).

Results

My first aim was to confirm Tie2 expression in bone marrow derived mast cells (BMMC) at the protein level. To this end, I employed flow cytometry to confirm surface expression of Tie2, in addition to immunoprecipitation (Figure 25 A, B,C). I utilized bEND3 cells (an endothelial cell line) as a positive control for Tie2 expression.

Angiopoietin-1 (Ang-1) is known ligand for the Tie2 receptor in endothelial cells, so we sought to determine if Tie2 is a functional receptor for Ang-1 in BMMC. I observed that stimulation of BMMC with Ang-1 resulted in phosphorylation of Akt and Erk1/2 pathways (Figure 26).

To probe the necessity of Tie-2 expression in mast cells, I utilized a genetic approach. The simplest method would entail the derivation of mast cells from the bone marrow of *Tie2-/-* mice. However, *Tie2-/-* mice are embryonic lethal; they die at mid-gestation from an inability to maintain the endothelial cell integrity of microvasculature (Puri, et al., 1995). Therefore, I utilized a technique where transgenic embryonic stem cells can be guided to differentiate into mast cells (ESMC) through the addition of specific cytokines and growth factors (Tsai et al., 2000). I observed that *Tie2-/-* and *Tie2+/-* ESMC were viable and did not display any morphological abnormalities (Figure 27A). Furthermore, mature mast cell surface markers (c-kit and FcεRI) were present in *Tie2-/-* ESMC, 6 weeks following the initiation of differentiation (Figure 27B). Expanding the ESMC cultures presented us with numerous difficulties, thus we were unable to perform any functional assays with these cells.

