INTERLEUKIN-10 INHIBITION OF TUMOR NECROSIS FACTOR ALPHA PRODUCTION IN ACTIVATED MACROPHAGES REQUIRES SHIP1 AND BTK

by

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Abstract

Inflammation is a physiological process required for defense against pathogens and the repair of damaged tissues. However, excessive or improper inflammation can be detrimental and results in a number of diseases such as rheumatoid arthritis and inflammatory bowel disease. To prevent the negative effects of inflammation, the inflammatory response is tightly regulated by the anti-inflammatory cytokine interleukin-10 (IL-10). The main target of IL-10 are activated macrophages whose exposure to IL-10 results in the anti-inflammatory response (AIR) characterized by depressed antigen presentation and the inhibited secretion of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα). To induce the AIR in macrophages, IL-10 binds to cell surface receptors which activate the transcription factor STAT3 leading to the transcription of gene products responsible for carrying out the AIR. However, we hypothesized that IL-10 also uses a STAT3-independent mechanism to induce the AIR. Here we demonstrate that IL-10 utilizes the lipid phosphatase SHIP1 to inhibit TNFα production in activated macrophages. SHIP1 is responsible for dissociating TNFα mRNA from polysomes leading to inhibited translation of TNFα mRNA during the AIR. This effect of SHIP1 occurs early in the AIR and helps to immediately halt TNFα production. We also demonstrate that the tyrosine kinase Btk, a reported positive regulator of TNFα production in macrophages, is also utilized by IL-10 in the early AIR to inhibit TNFα production. However, Btk is not required for IL-10 to dissociate TNFα mRNA from polysomes suggesting that Btk and SHIP1 are involved in distinct IL-10 signalling pathways. Finally we show that TIA-1, a RNA binding protein that silences TNFα mRNA translation is not involved in the IL-10 AIR. These results clearly demonstrate the existence of non-STAT3 signalling pathways in the IL-10 AIR and suggest that SHIP1 and Btk activators could be used as potential therapeutics in the treatment of inflammatory disorders.

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List of abbreviations

AIR	anti-inflammatory response
ARE	AU-rich elements
BCR	B-cell receptor
BLNK	B-cell linker protein
BMDM	bone-marrow derived macrophage
Bmx	bone marrow kinase on chromosome X
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle medium
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green flourescent protein
IBD	inflammatory bowel disease
IBtk	Inhibitor of Btk
IFN	interferon
IκB	inhibitor of nuclear factor-kappa B
Iκk	inhibitor of nuclear factor-κB kinase
IkκB	inhibitor of nuclear factor kappa B kinase
IL-10	interleukin-10
IL-10R1	interleukin-10 receptor subunit 1
IL-10R2	interleukin-10 receptor subunit 2

IL-1RA	interleukin-1 receptor agonist
iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor-associated kinase
Itk	interleukin-2 inducible T-cell kinase
Jak	janus associated kinase
LAT	linker for activation of T cells
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
Mal	myeloid differentiation primary-response protein 88 adaptor like protein
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MHC II	major histocompatability complex II
miR	microRNA-like
miR-155	microRNA-155
miRNA	microRNA
Myd88	myeloid differentiation primary-response protein 88
NF-κB	nuclear-factor kappa B
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PH	Pleckstrin homology
PI3K	phosphoinositide-3-kinase
Pin1	protein interacting with NIMA1

PIP3	phosphatidylinositol-(3,4,5)-triphosphate
PKC	Protein kinase C
PLCγ	phospholipase C gamma
PTEN	phosphatase and tensin homologue deleted on chromosome 10
qPCR	quantitative PCR
RANKL	receptor activator for nuclear factor κ B ligand
RRM	RNA recognition motif
Sab	SH3-domain binding protein that preferentially associates with Bruton's tyrosine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH1	Src homology 1
SH2	Src-homology 2
SH3	Src homology 3
SHIP1	Src homology 2-containing inositol-5′-phosphatase 1
siRNA	small inhibitory RNA
SLP	signal linker protein
SLPI	secretory leukocyte protease inhibitor
STAT3	signal transducer and activator of transcription 3
TAB	transforming-growth factor-β-activated kinase 1 binding protein
TAK1	transforming-growth factor-β-activated kinase 1
TBS-T	tris-buffered saline with tween
TCR	T-cell receptor

TFK	Tec family kinase
TGFβ	transforming growth factor beta
TIA-1	T-cell intracellular antigen 1
TIAR	T-cell intracellular antigen 1 related protein
TIR	toll/IL-1R
TLR	toll-like receptor
TLR4	toll-like receptor 4
TNFα	tumor necrosis factor alpha
TRAF6	tumor-necrosis-factor receptor-associated factor 6
TRE	tetracycline response element
TTP	Tristetraprolin
UTR	untranslated region
Xid	x-linked immunodeficiency
XLA	x-linked agammaglobulinemia

1 Introduction

1.1 Inflammation

Inflammation was originally described in the first century as the combined symptoms of heat, pain, redness and swelling[1]. Today, it is known that inflammation is a complex physiological process which combines the responses of the immune system as well as the circulatory system. The inflammatory response is believed to have evolved as a way for the body to fight infection and repair tissue damage and therefore it is generally considered to be a beneficial response [2]. However, chronic or excessive inflammation can be detrimental as seen in the case of a number of pathologies such as inflammatory bowel disease, rheumatoid arthritis, anaphylaxis and atherosclerosis [2].

Inflammation can be initiated by mast cells and macrophages residing in the tissues of the body [3]. These cells respond to a variety of stimuli including pathogen associated molecular patterns (PAMPS), virulence factors, allergens and intracellular contents from damaged cells. Once stimulated, these resident macrophages and mast cells produce a plethora of inflammatory mediators including cytokines like tumor necrosis factor alpha (TNFα), chemokines, histamine and eicosanoides[1]. The release of these mediators causes localized inflammation characterized by vasodilation of the circulatory system and extravasation of leukocytes from the blood into the tissue. Among the first leukocytes to migrate into the inflamed tissue are neutrophils which become activated by the secretions of the resident mast cells and macrophages such as TNFα and CXCL8 with TNFα being the major activator [1]. The activated neutrophils then release their own inflammatory mediators as well as the cytotoxic contents of their granules, such as hydrogen peroxide, in an attempt to kill any surrounding pathogens. If this is successful, the inflammatory

stimulus will be removed and the inflammatory response switches into a repair response [2]. In this way, inflammation is an important first step in the repair of damaged tissues. If the initial action of the recruited neutrophils does not successfully clear the pathogen, then the inflammatory stimulus persists and the response heightens with the recruitment of additional leukocytes such as macrophages and T-cells. The recruitment of T-cells to the site of inflammation is an important first step in the activation of the adaptive immune response and consequently the inflammatory process is also important for the initializing of the adaptive immune system.

1.1.2 Regulating inflammation

Despite the overall beneficial effect of the inflammatory response, many of the products released by activated leukocytes during inflammation are damaging not only to pathogens but also to host cells. These include reactive oxygen and nitrogen species, proteinases and hydrogen peroxide[1]. Therefore, the inflammatory response also damages the tissues of the host. To minimize this damage, inflammation is tightly regulated through the secretion of anti-inflammatory molecules such as secretory leukocyte protease inhibitor (SLPI) [4]. In the early stages of inflammation, lipoxins, derived from the arachadonic acid released by activated neutrophils[1], are particularly important for controlling the inflammatory response. In the later stages of inflammation, the main regulators are the anti-inflammatory cytokines transforming growth factor β (TGFβ) and interleukin-10 (IL-10), with IL-10 being the best characterized. Finally, the overall immune response in the body is also regulated by the actions of immunosuppressive regulatory T-cells and B-cells[5, 6], which often produce their immunosuppressive effects through the release of IL-10 and TGFβ. Without the proper regulation of inflammation by these factors the body is prone to developing a number of diseases

associated with a hyperactive inflammatory response such as Alzheimer's disease, asthma, chronic obstructive pulmonary disease, type I diabetes and atherosclerosis [1]. Inflammation is important in atherosclerosis pathogenesis as inflammatory macrophages accumulate at atherosclerotic plaques and help to drive the progression of these plaques and the formation of additional plaques [7].

1.2 Interleukin-10

Interleukin-10 (IL-10) was first discovered as a cytokine synthesis inhibitory factor secreted from stimulated T_{H2} cells that could inhibit the production of cytokines from T_{H1} cells[8]. After its initial discovery, it was quickly realized that IL-10 also affects the activity of many other immune cells making it a broad range modulator of immune function. The IL-10 gene is found on chromosome 1 in both humans and mice and shares a similar structure between the species with both mouse and human genes being composed of 5 exons separated by 4 introns[9]. The IL-10 gene encodes for a 17 kDa protein that forms a non-covalent homodimer in its active secreted form. Based on its structure IL-10 protein is a member of the class 2 α-helical cytokine family which also includes IL-19, IL-20, IL-22, IL-24, IL-26, interferon (IFN) alpha (IFNα) and IFNβ [10]. While many immune cells can respond to IL-10, activated macrophages and dendritic cells are believed to be the major target of IL-10 as these cell types express the highest levels of the IL-10 receptor complex and undergo profound changes upon IL-10 stimulation.

1.2.2 Interleukin-10 signalling

IL-10 binds to a receptor complex composed of two subunits: IL-10 receptor 1 (IL-10R1) and IL-10 receptor 2 (IL-10R2) [11]. IL-10R1 is expressed constitutively at low levels on most cells and IL-10R2 also has a broad range of expression suggesting that many cells are capable of responding to IL-10 [12]. The binding of IL-10 to its receptor complex is mediated primarily

through IL-10R1 as it has a much higher affinity for the IL-10 protein than does the IL-10R2 subunit[11] but IL-10R2 is required for signal transduction. The binding of the IL-10 homodimer to the receptor complex leads to the transphosphorylation and activation of the Janus associated kinases (Jak) Jak1 and Tyk2 that are associated with the IL-10R1 and IL-10R2 subunits, respectively [13]. The activation of these kinases allows them to phosphorylate residues Y446 and Y496 on the IL-10R1 subunit and once phosphorylated, these residues can then be bound by Src homology 2 (SH2) domains found on signal transducer and activator of transcription 3 (STAT3) and STAT1. Once bound, STAT3 and STAT1 become phosphorylated by the receptor associated kinases[14] causing homodimerization and/or heterodimerization and subsequent dissociation of STAT3 and STAT1 from the IL-10 receptor complex. This allows for the translocation of the STAT homodimers from the cytosol of the cell into the nucleus where they then regulate the transcription of gene targets. The gene targets, once transcribed and translated, then mediate the effects of IL-10 within the cell. Through analyses of STAT1 deficient cells, it has been shown that STAT1 is dispensable for the anti-inflammatory effects of IL-10 [15] but is still important in proliferative effects of IL-10.

1.2.3 IL-10 function in macrophages

Activated macrophages are one of the main targets of IL-10 as they express high levels of the IL-10 receptor complex. In activated macrophages, IL-10 induces what is termed the anti-inflammatory response (AIR) which is characterized primarily by depressed antigen presentation and the inhibited secretion of inflammatory mediators. IL-10 stimulation of macrophages leads to downregulation of the major histocompatability complex II (MHC II) as well as the CD80 and CD86 costimulatory molecules [16, 17]. The downregulation of ICAM-1 on macrophages by IL-10 is also reported suggesting that IL-10 can affect macrophage adhesion properties [18]. The other

major effect of IL-10 is to inhibit the secretion of a number of inflammatory cytokines by activated macrophages including TNFα, IL-1α, IL-1β, IL-6, IL-12 and IL-18 [12]. Chemokine secretion is also inhibited with IL-10 decreasing the secretion of MCP1, MCP5 and CXCL8 [12]. IL-10 also downregulates cyclooxygenase expression [19] and decreases the secretion of nitric oxide (NO) by activated macrophages [20]. Finally IL-10 stimulation leads to the production of anti-inflammatory molecules such as IL-1 receptor agonist (IL-1RA) [21] and soluble TNF receptor [22]. Overall the effects of IL-10 on activated macrophages are quite profound and serve to inhibit macrophage effector function.

1.2.4 IL-10 in disease

As IL-10 is a potent anti-inflammatory cytokine, it is not surprising that IL-10 plays a key role in a number of diseases, particularly in inflammatory and autoimmune disease. IL-10 knockout mice spontaneously develop inflammatory bowel disease (IBD) and arthritis highlighting the importance of IL-10 in the normal prevention of these diseases[23]. Interluekin-10 is also protective in models of endotoxic shock [24] and helps to prevent systemic lupus erythematosis, rheumatoid arthritis and allergic inflammation [12], demonstrating the importance of IL-10 in the regulation of autoimmune disease. IL-10 has also been found to inhibit allograft rejection in organ transplantation [25]. The importance of IL-10 in the prevention of these diseases demonstrates that IL-10 function is usually beneficial in the body. However, increased IL-10 levels have also been associated with chronic infections and some types of cancer [10, 12] due to the inhibition of the immune response by IL-10. Therefore, IL-10 function is important in both the prevention and the progression of a number of diseases.

1.2.5 IL-10 independent STAT3 signalling

While STAT3 is required for the anti-inflammatory effects attributed to IL-10, there is evidence for STAT3 independent IL-10 signalling. IL-10 has been reported to modulate both the phosphoinosotide-3-kinase (PI3K) pathway and the p38 mitogen activated protein kinase (MAPK) pathway [24, 26]. In fact, it is known that IL-10 is able to dissociate TNFα mRNA from polysomes in a p38 dependent fashion suggesting that this function of IL-10 may be STAT3 independent [26]. Studies using a STAT3 dominant negative isoform expressed in monocytes have also shown that IL-10 can still inhibit TNFα production in LPS activated macrophages in the first hour of IL-10 stimulation. This suggests that in the early phase of the IL-10 response in macrophages, STAT3 is dispensable and further suggests that other pathways must be mediating the inhibitory effects of IL-10 on TNFα production. Finally, IL-10 has also been shown to inhibit nuclear factor kappa B (NF-κB) activity through two mechanisms: STAT3 dependent expression of inhibitor of nuclear factor kappa B kinase (IκK), and the rapid and apparently STAT3 independent decrease in NF-κB DNA binding [27, 28].

1.3 Macrophages

Macrophages were originally identified as a subset of cells in the blood which had the capability to engulf large particles. While macrophages are now recognized as being an integral part of the innate immune system, their primary role is to engulf apoptotic cells in the blood such as red blood cells[29] and therefore one of the most important roles of macrophages is to maintain cell homeostasis in the body. Macrophages are derived from circulating blood monocytes and reside in every tissue in the body which helps them to serve as sentinels for the detection of pathogens and injury in the body. Macrophages serve an important role in immune responses as

they secrete a wide range of autocrine and paracrine factors and are also critical for antigen presentation and activation of the adaptive immune system [29].

1.3.2 Macrophage activation

Normally macrophages are found in a resting inactive state in which they can phagocytose apoptotic cells but do not produce inflammatory mediators. However, upon proper stimulation macrophages can become "activated" leading to profound changes in their behaviour. The activation of macrophages was originally described as a response to interferon gamma (IFNγ) exposure [30], but it is now known that macrophages can become activated through a number of other stimuli. One common stimulus is PAMPs which can be recognized by the toll-like receptors (TLRs) present on the outer cell membrane of macrophages. PAMPs can also be recognized by NOD-like receptors expressed inside cells. Macrophages activated through TLR signalling produce high levels of pro-inflammatory mediators such as TNFα, IL-1β, IL-6 and IL-23, secrete high levels of NO and also express high levels of MHC II and co-stimulatory molecules CD80 and CD86 [29]. While activated macrophages generally secrete these pro-inflammatory cytokines, certain stimuli such as IL-4 can lead to the "alternative" activation of macrophages which results in macrophages that do not produce large levels of inflammatory mediators, do not have increased levels of antigen presentation and do not produce NO[31]. These alternatively activated M2 macrophages are generally thought to be involved in wound repair processes and the promotion of healing[31]. As TNFα is one of the most important mediators of inflammation and activated macrophages are one of the largest producers of TNFα, macrophages are key players in the inflammatory response and therefore regulating macrophage response is important in controlling inflammation.

1.4 TLR4 signalling

Macrophages recognize PAMPS primarily through TLRs expressed on their outer cell membrane. TLRs were originally identified in *Drosophila melongaster* as a receptor important in embyrogenesis, but they were soon found to play a critical role in the innate immune system. TLRs all share a conserved toll/IL-1R (TIR) domain located on the cytoplasmic portion of the protein [32]. This TIR domain is approximately 200 amino acids and is critical for TLR signal transduction [33]. To date there have been 11 different TLRs identified in mammals which possess differential ligand specificity, cell localization and signalling pathways [32]. One TLR agonist is lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, that binds and signals in cells through TLR4. LPS is first bound outside the cell by LPS binding protein (LBP) creating a LPS-LPB complex that is then bound by CD14 on the surface of macrophages. CD14 then presents the LPS-LPB complex to the TLR4-MD2 receptor complex causing the dimerization of the TLR4 receptor [34]. The dimerized receptor will then recruit myeloid differentiation primary-response protein 88 (Myd88) that in turn recruits IL-1R-associated kinase 4 (IRAK-4) to the receptor. IRAK-4 then phosphorylates IRAK-1 causing the recruitment of tumor-necrosis-factor receptor-associated factor 6 (TRAF6). TRAF6 and IRAK-1 then dissociate from the receptor and form a complex with transforming-growth factor-β-activated kinase (TAK1) and TAK1-binding proteins 1 and 2 (TAB1 and TAB2). This leads to the degradation of IRAK-1 and the remaining proteins migrate into the cytosol where TRAF6 becomes ubiquitinated activating TAK1. Activated TAK1 then phosphorylates MAPKs as well as the inhibitor of nuclear factor-κB (IκB)-kinase (IκK) complex. This complex phosphorylates IκB causing it to release NF-κB which can then translocate to the nucleus and induce the transcription of pro-inflammatory genes [33]. In addition to the activation of NF-kB and MAPKs,

TLR4 signalling also leads to the activation of the PI3K pathway and Src kinases and Tec kinases [33].

1.5 SH2-containing inositol-5′-phosphatase 1 (SHIP1)

SHIP1 is a 145 kDa lipid phosphatase expressed exclusively in hematopoietic cells whereas the closely related SHIP2 protein is expressed ubiquitously in the body. SHIP1 contains an N-terminal SH2 domain, a C2 domian, a phosphatase domain, as well as a putative pleckstrin homology (PH) domain (data unpublished) (Figure 1). The primary function of SHIP1 is to antagonize PI3K signalling by converting the PI3K product, phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) into phosphatidylinositol-(4,5)-biphosphate by dephosphorylating the 3′ position of the lipid inositol ring[35]. The ability to antagnoize PI3K signalling is also shared by phosphatase and tensin homologue deleted on chromosome ten (PTEN), but SHIP1 and PTEN often have functions distinct from one another [36]. As SHIP1 is only expressed in hematopoietic cells, it is not surprising that SHIP1 has an important role in immune cell function. $SHIP1^{-/-}$ mice have increased numbers of macrophages and granulocytes and exhibit depressed Natural killer cell development [34, 37], highlighting the importance of SHIP1 in immune cell development. As SHIP1 antagonizes the PI3K pathway which can function as a survival and proliferation pathway, SHIP1 is also an important tumor suppressor [34].

1.5.2 SHIP1 function in macrophages

SHIP1 appears to play a very important role in the biology of macrophages. SHIP1 has been found to be a negative regulator of macrophage colony stimulating factor (M-CSF) signalling by preventing M-CSF induced Akt phosphorylation [38], suggesting that SHIP1 is important in regulating macrophage proliferation as Akt activity promotes cell survival and proliferation [39]. In $SHIP^{-/-}$ mice there is a dramatic increase in the levels of alternatively

A SHIP (145 kDa)

Btk (75 kDa)

B

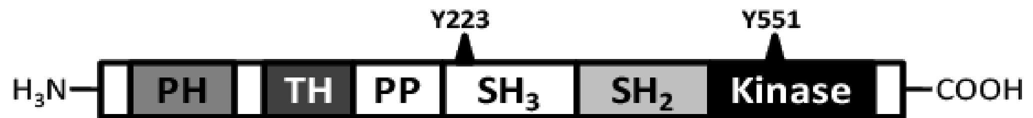


Figure 1 Domain structure of Btk and SHIP1 proteins. (A) Schematic diagram of the domain structure of SHIP1. **(B)** Schematic diagram of the domain structure of Btk. Y223 and Y551 represent tyrosine residues and phosphorylation sites 223 and 551 of human Btk. SH3 = Src homology 3 domain. SH2 = Src homology 2 domain. TH = Tec homology domain. PH = Pleckstrin homology domain. C2 = C2 domain. PR = Proline-rich region. Kinase = SH1 domain.

activated M2 macrophages. These M2 macrophages are considered to be healer macrophages as they produce considerably less NO than classical M1 macrophages, and they also express high levels of L-arginase, an enzyme which depletes arginine reserves required for the production of NO [34]. Also it has been found that SHIP$^{-/-}$ mice are much more susceptible to endotoxic shock, and SHIP1 activation during LPS induced endotoxic shock in mice leads to much higher survival rates and decreased TNFα serum levels [40]. These findings suggest that SHIP1 is an important negative regulator of the inflammatory pathway and TNFα production in macrophages. This notion is further supported by the finding that one of the anti-inflammatory effects of IL-10 on macrophages is to suppress microRNA(miRNA)-155 (miR-155) which is a suppressor of SHIP1 protein expression[41].

1.6 Bruton's tyrosine kinase and Tec kinase

Bruton's tyrosine kinase (Btk) and Tec kinase are non-receptor tyrosine kinases which are members of the Tec family kinases (TFKs) which also include interleukin-2 inducible T-cell kinase (Itk), Rlk/Txk and bone marrow kinase on chromosome X (Bmx) [42]. The TFKs are expressed almost exclusively in hematopoietic cells suggesting that they are important in immune cell function [43]. They share a structure similar to the related Src tyrosine kinases containing SH1, SH2 and SH3 domains, but they also possess an N-terminal PH domain (Figure 1). The SH3 and PH domain of Tec kinases are also separated by a Tec homology domain which contains proline-rich sequences likely involved in mediating protein interactions [44].

1.6.2 Role of Btk and Tec in B-cell and T-cell signalling

Of the Tec kinases, Btk was the first to be discovered as it was found that mutation of Btk causes X-linked agammaglobulinemia (XLA) in humans[45]. XLA is characterized by a severe deficiency in circulating B-cells which leads to frequent bacterial infections[45]. Mutations of Btk

in mice lead to a similar, although less severe condition in mice known as X-linked immunodeficiency (xid). Mutations in Btk result in XLA and xid due to the fact that Btk is required for B-cell maturation through B cell receptor (BCR) signalling [46]. Similarly, Tec is involved in T-cell receptor (TCR) signalling [47] and both Tec and Btk play an important role in phospholipase C gamma (PLCγ) activation and subsequent Ca^{2+} flux downstream of the TCR or BCR[47].

1.6.3 Role of Btk and Tec in macrophage function

The role of the TFKs in myeloid cells has been less studied but recent research has indicated an important role for the TFKs in various myeloid cell functions [48]. Macrophages express both Btk and Tec, and LPS stimulation of macrophages has been shown to lead to rapid phosphorylation of Btk and Tec [49]. Macrophages derived from XLA patients or xid mice produce less TNFα and IL-1β in response to LPS stimulation [49, 50] and have decreased expression of inducible nitric oxide synthase (iNOS) and produce less NO[51]. These results indicate that Btk function is important in LPS-TLR4 signalling in macrophages. Btk is also known to interact with a number of proteins in the TLR4 signalling pathway such as Myd88 adaptor like protein (Mal), IRAK-1, Myd88 and TLR4 itself [52] further suggesting that Btk can participate in TLR4 signalling. Btk appears to enhance LPS induced macrophage responses through two main mechanisms: p38 mediated mRNA stability and NF-κB activity. Btk overexpression in monocytes has been shown to increase the stability of TNFα mRNA and therefore lead to increased TNFα production in a p38 dependent manner [49]. Inhibition of Btk activity has also been found to result in decreased NF-κB activity following LPS stimulation in a number of studies [52-55]. Btk appears to modulate NF-κB activity through the phosphorylation of Mal [53]. The activation of NF-κB in B cells through BCR signalling is also dependent on Btk [46]. While

Tec is also activated by LPS in macrophages, there is little known about specific roles for Tec in LPS signalling in macrophages. Btk and Tec also appear to play a role in the development and survival of macrophages as Tec is required for proper macrophage signalling through the M-CSF receptor [56] and xid mice show reduced numbers of myeloid progenitors and monocytes [48]. Finally, Btk and Tec are also required for phagocytosis in macrophages as inhibiting Tec and Btk activity with the drug LFM-A13 leads to impaired phagocytosis of IgG opsonized beads in a mouse macrophage cell line, RAW264.7 [57].

1.6.4 Activation of Btk and Tec

The activation of Btk and Tec is generally considered to be a two step process. First, Btk or Tec must be localized to the membrane by the binding of their PH domain to PIP_3 [58]. This requirement of membrane localization indicates that Btk and Tec require PI3K activity for their own function however, it has been found that Btk can still be activated and function in PI3K p85α($^{-/-}$) B-cells suggesting that PIP_3 binding may not be a complete requirement for Btk activity [59]. The next step in Btk and Tec activation is their phosphorylation by an upstream kinase [58]. This is generally accomplished by the Src kinases Lyn or Syk [60, 61] and takes place on Tyrosine 551 (Y551) residue of human Btk located in the SH1 domain (Figure 1). Phosphorylation of this tyrosine residue is critical for kinase activation of Btk since phenylalanine substitution of Y551 (Y551F) showed substantially reduced enzymatic activity compared to wild-type Btk [62]. Once this tyrosine residue is phosphorylated, an α-helix that normally occludes the active site of Btk undergoes a conformational change exposing the active site and allowing kinase acitivity[63]. The activity of Btk can be further modulated by phosphorylation of Y223 of the SH3 domain, but this phosphorylation does not directly affect the active site of Btk but rather affects the association of Btk with other proteins [64].

1.6.5 Negative regulation of Btk and Tec

Btk is known to interact with a number of different proteins [44] and some of these proteins have been found to negatively regulate the activity of Btk. Inhibitor of Btk (IBtk) is a small protein that binds between the PH and TH domain of Btk resulting in inhibited Btk function[44]. Another negative regulator of Btk is SH3-domain binding protein that preferentially associates with Btk (Sab), a protein which binds to the SH3 domain of Btk resulting in the inhibited kinase activity of Btk [64]. Protein interacting with NIMA1 (Pin1) a peptidyl-prolyl cis-trans isomerase is another Btk inhibitor which can bind to Btk and promote the dephosphorylation of Btk [60]. The identification of these and other Btk interacting proteins suggest that protein-protein interaction is very important in modulating and regulating Btk activity.

As Btk and Tec traditionally require PIP_3 for their activity, negative regulators of the PI3K pathway might also inhibit Btk and Tec function. Indeed, SHIP1 has been found to negatively regulate the function of both Btk and Tec by inhibiting their membrane localization [65, 66]. Overexpression of SHIP1 in B-cells led to decreased Btk membrane association and attenuated BCR signalling [65]. The overexpression of SHIP1 in the Jurkat T-cell line also diminished function and membrane localization of Tec [66]. Interestingly, it was also found that the SH3 domain of Tec but not Btk could associate with SHIP1 [66], suggesting that SHIP1 may regulate Tec and Btk differently.

1.7 T-cell intracellular antigen

T-cell intracellular antigen 1 (TIA-1) is a small RNA-binding protein which is a member of the RNA-recognition motif (RRM) family of RNA binding proteins [67]. TIA-1 contains three RRMs which bind uridine rich sequences with high affinity [68], as well as a glutamine rich region at the carboxy-terminus which is important in mediating protein interactions[68]. Studies have

demonstrated a role for TIA-1 in a variety of processes. TIA-1 knockout mice have a high rate of embryonic lethality indicating a requirement of TIA-1 during development [69]. The splicing of various pre-mRNA molecules is also regulated by TIA-1 through its interaction with U1 small nuclear ribonucleoprotein [70]. TIA-1 function has been shown to be important in apoptosis [71], viral replication [72] and stress response in cells [73]. TIA-1 is also a well characterized translational regulator and can control the translation of target mRNA molecules which include β2 adrenergic receptor [74], β-F1 ATPase [70], mitochondrial cytochrome c [75], cyclooxygenase-2 [76] and TNFα [69]. TIA-1 specifically binds to the AU-rich element (ARE) in the 3′ untranslated region (UTR) of TNFα mRNA [69] and controls TNFα mRNA translation. Macrophages from TIA-1$^{-/-}$ mice overproduce TNFα in response to LPS and have increased levels of TNFα mRNA association with polysomes.