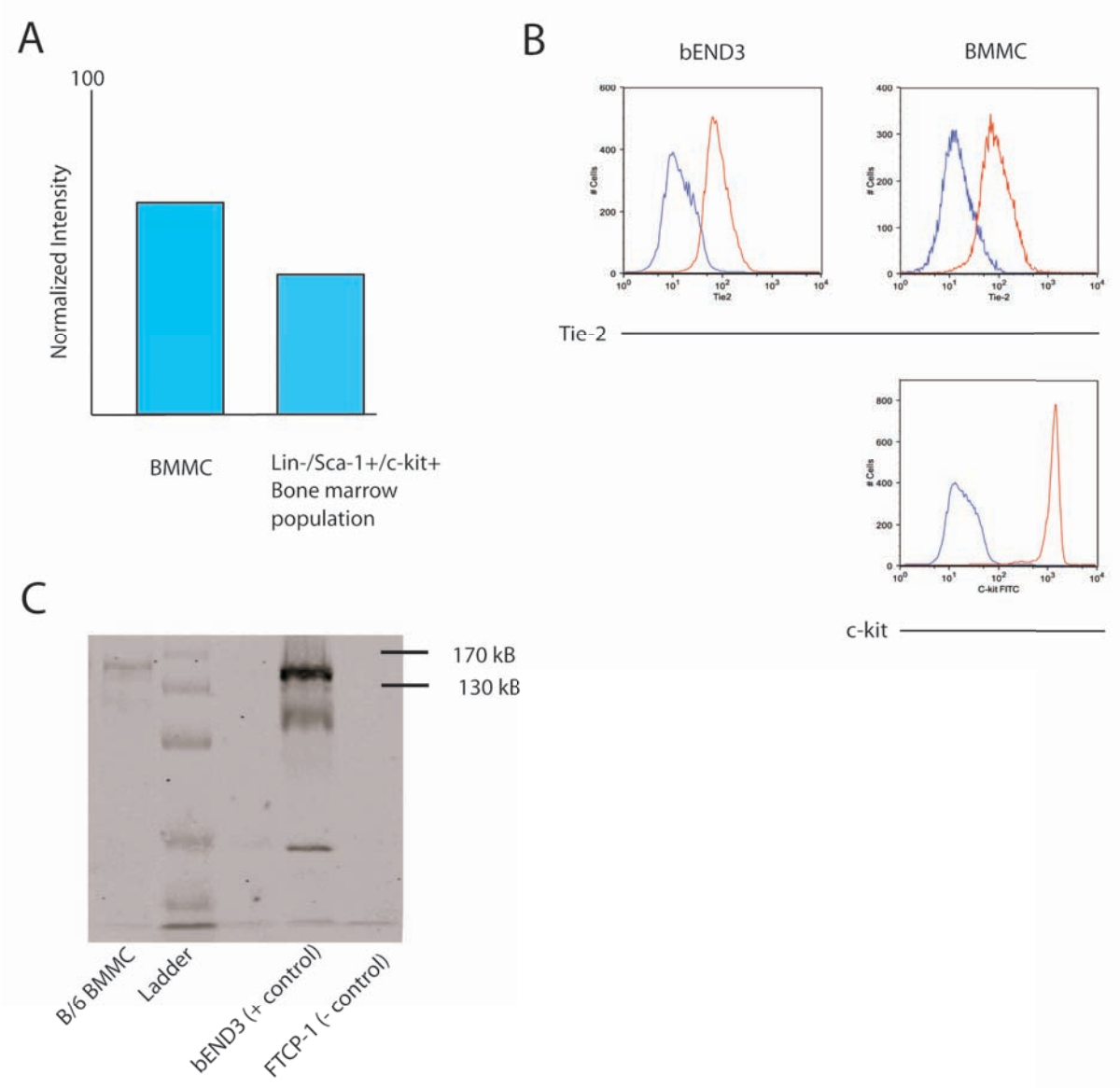


Figure 25. Tie2 expression at the transcript and protein level in BMMC

A, A microarray was employed to determine global gene expression of BMMC genes in relation to the Lin-Scal-1+/c-kit+ (LSK) bone marrow population. Higher Tie-2 expresion was identified in BMMC

B, Protein expression at the surface of BMMC was confirmed via immunoprecipitation. Total BMMC cells lysates were probed using anti-Tie2 antibody. Tie2 has a molecular weight of approximately 150 kDa. bEND3 positive control.

C, Protein expression at the surface of BMMC was also confirmed via flow cytometry, Cells were stained with anti-Tie2 antibody as well as anti-c-kit antibody, to confirm the mast cell profile of the cultures.

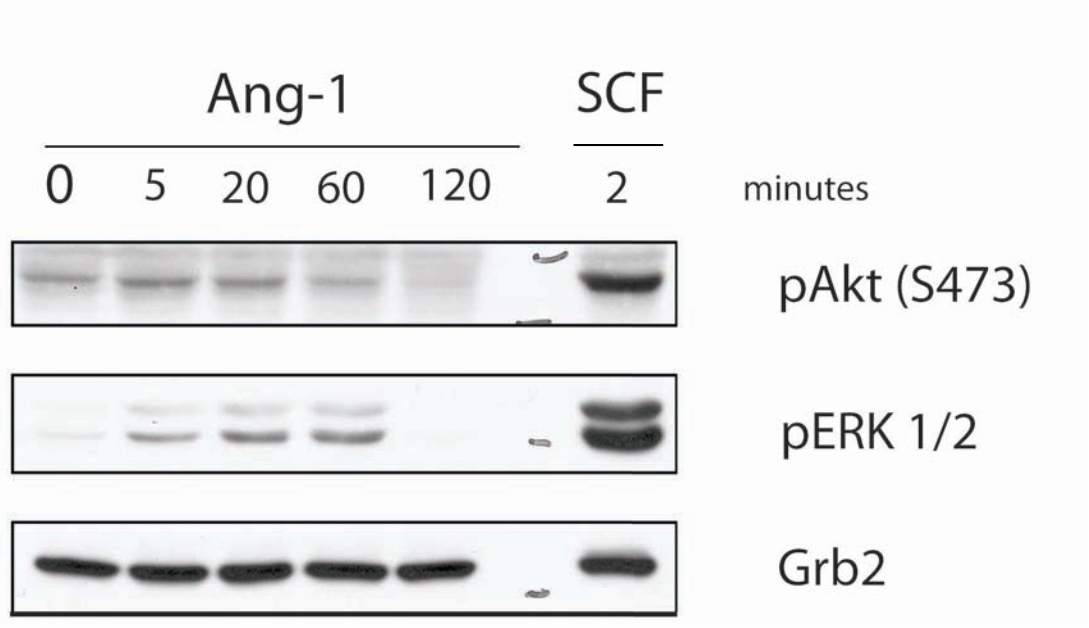
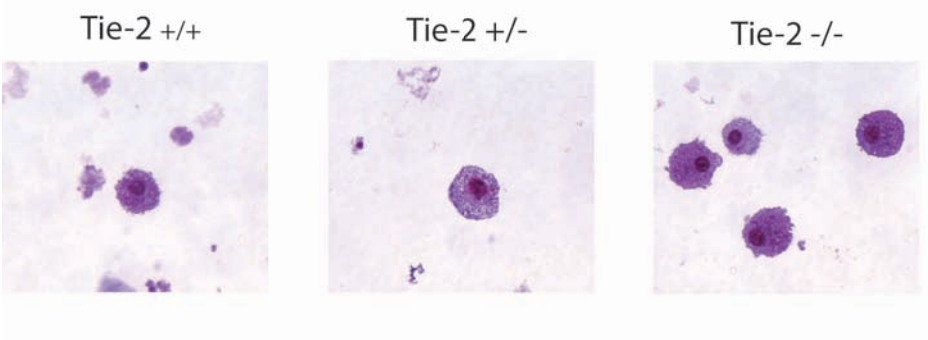


Figure 26. Tie2 expressed on BMMC is a competent signaling receptor that activates Akt and Erk1/2 pathways in response to Ang-1

BMMC were starved overnight and then stimulated with a vehicle control (0 min) or Ang-1 (100ng/ml) and SCF (10ng/ml) for the times indicated. Whole cell lysates were prepared in Laemmli sample buffer and loaded onto 10% SDS-PAGE gel for fractionation. The proteins were transferred to PVDF membrane, blocked and probed with antibodies to detected Grb2 (loading control), phospho-Erk1/2 and phospho-Akt (S473).