1.7.2 TIA-1 related protein

T-cell intracellular antigen related protein (TIAR) is also a member of the RRM family of RNA binding proteins and is highly similar to TIA-1, with 85% overall sequence similarity but much lower similarity in the C-terminal region of the protein [77]. Similar to TIA-1, TIAR binds to AREs in the 3′UTR of TNFα mRNA [78] and regulates TNFα translation. TIAR knockout mice also have a high rate of embryonic lethality and macrophages from TIAR$^{-/-}$ mice produce excess amounts of TNFα in response to LPS. One important function of TIAR is its ability to regulate the alternate splicing of TIA-1 pre-mRNAs which causes preferential levels of one TIA-1 isoform over the other [79].

1.7.3 TIA-1 and TIAR activation

The mechanism of activation of TIA-1 and TIAR is still largely unknown. During times of cell stress, phosphorylation of the translation initiation factor eIF-2a causes TIA-1 and TIAR

to shuttle target mRNAs to stress granules resulting in translational silencing [80]. However, TIA and TIA-R function in non-stressed cells as well but how their activity is regulated under these conditions is not yet known.

1.8 Translation

The translation of messenger RNA is a critical step in the regulation of protein production. While the initiation of transcription is the key first step in protein production, transcriptional regulation is complex and not rapidly modulated meaning that transcription is not a means whereby rapid changes in a cell can be achieved[81]. Translation on the other hand allows for quick regulation and therefore translational control is a mechanism allowing for rapid changes within a cell[81]. This rapid response has been taken advantage of by a number of processes in the immune system[82]. Translation can be controlled on a global scale in a cell by the regulation of translation initiation factors, or can be specific to a subset of mRNAs through the presence of regulatory elements within the mRNA molecule[83]. All mRNAs contain the same basic structure comprising a 5' 7-methylguanylate cap and a 3' poly(A) tail, both of which are important in overall mRNA stability [83]. In between these two structures exist a cap structure near the 5' end important in translation initiation, internal ribosome entry sites, and one or more open reading frames which code for the protein [83]. These basic elements of an mRNA molecule are important in controlling the three basic steps of translation: initiation, elongation and termination.

1.8.2 Initiation

Translation initiation is the most complex process of translation and therefore is the main mechanism of translational regulation. Translation itself is carried out by ribosomes which are composed of 40S and 60S subunits. Initially, the 40S subunit forms a 43S pre-initiation complex

by binding to global translation initiation factors [83]. These initiation factors interact with the 5' 7-methylguanylate cap and facilitate the loading of the 43S pre-initiation complex onto the mRNA molecule[84]. Once loaded, the 43S complex will scan along the mRNA molecule in a 5' to 3' direction until it encounters an initiation codon which is usually the first AUG found on the mRNA[85]. However, sometimes the 43S complex will bypass the first initiation codon and instead continue to scan the mRNA until it encounters an internal ribosome entry site[86]. Once at the initiation site, an initiator tRNA will bind to the AUG sequence which then allows for the binding of the 60S ribosomal subunit to form the 80S ribosome complex [83]. During this process of tRNA binding and 60S binding, many of the initiation factors associated with the 43S pre-initiation complex dissociate. At this stage translation elongation will begin and the protein will start to be produced.

1.8.3 Elongation and termination

Once the 80S ribosome complex is associated with an mRNA, an amino-acyl tRNA will bind in the A-site of the ribosome based upon the sequence of the next codon of the mRNA molecule. A peptide bond is then formed between the preceding amino acid located in the P-site of the ribosome complex and the amino acid in the A-site [83]. After this, the tRNA in the P-site, which is no longer linked to its amino acid, will then migrate to the E-site of the ribosome allowing it to dissociate from the 80S ribosome complex, while the tRNA in the A-site moves to the P site. This opens up the A-site for a new tRNA to bind based upon the sequence of the next codon of the mRNA. This process of elongation continues until a stop codon is reached which causes release factors to bind to the A-site of the 80S ribosome complex leading to the dissociation of the ribosome subunits from the mRNA molecule. In addition to these stop codons, other proteins have been found to be able to cause the release of the 60S ribosome

subunit halting translation [83]. However, in this case of termination the 40S subunit continues to scan along the mRNA [83].

1.8.4 Regulation of translation

As initiation is the key step in translation, the majority of translational regulation occurs at this initiation step. Because of this, the number of ribosomes associated with a particular mRNA molecule is generally a good indicator of the level of translation of that mRNA. Following from this, mRNA molecules associated with only one ribosome (monosomes) are thought to be less translationally active than mRNA molecules associated with multiple ribosomes (polysomes). The main mechanism of translation initiation regulation is through the control of the initiation factors. This is achieved by modulating their phosphorylation state [83]. However, the degradation of initiation factors has also been found to be important in regulating translational activity[87]. As the same set of initiation factors are involved in the initiation of translation for all mRNA molecules, changes in the activity of these factors lead to the global translational regulation of all mRNA molecules[83].

Specific regulation of mRNA molecules often involves the sequences found in the 3' UTR of a mRNA molecule. Sequences in this region are bound by regulatory proteins which interfere with the loading of the 43S pre-initiation complex, often through steric hinderence of the initiation factors[88]. Aside from their role in inhibiting initiation, these sequences can also be bound by proteins which regulate the stability of a particular mRNA, and this has direct consequences on the amount of protein produced by that mRNA. These 3'UTRs are particularly important in inflammation as a large number of pro-inflammatory mediators contain 3'UTRs in their mRNA [89]. Finally, there can be spatial regulation of mRNA translation whereby specific

mRNA molecules are transported to a particular location of a cell before translation occurs. This can help lead to cell polarization and facilitate asymmetric cell division[90].

1.9 Hypothesis

IL-10 function is pivotal in the body for the regulation of the inflammatory process as well as the control and suppression of multiple diseases. Activated macrophages are one of the main targets of IL-10 and the inhibited production of TNFα in activated macrophages by IL-10 contributes largely to its ability to regulate inflammation. While the STAT3 pathway is important in IL-10 induced suppression of TNFα production, there is reason to believe that IL-10 also signals through additional STAT3-independent pathways. We believe that one of these STAT3 independent pathways utilized by IL-10 involves the protein SHIP1 as SHIP1 activity can suppress TNFα production in LPS stimulated macrophages. Specifically, we hypothesize that IL-10 utilizes SHIP1 to induce the dissociation of TNFα mRNA away from polysomes in LPS activated macrophages, thereby leading to decreased TNFα production. This IL-10-SHIP1 pathway should function independent of STAT3. Herein we demonstrate that IL-10 requires SHIP1 protein to dissociate TNFα mRNA from polysomes in activated macrophages and that this occurs early in the IL-10 AIR. We also identify Btk as another protein utilized by IL-10 to suppress TNFα production in activated macrophages.

2 Materials and methods

2.1 Reagents and cell lines

All reagents used in the described experiments were obtained from Sigma-Aldrich (Oakville ON, Canada) unless otherwise stated. The RAW264.7 mouse macrophage cell line was obtained from Dr. Neil Reiner (University of British Columbia). The following primary antibodies were used: SHIP1 p150 (BD Biosciences, Mississauga ON, Canada) Catalogue # 611334, STAT3 (BD Biosciences, Mississauga ON, Canada) Catalogue #S21320, p38 MAPK (Santa Cruz Biotechnology, Santa Cruz CA) Catalogue # sc-535, Btk (Santa Cruz Biotechnology, Santa Cruz CA) Catalogue # sc-1696, TIA-1/TIAR (Santa Cruz Biotechnology, Santa Cruz CA) Catalogue # sc-48371, Tec (Millipore, Etobicoke ON, Canada) Catalogue # 06-561.

2.2 Cell culture

All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Fisher Scientific, Toronto ON, Canada) supplemented with 9% (v/v) fetal calf serum (FCS) (Fisher Scientific, Toronto ON, Canada) which had been heat inactivated by incubation at 56°C for 90 minutes to inactive complement proteins contained in the serum. Cells were passaged every 48 hours and plated at $2.5x10^{6}$ cells in 10 mL of medium on a 10 cm tissue culture dish (Fisher Scientific, Toronto ON, Canada). All cells were maintained at 37°C and 5% CO_2.

2.3 Bacterial transformation and vector purification

For all transformations 2 μL of plasmid was added to one vial of DH5α chemically competent *Escherichia coli* (Invitrogen, Burlington, ON, Canada) and incubated for 5 minutes on ice. The mixture was then heat shocked for 30 seconds at 42°C and placed on ice. 250 μL of

room temperature Luria-Bertani medium was then added and the mixture was incubated for 60 minutes at 37°C on a shaker at 200 revolutions per minute (rpm). The mixture was then plated onto LB-Agar plates and grown overnight at 37°C to produce colonies. For plasmid purification QIAprep Spin Miniprep Kit (QIAGEN, Mississauga ON, Canada) was used according to the manufacturers' protocol.

2.4 Generation of pTRIPZ-siRNA vectors

siRNA oligonucleotides targeting mRNA of interest were designed using BLOCK-iT™ siRNA online design software (Invitrogen, Burlington, ON, Canada) and obtained as single stranded oligonucleotides (Invitrogen, Burlington, ON, Canada). An annealing reaction was performed between single stranded oligonucleotides by incubating 10 μM of each oligonucleotide for 4 minutes at 95°C in annealing buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 100 mM NaCl) and then letting the resulting double stranded oligonucleotide cool to room temperature. The double stranded oligonucleotide was then used in a ligation reaction with a modified pcDNA6.2-EmGFP (Dr. Deider Fink, University of British Columbia) that had been linearized by a restriction enzyme digestion with Bsa I (New England Biolabs, Pickering ON, Canada). 10 ng of linearized pcDNA6.2-EmGFP vector was incubated with 1 ng of double stranded oligonucleotide and 1 unit of T4 DNA Ligase (Invitrogen, Burlington, ON, Canada) in T4 Ligation Buffer (Invitrogen, Burlington, ON, Canada) for 2 hours at 24°C. Ligated plasmids were then transformed into DH5α component *E. coli* and purified to obtain pcDNA6.2-EmGFP-siRNA vectors. 300 ng of pcDNA6.2-EmGFP-siRNA vector and 300 ng of pDONR-221 vector (Invitrogen, Burlington, ON, Canada) were used in a BP recombination reaction by adding BP-Clonase™ mix (Invitrogen, Burlington, ON, Canada), incubating at 24°C for 1 hour and then adding 1 unit of Proteinase K (Invitrogen, Burlington, ON, Canada) and incubating for 10

minutes at 37°C. Recombined plasmids were then transformed into DH5α component *E. coli* and purified to obtain pENTR-221-siRNA vectors. 150 ng of pENTR-221-siRNA vector was combined with 150 ng of pTRIPZ-Dest vector (Fisher Scientific, Toronto ON, Canada) and LR Clonase™ mix (Invitrogen, Burlington, ON, Canada) and incubated for 60 minutes at 24°C in a LR recombination reaction. 1 unit of Proteinase K was then added and the mixture was incubated at 37°C for 10 minutes. Recombined plasmids were transformed into DH5α competent *E. coli* and purified to obtain pTRIPZ-siRNA vectors. BP and LR Clonase™ technology is based upon *E. Coli* bacteriophage lambda recombination which uses integrase enzyme to excise and integrate the bacteriophage genome into host bacterial genomes.

2.5 Lentiviral transduction

Lentivirus was produced from generated pTRIPZ-siRNA vectors by Mr. Rupinder Dhesi (Vancouver Costal Health Research Center, Vancouver Canada). RAW264.7 cells were seeded at $7.5x10^{4}$ cells per well onto 96 well plates in 200 μL of medium and grown overnight. The next day, lentivirus was diluted 1:20, 1:40 or 1:80 in DMEM supplemented with 9% (v/v) FCS and 8 μg/mL protamine sulphate. Media was removed from each well containing RAW264.7 cells and 50 μL of diluted lentivirus was added and cells incubated for 6 hours. Medium was then removed and 200 μL DMEM medium supplemented with 9% (v/v) FCS was added and cells were grown overnight. The next day cells in each well were transferred into 24 well plates containing 1 mL of DMEM medium supplemented with 9% (v/v) FCS and 3 μg/mL puromycin. Cells were maintained in puromycin-containing medium for 5 days to allow for drug selection. Cells were then incubated for 24 hours with 9% (v/v) FCS DMEM supplemented with 2 μg/mL doxocycline to induce expression of GFP-siRNA constructs. These cells were then sorted for

GFP fluorescence with the top 10% GFP fluorescent cells being kept. These cells became the siRNA transduced cell lines used in experiments.

2.6 Immunoblotting and analysis

Cells were seeded at 6.25×10^5 cells per well on 6-well plates and grown for 48 hours in either the presence or absence of 2 μg/mL doxocycline. Media was removed and cells were lifted in 1 mL of cold Dulbecco's Phosphate Buffered Saline (PBS) (Fisher Scientific, Toronto ON, Canada). Cells were pelleted by centrifugation at 1,500 rpm for 5 minutes at 4°C. The PBS supernatant was removed and cells were resuspended in 150 μL of PSB (50 mM HEPES, 100 mM NaF, 10 mM NaPPi, 2 mM Na_3VO_4 , 2 mM MoO_4 and 5 mM EDTA, pH 7.5) supplemented with 1% Nonidet P-40 (Roche Diagnostics, Mississauga ON, Canada) and Protease Inhibitor Cocktail (Roche Diagnostics, Mississauga ON, Canada) and incubated for 30 minutes on a rocker at 4°C. Samples were then centrifuged at 14,000 rpm for 10 minutes at 4°C to pellet cell debris and nucleii. 125 μL of supernatant was added to 125 μL of sodium dodecyl sulfate (SDS) sample buffer (35% glycerol, 20% 2-Mercaptoethanol (BIO-RAD, Mississauga ON, Canada) and 45% SDS). Equal volumes of samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto Immobilon polyvinylidene difluoride membrane (Millipore, Etobicoke ON, Canada) at 3 mAmp/cm^2 of membrane for 35 minutes. Membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered Saline supplemented with 0.1% Tween (TBS-T) for 60 minutes at 24°C. Membranes were then rinsed in TBS-T and incubated with primary antibody overnight at 24°C. Membranes were washed 3 times in TBS-T for 5 minutes and incubated with Alexa-Fluor 680® secondary antibodies (Invitrogen, Burlington, ON, Canada) diluted 1:10,000 in TBS-T for 45 minutes. Membranes were then washed 3 times in TBS-T for 5 minutes and imaged on a LI-COR

Odyssey system (LI-COR, Lincoln NB, USA). Densitometrical analysis was performed using Odyssey 2.1 software by taking the integrated intensity of each protein band, normalizing to the integrated intensity of an appropriate endogenous control (p38 or STAT3) and calculating the normalized value relative to samples from non doxocycline treated cells.

2.7 Sucrose gradient fractionation and RNA purification

Two days prior to stimulation, cells were seeded at $8x10^6$ cells onto a 10 cm plate in 10 mL of media supplemented with 2 μg/mL doxocycline and grown overnight. Cells were then lifted and seeded at $8x10^6$ cells onto a 10 cm plate in 10 mL of media supplemented with 2 μg/mL doxocycline and grown overnight for stimulation the next day. For stimulations, media was removed and 5 mL of fresh 9% (v/v) FCS DMEM was added and cells were incubated for 60 minutes. LPS was then added to a final concentration of 1 ng/mL and cells were incubated for 45 minutes. For IL-10 stimulated cells, IL-10 was then added to a final concentration of 100 ng/mL and cells were incubated for an additional 15 minutes. For cells stimulated with LPS alone, IL-10 was not added and cells were incubated for 15 minutes. After stimulation, media was removed and cells were washed in 5 mL cold PBS. Lysates were prepared by adding 500 uL of Lysis Buffer (10 mM KCl, 10 mM Tris-Cl, 10 mM $MgCl_2$, 20 mM Dithiothreitol, 150 ug/mL cycloheximide, 0.5% NP-40, and 500 Units of RNase Protector (Roche Diagnostics, Mississauga ON, Canada)) to each plate and lifting cells using a cell scraper. Lysates were then incubated for 30 minutes at 4°C on a rocker and then centrifuged at 14,000 rpm for 10 minutes at 4°C. 70%, 50% or 30% sucrose solutions (10 mM KCl, 10 mM Tris-Cl, 10 mM $MgCl_2$, 20 mM DTT, 150 μg/mL cycloheximide, 100 U/mL RNase Protector and sucrose) were prepared. 500 μL of lysate supernatants were added to 125 μL of 50% sucrose solution to make 10% sucrose lysates. 450 μL of this solution was then layered onto the top of a sucrose step gradient (450 μL

of 30% sucrose solution on top of 250 μL of 50% sucrose solution on top of 200 μL of 70% sucrose solution in a 1.5mL ultracentrifuge tube (Beckman, Mississauga ON, Canada)). Gradients were centrifuged at 60,000 rpm for 35 minutes in a TL-100.4 rotor using a TL-100 Tabletop Ultracentrifuge (Beckman, Mississauga ON, Canada). Starting from the top, 10 125 μL fractions were collected from the sucrose gradients. For each fraction, 150 ng of human RNA was then added as well as 950 μL of TRIZOL® Reagent (Invitrogen, Burlington, ON, Canada). RNA was then purified from TRIZOL® solution according to manufacturer's protocol and resuspended in 15 μL of diethyl pyrocarbonate (DEPC) treated water.

2.8 DNase treatment and cDNA synthesis

For each sample, 7 μL of purified RNA was combined with 2.5 μL of 5X DNase I Incubation Buffer (Roche Diagnostics, Mississauga ON, Canada), 10 units of DNase I Enzyme (Roche Diagnostics, Mississauga ON, Canada) and 14.5 μL of DEPC water and this mixture was incubated for 30 minutes at 30°C. 2 μL of 0.1 M EDTA was added and the mixture was then incubated at 75°C for 10 minutes to terminate the reaction. For cDNA synthesis reactions, Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mississauga ON, Canada) was used according to manufacturer's protocol. 5 μL of DNAse treated samples was used as a template for all cDNA synthesis reactions. The resulting cDNA was diluted 1:4 in DEPC water prior to use in quantitative PCR.

2.9 Quantitative-PCR and analysis

Quantitative PCR (qPCR) analysis of cDNA samples was done to determine levels of mouse TNFα mRNA and human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. For detection of TNFα mRNA levels, Taqman® probe-based detection was used. For each

reaction, 5 μL of template was added to 250 nM Universal ProbeLibrary Probe #49 (Roche Diagnostics, Mississauga ON, Canada), 900 nM of TNFα forward primer (5`TCTTCTCATTCCTGCTTGTGG3′), 900nM of TNFα reverse primer (5`GGTCTGGGCCATAGAACTGA3′), 1X Faststart Universal Probe Master (Rox) mix (Roche Diagnostics, Mississauga ON, Canada) and DEPC water in 15 μL total volume. For detection of GAPDH, SYBR green based detection was used. 5 μL of template was added to 300 nM of GAPDH-forward primer (5′AGCCACATCGCTCAGACAC3′), 300 nM of GAPDH-reverse primer (5′GCCCAATACGACCAAATCC3′), 1X Faststart SYBR Green (Rox) (Roche Diagnostics, Mississauga ON, Canada) and DEPC water in 15 μL total volume. For both TNFα mRNA and GAPDH mRNA detection, each sample was run in triplicate on a 96-well optical plate (Applied Biosystems, Foster City CA) using an ABI 7300 Real Time PCR System (Applied Biosystems, Foster City CA). Ct values for all samples were determined using ABI 7300 Real Time PCR Software (Applied Biosystems, Foster City CA). To quantify levels of TNFα mRNA the Comparative Ct method of quantification was used [91]. Briefly, for each sample the Ct value for GAPDH was subtracted from the Ct value for TNFα of the same sample to give ΔCt. This normalized value was then compared to the normalized value of an external standard (cDNA from RNA purified from RAW264.7 cells stimulated for 2 hours with 1 ng/mL LPS) by subtracting the normalized value of the standard from the normalized value for all other samples to give ΔΔCt. The level of TNFα mRNA in each sample relative to the external standard was then determined by taking $2^{-\Delta\Delta Ct}$. To calculate percent TNFα mRNA in each fraction, the sum of the relative values of TNFα mRNA in all 10 fractions were added to give the total level of TNFα mRNA recovered. The level of TNFα mRNA in each fraction was then divided by this value to give percent TNFα mRNA as previously described[92]. A validation experiment between mouse

TNFα primers and human GAPDH primers was also performed to ensure that PCR efficiencies were within 5% of each other [91]. All qPCR reations were performed with both a no template control (DEPC Water Only) and a No Reverse-transcriptase control (DNase treated RNA incubated without reverse transcriptase in cDNA synthesis reaction) to control for the presence of genomic DNA.

2.10 Quantification of TNFα production in LPS and IL-10 stimulated macrophages

Cells were seeded at 5×10^6 cells onto a 10 cm plate in 10 mL of 9% (v/v) FCS DMEM and grown overnight either in the presence or absence of 2 μg/mL doxocycline. Cells were then lifted and seeded at 5×10^4 cells/well in a 96-well plate in 200 μL of 9% (v/v) FCS DMEM supplemented with or without 2 μg/mL doxocycline and grown overnight. For stimulations, medium was removed and 250 μL of 9% (v/v) FCS DMEM was added to each well and cells were incubated for 45 minutes. 50 μL of medium containing LPS or LPS and IL-10 was then added to each well to give the desired final concentration of LPS and IL-10. Cells were then incubated for 60 minutes and 100 μL of cell supernatants were collected. These supernatants were diluted 1:1 in blocking buffer (10% FCS in PBS) and assayed for TNFα concentration by enzyme-linked immunosorbant assay (ELISA) using BD OptEIA™ Mouse TNF ELISA Set II kit (BD Biosciences Mississauga ON, Canada) according to manufacturer's protocol. For IL-10 stimulations, percent maximum TNFα was calculated by taking the concentration of TNFα produced in the presence of LPS and IL-10 and dividing by the concentration of TNFα produced in the presence of LPS alone.

2.11 Statistical analysis

ANOVA analyses and students t-tests were performed using GraphPad Prism4 software.

3 Results

3.1 Inducible knockdown of SHIP1 and STAT3

To investigate the role of SHIP1 and STAT3 in the IL-10 AIR, RAW264.7 mouse macrophages with small interfering RNA (siRNA) mediated knockdown of SHIP1 or STAT3 were generated. As STAT3 is required for cell growth and survival [93], and over time, cells can compensate for the loss of a particular protein, the knockdown of SHIP1 and STAT3 was required to be inducible. To accomplish this, siRNA targeting SHIP1 or STAT3 was designed and cloned into a miRNA expression vector (pTRIPZ) under the control of a doxocycline/tetracycline inducible promoter. BLOCK-iT™ siRNA design software from Invitrogen was used to create siRNA against target protein SHIP1 or STAT3 mRNA. The siRNA designed by this program mimics the structure and the function of miRNA, a type of of siRNA found in cells. miRNAs bind to target mRNA through sequence complementarity and either repress the translation of the mRNA or target the mRNA for degradation leading to a decrease in the levels of target protein over time. A non-specific siRNA (Scrambled siRNA) which does not target any mRNA was also designed to serve as a control for siRNA expression. The single stranded oligonucleotides designed by the BLOCK-iT™ software (Table 1) were annealed together to create double stranded siRNA with single stranded RNA overhangs on both the 5′ and 3′ end of the siRNA. These double stranded siRNAs were ultimately inserted into the pTRIPZ lentiviral vector using the cloning scheme outlined in Figure 2. Briefly, a ligation reaction between the double stranded siRNAs and linearized pcDNA 6.2-GW/EmGFP-miR vector was performed to create pcDNA 6.2-GW/EmGFP-miR-siRNA vectors. The pcDNA 6.2-GW/EmGFP-miR was used because the insertion site for the siRNA is flanked by 5′ and 3′ microRNA-like (miR) sites which facilitate the expression and processing of the inserted siRNAs

Target	Top Oligonucleotide	Bottom Oligonucleotide
SHIP1	5'-TGCTGATTCTCTCCTTCCTGACTCTTGTTTTGGCCACTGACTGACAAGAGTCAAAGGAGAGAAT-3'	5'-CCTGATTCTCTCCTTTGACTCTTGTCAGTCAGTGGCCAAAACAAGAGTCAGGAAGGAGAGAATC-3'
STAT3	5'-TGCTGGTTGTTAGACTCCTCCATGTTGTTTTGGCCACTGACTGACAACATGGAGTCTAACAACC-3'	5'-CCTGGTTGTTAGACTTCCATGTTGTCAGTCAGTGGCCAAAACAACATGGAGGAGTCTAACAACC-3'
Scrambled	5'-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3'	5'-CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTC-3'

Table 1 siRNA Oligonucleotides targeting SHIP1, STAT3 or Scrambled Control. The sequences of oligonucleotides used to generate SHIP1, STAT3 or Scrambled siRNAs.

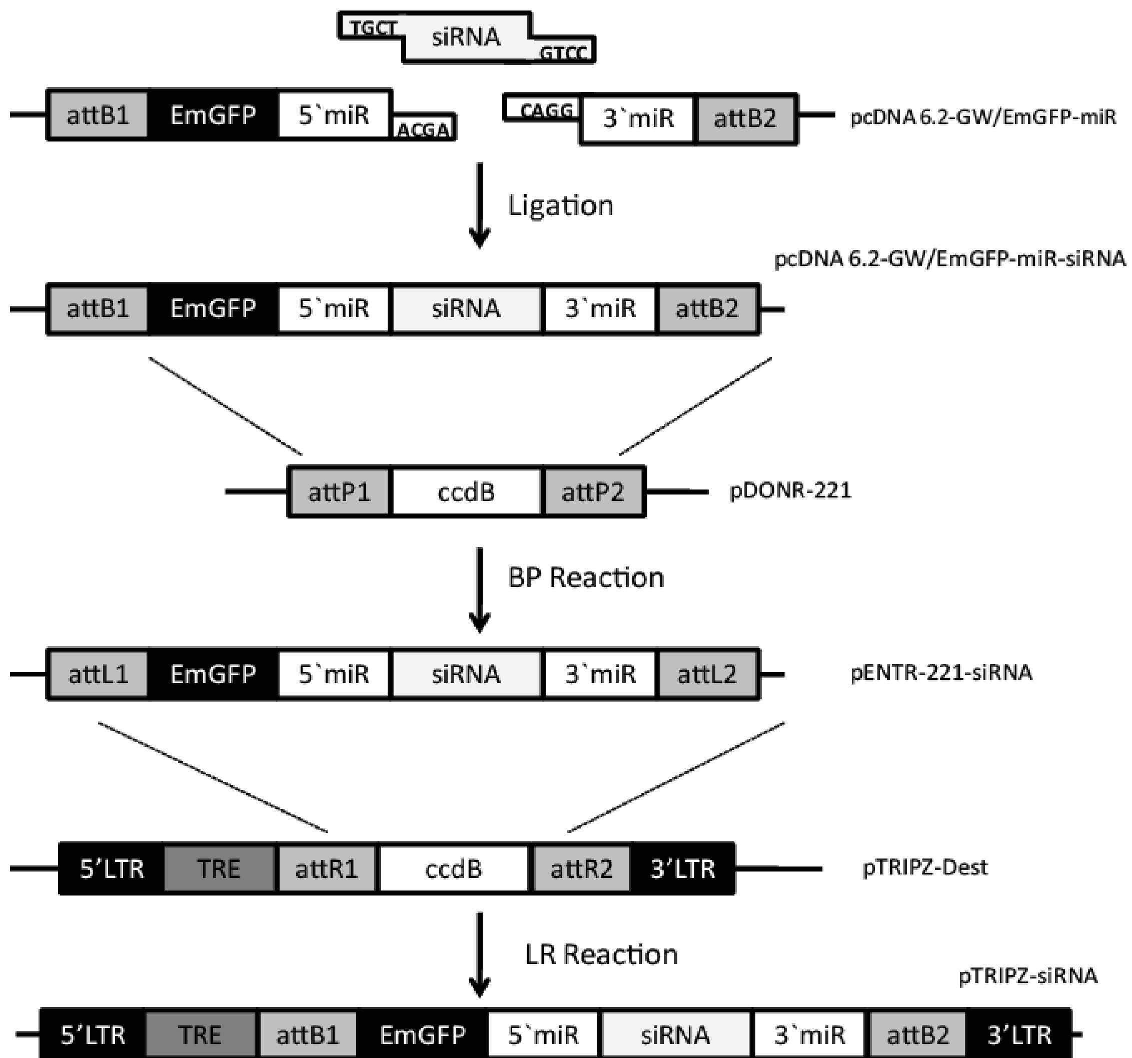


Figure 2 Cloning strategy for generation of drug inducible siRNA lentiviral vectors. Double stranded siRNA designed using BLOCK-iT design software was annealed into linearized pcDNA 6.2-GW/EmGFP-miR vector via complementary overhanging nucleotides to create pcDNA 6.2-GW/EmGFP-miR-siRNA. A BP recombination reaction was then performed between pcDNA 6.2-GW/EmGFP-miR-siRNA and pDONR-221 to generate the pENTR-221-siRNA vector. A LR recombination reaction between the generated pENTR-221-siRNA vector and pTRIPZ-Dest vector was then performed to create the pTRIPZ-siRNA vector used for lentiviral production. attB = attB recombination site. EmGFP = GFP gene. miR = microRNA-like sequence. attP = attP recombination site. attL = attL recombination site. attR = attR recombination site. TRE = tetracycline response element.

as miRNA. The insertion site also contains an upstream gene encoding for green fluorescent protein (GFP) which allows co-expression of both GFP and the siRNA resulting in the ability to track siRNA expression through GFP fluorescence. Once the pcDNA 6.2-GW/EmGFP-miR-siRNA vectors were generated they were used in a BP recombination reaction with pDONR-221 vector to create pENTR-221-siRNA vectors which contained attL recombination sites flanking the siRNA insert. These sites allowed for a LR recombination reaction to be performed with a pTRIPZ vector modified to contain attR recombination sites (pTRIPZ-Dest) to create pTRIPZ-siRNA vectors. The pTRIPZ vector is a lentiviral vector and contains a tetracycline response element (TRE) which in the presence of tetracycline or doxocycline allows for the induced expression of the GFP-siRNA insert.