A.



B.

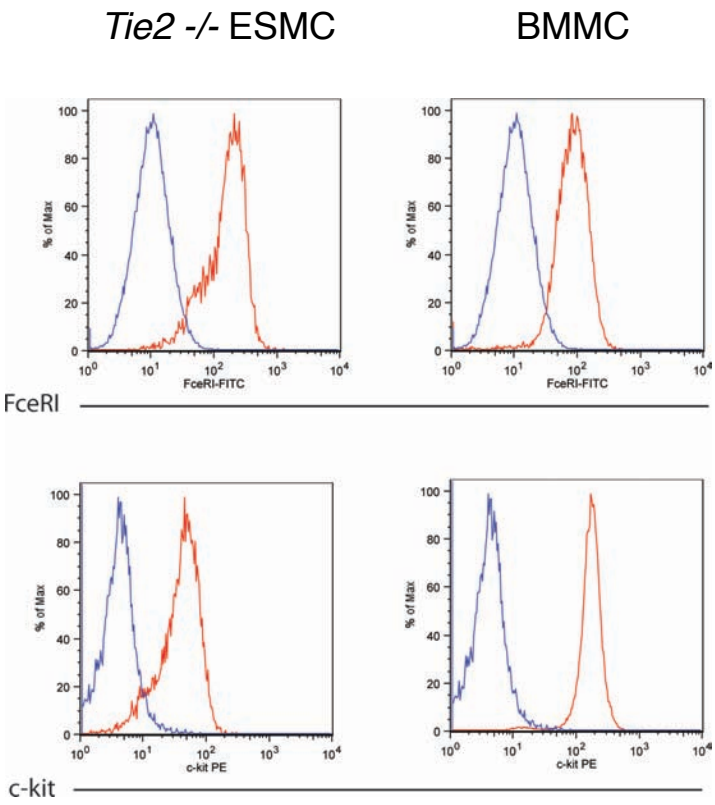


Figure 27. Characterization of Tie2 deficient ESMC

A, Giemsa staining of ES cell derived mast cells (ESMC), 6 weeks after initiation of differentiation. Images taken at 200x objective.
B, Evaluation of the mast cell profile of of 6 week of Tie2 -/- ESMC cultures versus 8 week BMMC cultures, via flow cytometry. Cells were stained with anti-FcεRI and anti-c-kit antibodies.
Discussion

Discussion

In this study I demonstrated that BMMC express Tie2, a receptor tyrosine kinase, and that Tie2 is a functional receptor for Ang-1 in these cells. Finally, I derived Tie2 deficient ESMC from *Tie2-/-* ES cells, and observed that cells appear morphologically normal. What remains to be determined is the relationship of Tie2 to biological functions of mast cells. Previously, our lab compared degranulation, adhesion and survival of wild type mast cells in the presence or absence of Ang-1, but did not observe a significant difference between the conditions.

The ESMC derivation technique was difficult to execute, and provided a very low yield of ESMC. Thus, I propose several alternative methods for examining loss of Tie2 in mast cells. First, mast cells could be transfected with small interfering RNA (siRNA) to knock down Tie2 expression. Alternatively, the are a number of pharmacological Tie2 inhibitors commercially available. Another technique involves the derivation of mast cells from the para-aortic-splanchnopleura (Psp). The Psp is an embryonic structure which facilitates primitive hematopoiesis during early development. The Psp can be cultured, *ex vivo*, and in the presence of certain cytokines, can produce hematopoietic cells of the mast cell lineage. (Takakura et al., 1998). Since hematopoietic cells appear in the Psp between 8.5-9.5 days post coitus (dpc) (Godin, Dieterlen-Lièvre, & Cumano, 1995) and *Tie2-/-* mice survive until 10.5 dpc, this method could be used to isolate Tie2 ablated mast cells. Alternatively, the yolk sac can also be cultured *ex vivo* to yield hematopoietic populations. A number of *in vitro* assays could then be performed to assess how loss of Tie2 in mast cells affects cellular function and development. These include degranulation, survival and proliferation, and adhesion. To explore the function of *Tie2 -/-* mast cells *in vivo*, we would evaluate their ability to reconstitute $Kit^{W}/Kit^{W\text{-}v}$ mice, a strain that is deficient in mast cells, and then perform histological analysis of their tissues. To address, *in vivo*, Ang-1 ligation of Tie2, we could treat the reconstituted mice with recombinant Ang-1, and then evaluate mast cell recruitment and activity.

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