Lentivirus was produced from the pTRIPZ-siRNA vectors and this was used to transduce the immortalized mouse macrophage cell line RAW264.7. After transduction, cells were drug selected in 3 μg/mL puromycin for five days to allow for the death of any non-transduced cells. Drug selected cells were treated for 24 hours with 2 μg/mL of doxocycline to induce GFP and siRNA expression and cells were then sorted for the highest GFP expressing cells (top 10%) as it was found that cells with high levels of GFP expression resulted in greater protein knockdown. This may be due to multiple insertions of the GFP-siRNA constructs into the host genome resulting in greater siRNA and GFP expression. The resulting cells became the SHIP1 siRNA, STAT3 siRNA and Scrambled siRNA transduced cell lines used in subsequent experiments.

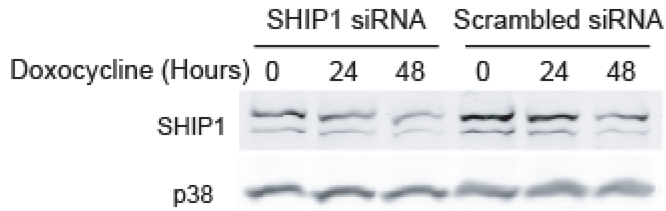
To determine the amount of protein knockdown by expression of the siRNA constructs, SHIP1 siRNA, STAT3 siRNA and Scrambled siRNA transduced cells were treated with 2 μg/mL doxocycline for 0, 24 or 48 hours. Treatment of the STAT3 siRNA cells with doxocycline for longer periods of time (72 hours or more) resulted in decreased cell viability

based upon trypan blue exclusion staining. This suggests that cells cannot survive with prolonged knockdown of STAT3. Lysates from the siRNA transduced cells were analyzed by immunoblotting for SHIP1 and STAT3 protein as well as p38 MAPK as an endogenous control (Figure 3). It was found that induction of the SHIP1 siRNA resulted in approximately 70% reduction in SHIP1 protein levels compared to untreated cells after 48 hours of doxocycline treatment. However, expression of the STAT3 siRNA only resulted in a 50% reduction in STAT3 protein levels as compared to untreated cells. This modest degree of STAT3 knockdown makes it hard to rule out the ability for STAT3 to still function in STAT3 siRNA transduced cells and therefore any attempts to identify the individual role of STAT3 in the IL-10 AIR using this cell line would be subject to the criticism that STAT3 may still function in the cells. This is especially important as STAT3 is widely regarded as being necessary and sufficient for all of the effects of the IL-10 AIR. Therefore, without complete knockdown of STAT3 protein, the individual role of STAT3 in the IL-10 AIR could not be adequately probed using the STAT3 siRNA transduced cell line.

3.2 SHIP1 is required for IL-10 to dissociate TNFα mRNA from polysomes.

IL-10 has previously been reported to dissociate TNFα mRNA from polysomes in activated mouse bone marrow derived macrophages (BMDMs) [26]. To see if IL-10 has the same effect in RAW264.7 cells, small scale sucrose gradient fractionation of total cell lysates was used to fractionate RNA associated with monosomes and polysomes. To look specifically at TNFα mRNA in these fractions, RT-qPCR was used to measure the amount of TNFα mRNA in each fraction as described in Materials and Methods. In RAW264.7 cells stimulated with 1 ng/mL LPS for 60 minutes, the majority of TNFα mRNA was detected in the polysome associated fractions 8 and 9 as well as the monosome associated fraction 4 (Figure 4). However, in

A



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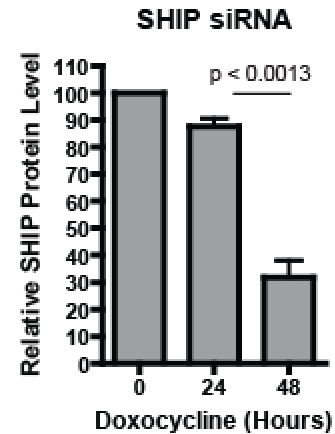

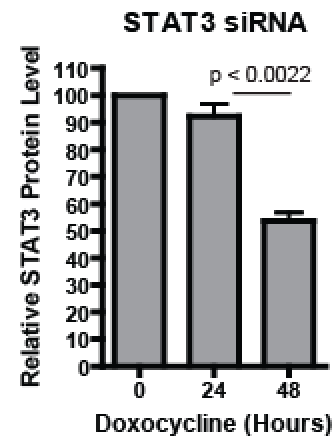

Figure 3 Drug inducible knockdown of SHIP1 and STAT3 protein. **(A)** Immunoblot analysis of SHIP1 and STAT3 protein knockdown. SHIP1 siRNA, STAT3 siRNA or Scrambled siRNA transduced macrophages were treated with 2 μg/mL doxocycline for 0, 24 or 48 hours. Cell lysates were prepared as described in Materials and Methods and analyzed by immunoblot for SHIP1, STAT3 and p38 protein levels. **(B)** Quantification of SHIP1 and STAT3 knockdown. Densitometry was performed using Odyssey 2.1 software on immunoblots to quantify the levels of SHIP1, STAT3 and p38 protein. Levels of SHIP1 or STAT3 protein were normalized to levels of p38 protein. Normalized values were then determined relative to siRNA transduced cells treated with 2 μg/mL doxocycline for 0 hours. Results represent 3 independent experiments. P-values are based on a two tailed unpaired Students t-test with a confidence interval of 95%.

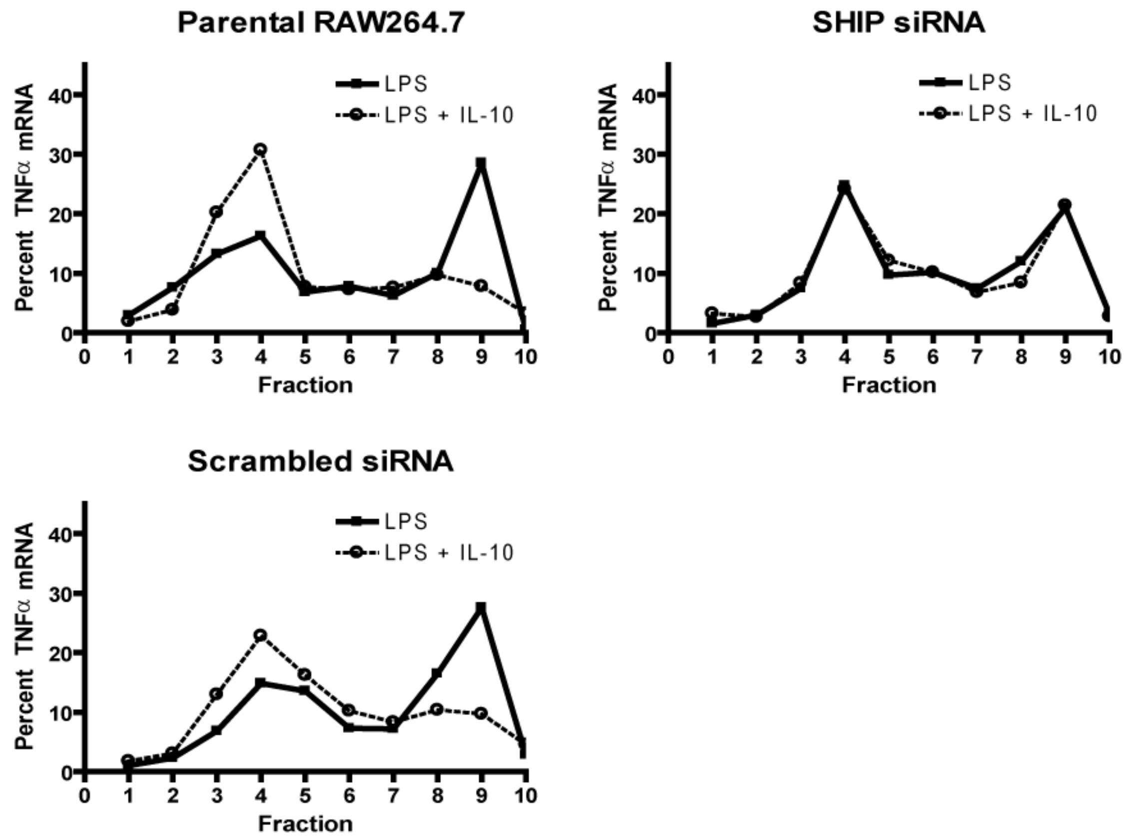

Figure 4 Polysome fractionation of RNA from RAW264.7, SHIP1 siRNA and Scrambled siRNA transduced cells. Cells treated for 48 hours with 2 μg/mL doxocycline were stimulated with 1 ng/mL LPS for 45 minutes. IL-10 was then added to a final concentration of 100 ng/mL or not added and cells were stimulated for an additional 15 minutes. Cell lysates were then prepared and subjected to sucrose gradient fractionation and RT-qPCR analysis as described in Materials and Methods. Percent TNFα mRNA in each fraction was determined by taking the relative TNFα signal from each fraction as a percent of the sum of the TNFα mRNA signal from all 10 fractions. Fraction 1 indicates the top of the sucrose gradient. Results are representative of at least 2 independent experiments.

macrophages stimulated with 1 ng/mL LPS for 45 minutes and then treated with 100 ng/mL IL-10 for 15 minutes, no TNFα mRNA was found associated with the polysomal fractions and there was a corresponding increase in the TNFα mRNA associated with monosomal fractions. This confirmed that IL-10 had the ability to dissociate TNFα mRNA away from polysomes in RAW264.7 macrophages. Importantly, this effect of IL-10 was rapid, occurring within 15 minutes of IL-10 stimulation. This shows that this is an early effect of IL-10 on macrophages which suggests STAT3 independence. Longer periods of IL-10 stimulation were not tested.

To determine the potential contribution of SHIP1 to the IL-10 induced dissociation of TNFα mRNA from polysomes in activated macrophages, lysates from SHIP1 siRNA transduced cells treated for 48 hours with doxocycline to induce SHIP1 knockdown were analyzed by sucrose gradient fractionation as described for parental RAW264.7 cells. LPS stimulation of these knockdown cells once again led to association of TNFα mRNA with both polysomes and monosomes. However, when these cells were then stimulated with IL-10 it was found that IL-10 was no longer able to dissociate TNFα mRNA away from polysomes as indicated by the high level of TNFα mRNA associated with the polysome fraction (Figure 4). To ensure that the expression of the siRNA-GFP constructs within the macrophages was not the reason for IL-10's inability to dissociate TNFα mRNA from polysomes, the same experiment was performed with lysates from Scrambled siRNA transduced cells treated with doxocycline for 48 hours. The ability for IL-10 to dissociate TNFα mRNA from polysomes was found to be fully functional in these Scrambled siRNA transduced cells as compared to Parental RAW 264.7cells (Figure 4). Taken together, these results demonstrate that IL-10 requires SHIP1 to induce the dissociation of TNFα mRNA from polysomes in LPS activated macrophages.

3.3 SHIP1 is required for early IL-10 inhibition of TNFα production in LPS activated macrophages

A decrease in the association of a particular mRNA with polysomes diminishes the translational efficiency of that mRNA molecule. Therefore, a decrease in the amount of TNFα mRNA associated with polysomes in RAW264.7 macrophages should be reflected in decreased TNFα protein being produced and secreted by these cells. Since SHIP1 is required for the dissociation of TNFα mRNA from polysomes by IL-10, we expect that SHIP1 will also be required for the decrease in TNFα secretion in response to IL-10 treatment. To investigate this, RAW264.7, SHIP1 siRNA transduced cells and Scrambled siRNA transduced cells were treated with doxocycline for 48 hours to induce protein knockdown or were incubated without doxoxcycline treatment. Equal numbers of these cells were then stimulated with LPS ± IL-10 for one hour and cell supernatants were collected and analyzed for TNFα concentration by ELISA. This one hour time point was chosen as LPS induced TNFα production is only detectable by ELISA at time points of 30 minutes or later. In parental RAW264.7 cells and Scrambled siRNA transduced cells it was found that IL-10 inhibited TNFα production by approximately 15% and 30% respectively, and that treatment with doxocycline did not affect this inhibition by IL-10 (Figure 5). However, in SHIP1 siRNA transduced cells treated with doxocycline for 48 hours, IL-10 was only able to inhibit TNFα production by approximately 5%. This is in contrast to SHIP1 siRNA-transduced cells not treated with doxocycline where IL-10 was still capable of inhibiting TNFα production by about 25%. This finding indicates that IL-10 requires SHIP1 to inhibit TNFα production in activated macrophages early on in IL-10 stimulation and is also in agreement with experiments using SHIP^{-}/$^{-}$ peritoneal macrophages (data unpublished). It also

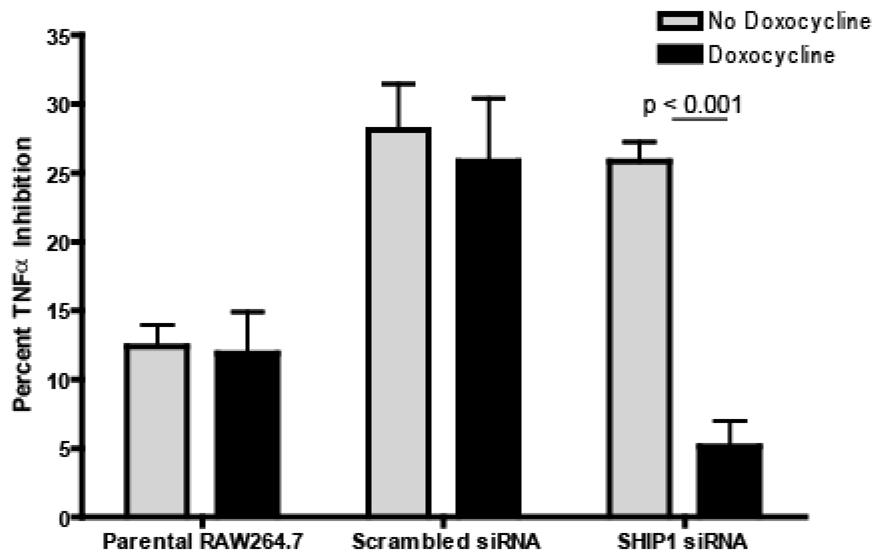

Figure 5 IL-10 responsiveness in Parental RAW264.7, SHIP1 siRNA and Scrambled siRNA transduced cells. Cells treated with or without 2 μg/mL doxocycline for 48 hours were stimulated with 1 ng/mL LPS or 1 ng/mL LPS and 15 ng/mL IL-10 for 1 hour. Cell supernatants were then collected and the concentration of TNFα in the supernantants was determined by ELISA. Percent inhibition TNFα was calculated by dividing the amount of TNFα produced in the presence of LPS + IL-10 by that produced in the presence of LPS alone. Each experiment was carried out in biological quadruplicates and results are based upon the average ± SEM of 6 independent experiments. Total average TNFα production ± SEM in response to LPS stimulation for Parental RAW264.7, Scrambled siRNA and SHIP1 siRNA transduced cells not treated with doxoxycline is 725 ± 197 pg/mL, 1074 ± 269 pg/mL and 734 ± 137 pg/mL respectively. Total average TNFα production ± SEM in response to LPS stimulation for Parental RAW264.7, Scrambled siRNA and SHIP1 siRNA transduced cells treated doxoxycline is 569 ± 154 pg/mL, 946 ± 251 pg/mL and 514 ± 80 pg/mL respectively. P-values are based upon two-way ANOVA analysis.

correlates with the requirement of SHIP1 by IL-10 to dissociate TNFα mRNA from polysomes suggesting that the decrease in early TNFα production by IL-10 is at least in part through a translational mechanism. The ability for IL-10 to inhibit TNFα production in STAT3 siRNA transduced cell lines was not tested due to the modest level of STAT3 protein knockdown.

3.4 Inducible knockdown of Btk and TIA-1

Having determined that SHIP1 is required for IL-10 to induce the dissociation of TNFα mRNA from polysomes in LPS activated macrophages, the potential downstream target of SHIP1 in this response was next investigated. Four proteins were evaluated as potential SHIP1 downstream mediators: Btk, Tec, TIA-1 and TIAR. Btk and Tec are both known to be activated by LPS and Btk activity can enhance TNFα production in LPS stimulated macrophages through the p38 MAPK signalling pathway [49], the same pathway utilized by IL-10 to regulate TNFα mRNA association with polysomes [26]. Additionally, SHIP1 is known to negatively regulate both Btk and Tec function [66]. TIA-1 and the highly related TIAR silence the translation of TNFα mRNA, while TIA-1$^{-/-}$ macrophages display greatly enhanced TNFα mRNA association with polysomes [69]. Therefore, SHIP1 may inhibit TNFα production by inhibiting Btk/Tec activity. Alternatively, SHIP1 may affect association of TNFα mRNA with polysomes through the activation of TIA-1/TIAR.

To assess the specific function of these four proteins in the IL-10 response in macrophages, the same technique used to knockdown SHIP1 and STAT3 protein through drug inducible expression of siRNA was employed. For each target protein two siRNAs were generated to increase the chances of successful knockdown (Table 2). These siRNAs were used to generate siRNA transduced cell lines using the same technique as described with SHIP1, STAT3 and Scrambled siRNAs. The resulting cells lines were then analyzed by immunoblot for

Target	Top Oligonucleotide	Bottom Oligonucleotide
TIA-1 Construct 3	5'-TGCTGACAAGGTCCAATCTGGCTAAAGTTTTGGCCACTGACTGACTTTAGCCATTGGACCTTGT-3'	5'-CCTGACAAGGTCCAATGGCTAAAGTCAGTCAGTGGCCAAAACTTTAGCCAGATTGGACCTTGTC-3'
TIA-1 Construct 4	5'-TGCTGTTGAGGTGGTGGCACACTGTAGTTTTGGCCACTGACTGACTACAGTGTCACCACCTCAA-3'	5'-CCTGTTGAGGTGGTGACACTGTAGTCAGTCAGTGGCCAAAACTACAGTGTGCCACCACCTCAAC-3'
TIAR Construct 1	5'-TGCTGAGAATAAGGACTTCTGTCACAGTTTTGGCCACTGACTGACTGTGACAGGTCCTTATTCT-3'	5'-CCTGAGAATAAGGACCTGTCACAGTCAGTCAGTGGCCAAAACTGTGACAGAAGTCCTTATTCTC-3'
TIAR Construct 5	5'-TGCTGAATCCTTGTTGGTTCCACGGCGTTTTGGCCACTGACTGACGCCGTGGACAACAAGGATT-3'	5'-CCTGAATCCTTGTTGTCCACGGCGTCAGTCAGTGGCCAAAACGCCGTGGAACCAACAAGGATTC-3'
Btk Construct 6	5'-TGCTGTTCACTAGACTCCTCACCTCTGTTTTGGCCACTGACTGACAGAGGTGAAGTCTAGTGAA-3'	5'-CCTGTTCACTAGACTTCACCTCTGTCAGTCAGTGGCCAAAACAGAGGTGAGGAGTCTAGTGAAC-3'
Btk Construct 8	5'-TGCTGTGACAATGAAACCTCCTTCTTGTTTTGGCCACTGACTGACAAGAAGGATTTCATTGTCA-3'	5'-CCTGTGACAATGAAATCCTTCTTGTCAGTCAGTGGCCAAAACAAGAAGGAGGTTTCATTGTCAC-3'
Tec Construct 7	5'-TGCTGTAAAGGGAGACAGTGTACAAGGTTTTGGCCACTGACTGACCTTGTACAGTCTCCCTTTA-3'	5'-CCTGTAAAGGGAGACTGTACAAGGTCAGTCAGTGGCCAAAACCTTGTACACTGTCTCCCTTTAC-3'
Tec Construct 9	5'-TGCTGTTAGCTTCCTCTATGAAATCCGTTTTGGCCACTGACTGACGGATTTCAGAGGAAGCTAA-3'	5'-CCTGTTAGCTTCCTCTGAAATCCGTCAGTCAGTGGCCAAAACGGATTTCATAGAGGAAGCTAAC-3'

Table 2 siRNA Oligonucleotides targeting TIA-1, TIAR, Btk or Tec. The sequences of oligonucleotides used to generate TIA-1, TIAR, Btk or Tec targeting siRNAs.

their ability to knockdown target protein after 24 and 48 hours of doxocycline induction of the siRNAs. For Btk, both siRNAs targeting Btk (Construct 6 and Construct 8) produced substantial decreases in Btk protein levels after 48 hours of doxocycline treatment (22% and 27% of normal levels respectively) (Figure 6). However, neither of the two siRNAs targeting Tec (Construct 7 and Construct 9) were capable of decreasing Tec protein levels. This lack of knockdown was also found with the siRNA constructs targeting TIA-1 (Construct 3 and Construct 4). For TIAR it was found that expression of either construct (Construct 1 and Construct 5) did not reduce the levels of TIAR protein. Surprisingly though, expression of the TIAR siRNAs did result in a substantial decrease in TIA-1 protein levels after 48 hours of doxocycline treatment (15% and 20% of normal levels for Construct 1 and Construct 5 respectively) (Figure 6). The ability for the siRNA targeting TIAR to knockdown TIA-1 can be rationalized in two ways. First, TIAR and TIA-1 mRNA are highly similar in sequence, and in the specific regions where the two siRNAs targeting TIAR target TIAR, the similarity between TIAR and TIA-1 is nearly 100% (Figure 7). Additionally, miRNAs are often bind without 100% sequence complementarity between the miRNA and the target mRNA molecule. This mismatch would allow siRNAs targeting one mRNA to also target mRNA molecules that are highly homologous, such as in the case of TIAR and TIA-1.

3.5 Btk but not TIA-1 is required for IL-10 to inhibit TNFα production in LPS activated macrophages

To investigate the potential contribution of Btk and TIA-1 to the IL-10 AIR response in macrophages, the ability for IL-10 to inhibit TNFα production in LPS-stimulated cells lacking Btk or TIA-1 protein was determined. Cells transduced with Btk siRNA or TIAR siRNA were treated with doxocycline for 48 hours to induce knockdown and then equal numbers of cells

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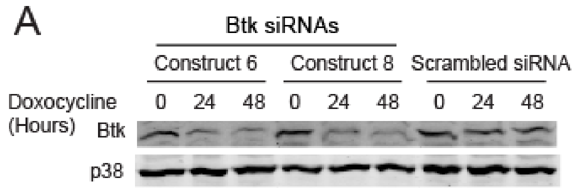

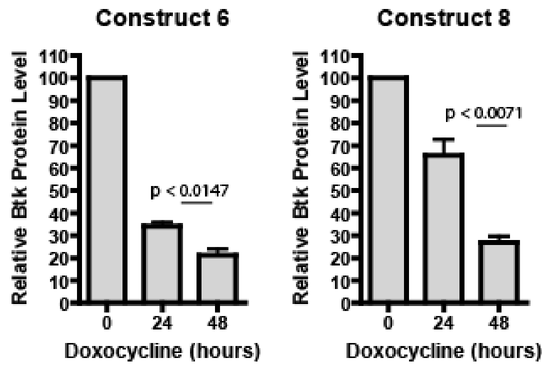

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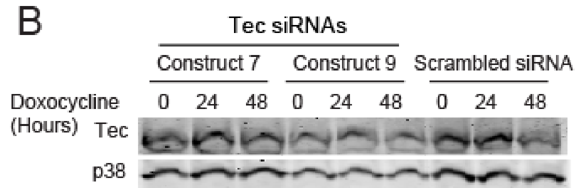

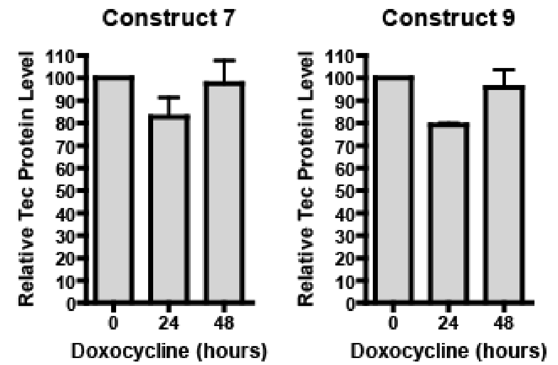

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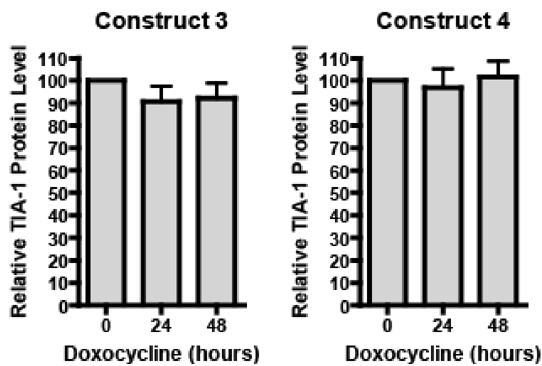

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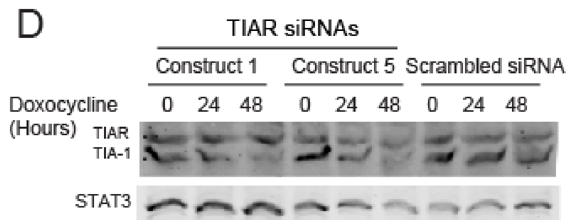

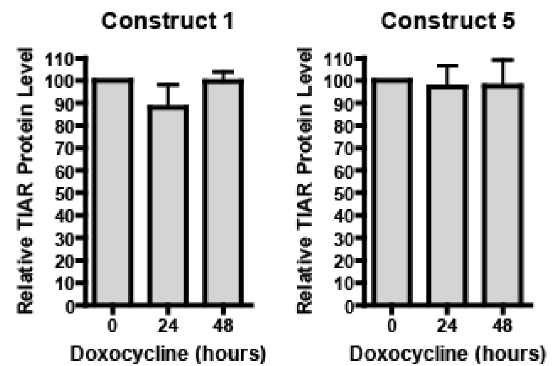

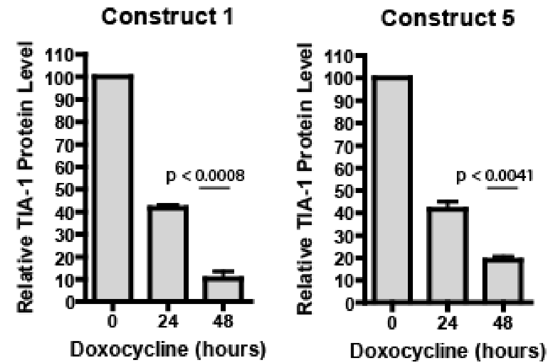

Figure 6 Drug inducible knockdown of Btk and TIA-1 protein. Immunoblot analysis and corresponding denistometrical analysis of Btk **(A)**, Tec**(B)**, TIA-1 **(C)** TIAR **(D)**, or Scrambled siRNA transduced macrophages were treated with 2 μg/mL doxocycline for 0 24 or 48 hours. Cell lysates were prepared as described in Materials and Methods and analyzed by immunoblot for Btk, Tec, TIA-1, TIA-R, p38 or STAT3 protein levels. Densitometry was performed using Odyssey 2.1 software on immunoblots to quantify the levels of Btk, Tec, TIA-1, TIAR, p38 or STAT3 protein. Levels of Btk and Tec protein were normalized to levels of p38 protein while levels of TIA-1 and TIA-R were normalized to levels of STAT3 protein. Normalized values were then determined relative to siRNA transduced cells treated with 2 μg/mL doxocycline for 0 hours. Results represent 3 independent experiments. P-values are based on a two tailed unpaired students t-test with a confidence interval of 95%.

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Construct 1          TGTGACAG--GTCCTTATTCT
TIAR 408        AGAGATGTGACAGAAGTCCTTATTCTTCAGT
TIA-1 327       CGAGATGTGACAGAAGCTCTCATCCTCCAGC
                     ********  *  ** ** **


Construct 5          GCCGTGGA--CAACAAGGATT
TIAR 1374       GGGCAGCCGTGGAACCAACAAGGATTTGGAG
TIA-1 1283      GGCCAGCCATGGAGCCAGCAGGGGTTCAATC
                     *** ****  ** ** ** **
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Figure 7 Sequence alignment between TIAR siRNA and TIA-1 and TIAR mRNA. Multiple sequence alignments were performed between TIA-1 and TIAR mRNA sequences as well as the mRNA targeting region of the TIAR siRNA Construct 1 or Construct 5. An * indicates 100% match between siRNA sequence, TIA-1 mRNA and TIAR mRNA. Sequence alignment was performed using ClustalW2 software.

were stimulated with LPS or co-stimulated with LPS and IL-10 for one hour, and the amount of TNFα produced in the cell supernatants was measured by ELISA. It was found that in cells lacking TIA-1, IL-10 was still able to inhibit TNFα production as effectively as in TIAR siRNA transduced cells not treated with doxocycline (Figure 8). This indicates that IL-10 does not use TIA-1 to inhibit TNFα production in the early phase of the IL-10 AIR in activated macrophages. Surprisingly, in Btk siRNA transduced cells treated with doxocycline for 48 hours, IL-10 was not able to inhibit TNFα production as compared to cells not treated with doxoxycline (Figure 8). This indicates that IL-10 requires Btk to inhibit TNFα production during the early (1 hour) response to IL-10 treatment of activated macrophages. Importantly, this effect was observed for both Btk-targeting siRNAs reducing the possibility that this effect was due to reducing the level of an off-target protein involved in the IL-10 AIR by the siRNAs targeting Btk. Taken altogether, this data demonstrates that Btk but not TIA-1 is required for IL-10 to inhibit TNFα production in LPS activated macrophages.

3.6 Btk is not required for IL-10 to dissociate TNFα mRNA from polysomes

The impaired ability of IL-10 to inhibit TNFα production in doxocycline treated Btk siRNA transduced cells was very similar to what was found with doxocycline treated SHIP1 siRNA transduced cells (Figure 9). As IL-10 utilizes SHIP1 to dissociate TNFα mRNA from polysomes in activated macrophages, we asked whether IL-10 might also require Btk for this process. To investigate this, small scale sucrose gradient fractionation was performed on Btk siRNA transduced cells as previously described for SHIP1 siRNA transduced cells. It was found that in Btk siRNA transduced cells treated for 48 hours with doxocycline to induce Btk knockdown, IL-10 was still able to dissociate TNFα mRNA from polysomes (Figure 9). This demonstrates that IL-10 does not require Btk to dissociate TNFα mRNA from polysomes and

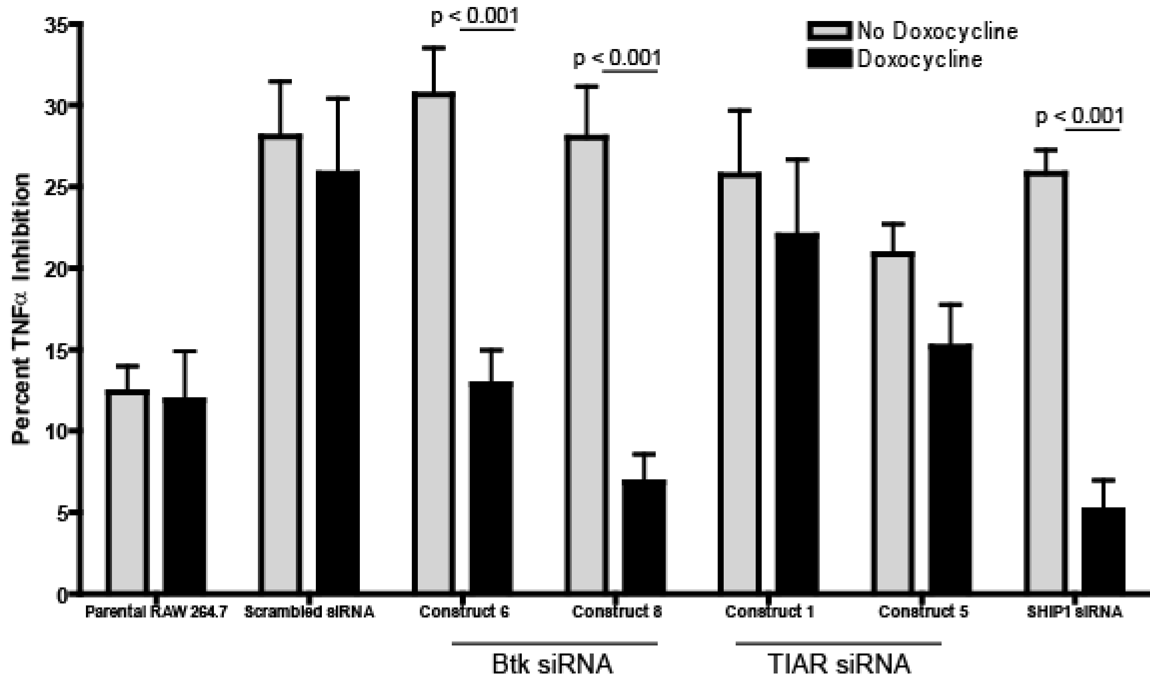

Figure 8 IL-10 responsiveness in Btk and TIAR siRNA transduced cell. Cells treated with or without 2 μg/mL doxocycline for 48 hours were stimulated with 1 ng/mL LPS or 1 ng/mL LPS and 10 ng/mL IL-10 for 1 hour. Cell supernatants were then collected and the concentration of TNFα in the supernantants was determined by ELISA. Percent inhibition TNFα was calculated by dividing the amount of TNFα produced in the presence of LPS + IL-10 by that produced in the presence of LPS alone. Each experiment was carried out in biological quadruplicates and results are based upon the average ± SEM of 6 independent experiments. Total average TNFα production ± SEM in response to LPS stimulation for Parental RAW264.7, Scrambled, Construct 6, Construct 8, Construct 1, Construct 5 and SHIP1 siRNA transduced cells not treated with doxocycline is 725 ± 197 pg/mL, 1074 ± 269 pg/mL, 834 ± 156 pg/mL, 724 ± 201 pg/mL, 780 ± 127 pg/mL, 826 ± 59 pg/mL and 734 ± 137 pg/mL respectively. Total average TNFα production ± SEM in response to LPS stimulation for Parental RAW264.7, Scrambled, Construct 6, Construct 8, Construct 1, Construct 5 and SHIP1 siRNA transduced cells treated with doxocycline is 569 ± 154 pg/mL, 946 ± 251 pg/mL, 579 ± 58 pg/mL, 523 ± 80 pg/mL, 691 ± 267 pg/mL, 741 ± 100 pg/mL and 514 ± 80 pg/mL respectively. P-values are based upon two-way ANOVA analysis.

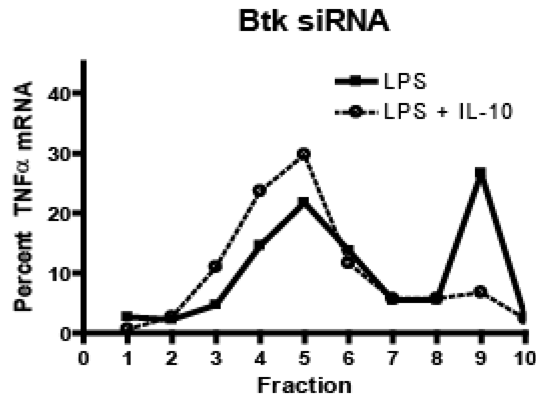

Figure 9 Polysome fractionation of RNA from Btk siRNA transduced cells. Cells transduced with Btk siRNA Construct 8 were treated for 48 hours with 2 μg/mL doxocycline and then stimulated with 1 ng/mL LPS for 45 minutes. IL-10 was then added to a final concentration of 100 ng/mL or not added and cells were stimulated for an additional 15 minutes. Cell lysates were then prepared and subjected to sucrose gradient fractionation and RT-qPCR analysis as described in Materials and Methods. Percent TNFα mRNA in each fraction was determined by taking the relative TNFα signal from each fraction as a percent of the sum of the TNFα mRNA signal from all 10 fractions. Fraction 1 indicates the top of the sucrose gradient. Results are representative of 2 independent experiments.

that the function of Btk in the IL-10-induced inhibition of TNFα production likely does not involve the translational regulation of TNFα mRNA. Instead Btk may regulate transcription of TNFα mRNA, TNFα mRNA stability or TNFα protein secretion in order to inhibit TNFα production.

3.7 Btk knockdown does not alter early LPS induced TNFα production in macrophages

IL-10's requirement of Btk for the inhibited production of TNFα in LPS-stimulated macrophages was surprising as this indicated that Btk is a negative regulator of TNFα production even though most research has found that Btk is a positive regulator of TNFα production [49-52]. This made us curious as to whether or not Btk is required for the production of TNFα in LPS stimulated macrophages. To determine this, Btk siRNA transduced cells were treated with doxocycline for 48 hours to induce knockdown of Btk. Cells were then stimulated with 1 ng/mL of LPS alone for one hour and the amount of TNFα produced by the cells was measured by ELISA. We found that cells lacking Btk produced the same amount of TNFα in response to LPS as compared to Btk siRNA transduced cells not treated with doxocycline (Figure 10). This indicates that Btk is not required for TNFα production during the first hour of LPS stimulation in RAW264.7 macrophages.

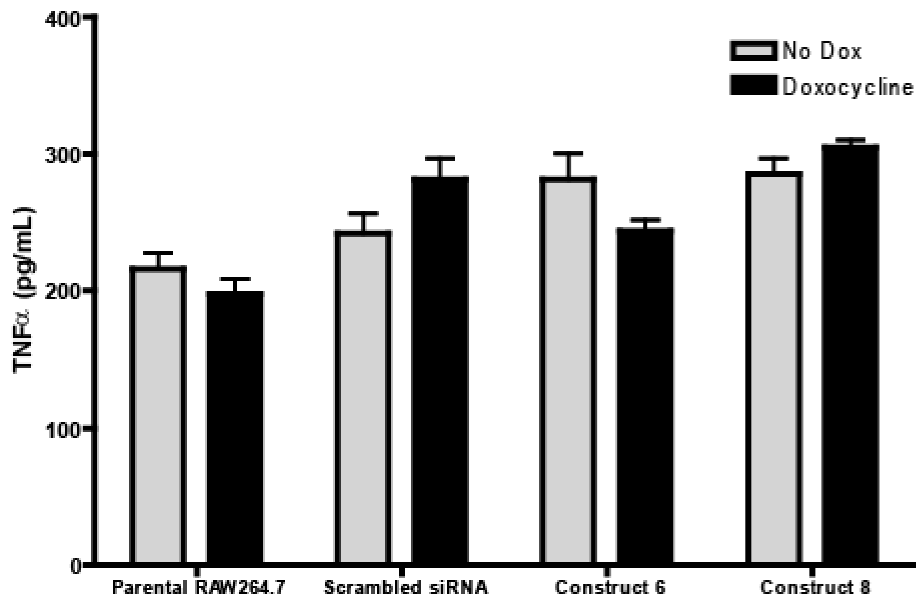

Figure 10 LPS induced TNFα production in Btk siRNA transduced cells. Parental RAW264.7, Scrambled siRNA or Btk siRNA transduced cells were treated with or without 2 μg/mL doxocycline for 48 hours. Cells were then stimulated with 1 ng/mL LPS for 60 minutes and cell supernatants were analyzed for TNFα concentration by ELISA. Each experiment was carried out in biological quadruplicates and error bars represent the deviation between these quadruplicates. Two-way ANOVA analysis was performed between cells treated with doxocycline and those not treated for each cell type. Results are representative of 5 independent experiments.

4 Discussion

4.1 The role of SHIP1 in the IL-10 AIR

The discovery that SHIP1 is required for IL-10 to promote the dissociation of TNFα mRNA from polysomes in activated macrophages is important for a number of reasons. First, the role of SHIP1 in the inhibition of TNFα production further supports the notion that SHIP1 is a negative regulator of TNFα production. Second, as SHIP1 is a negative regulator of PI3K signalling, the inhibition of TNFα production by SHIP1 suggests that PI3K is a positive regulator of TNFα production. This is important as there is still much debate as to whether or not PI3K is a positive or negative regulator of LPS-induced TNFα production in macrophages [94]. Likewise, this also helps to clarify the effect of IL-10 stimulation of macrophages on the PI3K pathway, suggesting that IL-10 inhibits PI3K signalling in activated macrophages. This could be confirmed by looking at downstream targets of PI3K such as Akt phosphorylation in response to IL-10 stimulation. Third, the finding that IL-10 uses SHIP1 to inhibit TNFα production in the early AIR strongly suggests the use of a STAT3-independent pathway by IL-10 in the AIR. The use of SHIP1 by IL-10, taken together with previous findings indicating STAT3-independent IL-10 signalling pathways [26, 95] provides strong evidence that the dogma that IL-10 signalling acts only through STAT3 is incorrect. This is not necessarily surprising as it is known that both IL-10 and the pro-inflammatory cytokine IL-6 signal in macrophages by activating STAT3 [96]. Therefore, the outcome of STAT3 activity in response to these two cytokines could be based at least in part upon non-STAT3 alternate pathways activated by IL-6 and IL-10. Alternatively, the timing or strength of STAT3 activity could account for the differences in IL-6 and IL-10 signalling. Finally, involvement of SHIP1 in the IL-10 AIR also supports the recent finding that

IL-10 stimulation of macrophages reduces miR-155 levels leading to increased SHIP1 levels[41] as signalling molecules often upregulate the expression of their downstream effectors [97].

Although SHIP1 is required to dissociate TNFα mRNA from polysomes in activated macrophages, it is still not clear how SHIP1 accomplishes this. TIA-1, one potential downstream target of SHIP1 in this response, was found not to be required for IL-10 to inhibit TNFα production in LPS activated macrophages indicating that TIA-1 is not the target of SHIP1. Interestingly Btk, another potential downstream target of SHIP1, was found to be required for IL-10 to inhibit TNFα production in LPS activated macrophages. However, it was found that Btk had no effect on TNFα mRNA association with polysomes in response to IL-10, demonstrating that Btk is not the target of SHIP1 in the IL-10 induced dissociation of TNFα mRNA from polysomes. As the knockdown of Tec and TIAR was not successful, these two proteins remain potential downstream targets of SHIP1 in the IL-10 AIR. The use of Tec$^{-/-}$ and TIAR$^{-/-}$ mice may allow an alternative means of investigating these proteins as a downstream target of SHIP1 in the IL-10 AIR. Also, since it is known that the IL-10-induced dissociation of TNFα mRNA from polysomes is dependent upon the p38 MAPK pathway [26], it is likely that the protein that SHIP1 modulates will be involved in p38 signalling. This notion is further supported by unpublished data from our lab indicating that IL-10 inhibits p38 MAPK in a SHIP1 dependent manner.

One interesting question raised by the involvement of SHIP1 in the IL-10 AIR is how IL-10 activates SHIP1. Like STAT3, SHIP1 contains an SH2 domain, so it is possible that SHIP1 could be recruited to the active IL-10 receptor complex through the binding of its SH2 domain to the phosphorylated tyrosine residues on IL-10R1. This question could be addressed through IL-10 receptor immunoprecipitation experiments or through the use of *in silico* protein interaction

prediction [98]. Alternatively, SHIP1 may be recruited to the IL-10 receptor through adaptor proteins that are known to interact with SHIP1 such as Shc [34]. If this is the case, then the knockdown of the adaptor protein involved in SHIP1 recruitment should yield a similar phenotype to SHIP1 knockdown in the IL-10 response.

4.2 Btk as a negative regulator of TNFα production

The finding that IL-10 requires Btk to inhibit TNFα production in LPS activated macrophages is surprising as it suggests that Btk is a negative regulator of TNFα production. This is contrary to many published papers which suggest that Btk is required for optimal TNFα production in macrophages in response to LPS [48-52, 55]. However, there is evidence to support a role for Btk in IL-10 signalling as it was found that B-cells from xid mice, a strain of mice with a naturally occurring mutation in Btk, were unresponsive to IL-10 stimulation and failed to upregulate MHC class II expression[99]. Some phenotypes associated with xid and XLA, a human disease based on mutations in Btk, also might suggest an involvement of Btk in IL-10 signalling. XLA patients frequently develop rheumatoid arthritis and type I diabetes mellitus[100, 101]. While this has been interpreted as a consequence of hyperactive T_{H1} responses in XLA patients, this could also be interpreted as a deficiency in IL-10 response as IL-10$^{-/-}$ mice also have increased incidence of rheumatoid arthritis and diabetes mellitus [102, 103]. Studies using T-cell depletion in xid mice might reveal whether the increased rates of autoimmune disease in these mice are in fact caused by a T_{H1} response.

While the data presented here clearly demonstrate a role for Btk in IL-10 signalling, how Btk helps to inhibit TNFα production remains unknown. Since the loss of Btk had no effect on the ability of IL-10 to dissociate TNFα mRNA from polysomes it seems likely that Btk does not affect TNFα production by regulating TNFα mRNA translation. Instead, Btk may affect the

transcription of TNFα mRNA or possibly the stability of TNFα mRNA. Both of these possibilities will need to be investigated. The possible effect of Btk on TNFα mRNA stability could be determined through actinomycin D mRNA stability experiments. The downstream target of Btk activity in the IL-10 inhibition of TNFα production in activated macrophages also remains elusive. In the context of BCR signalling, Btk is responsible for the activation of PLCγ and Protein Kinase C (PKC). While PLCγ is known to be a positive regulator of TNFα production in macrophages [104], activation of both PKCδ and PKCβ have been shown to lead to decreased TNFα mRNA levels by increasing the levels of tristetraprolin (TTP), a protein which binds to and destabilizes TNFα mRNA [105, 106]. This raises the possibility that activation of PKCβ/δ might be how Btk can suppress TNFα production in response to IL-10. Interestingly, PKCβ$^{-/-}$ mice have a very similar phenotype to xid mice with a severe reduction of circulating B-cells [107], highlighting the close connection between Btk activity and PKCβ activity.

Another puzzling question raised by the requirement of Btk in the IL-10 AIR is how IL-10 receptor engagement can lead to the activation of Btk. Since PI3K signalling is generally required for Btk activation [43, 44, 60], the finding that IL-10 utilizes the PI3K antagonist SHIP1 and previous research which has shown that IL-10 inhibits PI3K signalling [108], suggest that Btk must be recruited to the membrane and activated in a PI3K independent manner. In BCR and TCR signalling Btk is recruited to the receptor complex through protein interactions with BLNK/SLP65 in B-cells and LAT and SLP-76 in T-cells [109, 110]. Interestingly mouse macrophages express both BLNK/SLP-65 and SLP-76 [57], raising the possibility that these proteins are responsible for recruiting Btk to the activated IL-10 receptor. Recently it has been found that in osteoclasts Btk does form a complex with BLNK and SLP-76 [111] during Receptor Activator for Nuclear Factor κB Ligand (RANKL) signalling.

4.3 Requirement of Btk in TNFα production

Another interesting finding was that the loss of Btk did not affect TNFα production in LPS stimulated RAW264.7 macrophages. This goes against previous research which indicates that Btk is a positive regulator of TNFα production and is required for the LPS induced production of TNFα [48-51]. However, all of the studies reporting Btk to be required for LPS induced TNFα production have only examined TNFα production in the late stages of LPS stimulation (18 hours or later). The research presented here examined TNFα production in the early stages of LPS production (1 hour) and found no requirement for Btk in LPS induced TNFα production in macrophages. This might suggest that Btk is only required for LPS induced TNFα production at late timepoints, and that early on in the LPS stimulation of macrophages Btk is dispensible. This may not be surprising as one of the key molecules that is known to be regulated by Btk in LPS-TLR4 signalling is Mal, a protein which likely acts in the late stages of LPS signalling [112]. The notion that Btk is not involved in early LPS-induced TNFα production is further supported by the finding that early LPS induced activation of MAPKs was the same in wildtype human monocytes and XLA monocytes [113]. Similar results were found in bone marrow derived mast cells from xid mice [114]. Another factor to consider when looking at the role of Btk in LPS induced TNFα production in macrophages is that Btk is required for the induction by TLR4 of IL-10 production in macrophages [115]. This makes it hard to rule out the contribution of autocrine IL-10 effects in the phenotype of Btk deficient macrophages. Overall it seems that Btk may only be required for TNFα production in the late stages of LPS signalling, however further research will need to be done to confirm this.

4.4 Overall IL-10 response

The research presented here has further clarified how IL-10 signals and mediates the AIR in activated macrophages. The identification of SHIP1 and Btk as being required for IL-10 to inhibit TNFα production in macrophages clearly demonstrates that IL-10 uses signalling pathways in addition to STAT3 in the AIR. Interestingly, the loss of both SHIP1 and Btk affected the ability for IL-10 to inhibit TNFα production in the early phase of IL-10 stimulation suggesting that these two proteins are important in early IL-10 induced TNFα inhibition. As STAT3 function in the IL-10 AIR requires the transcription and translation of target genes, a process which takes time to occur, it may be that SHIP1 and Btk function to repress TNFα production when STAT3-dependent transcription and translation is occurring. In this way, IL-10 can rapidly inhibit TNFα production through SHIP1 and Btk before STAT3 function kicks in and the AIR is fully implemented. The idea that STAT3 independent pathways function in the early IL-10 response is supported by the finding that expression of a STAT3 dominant negative isoform in LPS-stimulated monocytes impaired the IL-10 inhibition of TNFα production only after two hours of IL-10 treatment while the effects of IL-10 in the first hour of stimulation were not impaired [95]. As inflammation can accelerate rapidly [1], this rapid SHIP1 and Btk-dependent decrease in TNFα production may be physiologically important for slowing down the initial inflammatory response before it becomes overwhelming.

The overall results of this research allow us to better understand how IL-10 signalling occurs in activated macrophages. Upon IL-10 receptor dimerization, SHIP1 is quickly recruited to the receptor complex, possibly through binding of its SH2 domain to the phosphorylated tyrosine resides on the IL-10R1 subunit. SHIP1 function at the membrane then leads to the dissociation of TNFα mRNA from polysomes possibly by inhibiting p38 MAPK, and this leads

to decreased TNFα translation and production (Figure 11). At the same time, IL-10 also activates Btk, perhaps through recruitment of the adaptor proteins BLNK/SLP-65 or SLP-76. Activated Btk then attenuates TNFα production, possibly through PKCβ induced TNFα mRNA destabalization by TTP. This combination of SHIP1 and Btk function leads to a rapid decrease in TNFα production. Meanwhile, STAT3 is activated by the IL-10 receptor complex and its Janus associated kinases and translocates to the nucleus where it regulates the transcription of gene targets such as SOCS3, which once translated, completely inhibit TNFα production and deactivate the macrophage.

4.5 Future directions

The research here has identified novel roles for both SHIP1 and Btk in the IL-10 AIR. While both of these proteins are required for IL-10 to inhibit early TNFα production, the downstream protein targets of SHIP1 and Btk in this response still need to be elucidated. As SHIP1 is required for IL-10 to dissociate TNFα mRNA from polysomes, a function regulated by the p38 MAPK pathway, it is likely that the downstream target of SHIP1 is involved in p38 MAPK signalling. Btk on the other hand is not involved in TNFα mRNA dissociation from polysomes suggesting that Btk inhibits TNFα production through non-translational mechanisms such as TNFα transcription, mRNA stability or secretion. Further studies will need to be done to determine at what level Btk inhibits the production of TNFα in macrophages.

Although the research here demonstrates a clear requirement for SHIP1 and Btk in the IL-10 AIR, the independence of STAT3 in the AIR remains unclear. Both Btk and SHIP1 are required for IL-10 to inhibit TNFα production in LPS-activated macrophages as early as one

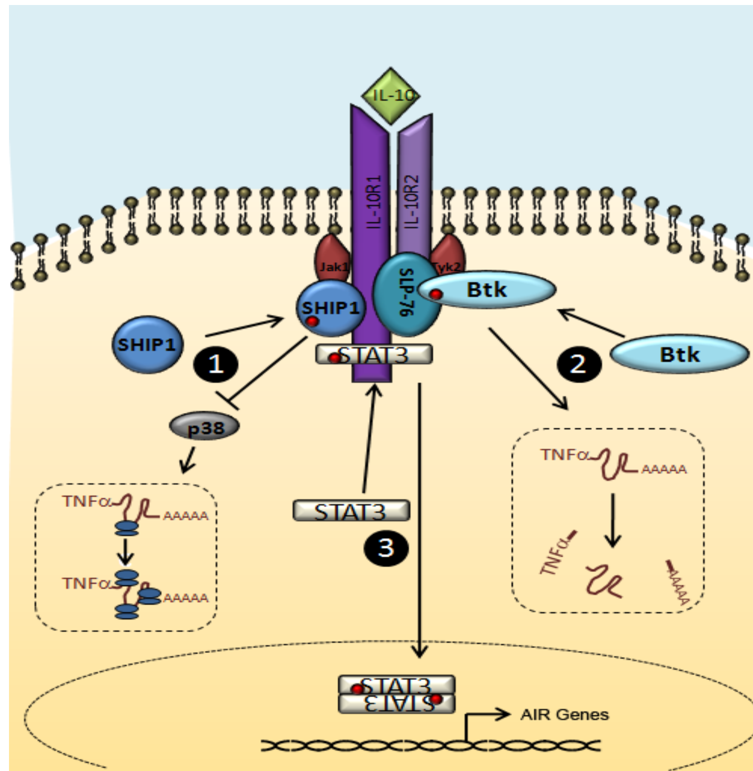

Figure 11 Proposed model of IL-10 signalling in macrophages. IL-10 receptor engagement and activation leads to three separate signalling pathways which help to inhibit TNFα production. **(1)** SHIP1 is recruited from the cytosol to the activated IL-10 receptor complex through its SH2 domain where it becomes phosphorylated and activated. Activated SHIP1 then inhibits the p38 MAPK pathway leading to the dissociation of TNFα mRNA from polysomes resulting in decreased translation of TNFα. **(2)** Btk is recruited to the IL-10 receptor complex by binding to the adaptor protein SLP-76 or BLNK. Once at the receptor Btk is phosphorylated on its active site leading to Btk kinase activation. Activated Btk then inhibits TNFα production by promoting the degradation of TNFα mRNA. **(3)** STAT3 is recruited to the activated IL-10 receptor by its SH2 domain binding to phosphorylated residues on the IL-10R1 subunit. STAT3 is then phosphorylated by the receptor associated Janus kinases Jak1 and Tyk2 leading to disengagement of STAT3 from the receptor. Phosphorylated STAT3 then homodimerizes and translocates to the nucleus where it controls the transcription of various gene targets which mediate the late stages of the AIR.

hour after IL-10 stimulation and SHIP1 is required for IL-10 to dissociate TNFα mRNA from polysomes, a function that occurs within 15 minutes after IL-10 stimulation. These early responses involving SHIP1 and Btk suggest STAT3 independence but cannot formally rule out a non-transcriptional role for STAT3 in the early IL-10 AIR. This will only be achieved after examining cells in which STAT3 is inducibly knocked down.

Finally, the exact role of Btk in the LPS induced production of TNFα must be further examined in order to determine definitely if Btk is required for TNFα production and at what stage, if any, in the LPS response is Btk required.

4.6 Conclusion

The results of the research presented here further our understanding of IL-10 signalling in cells and of the IL-10 AIR. A novel role for both SHIP1 and Btk in the IL-10 AIR has been found with both proteins being required for IL-10 to inhibit TNFα production in the early AIR. SHIP1 has been found to be required for IL-10 to induce the dissociation of TNFα mRNA from polysomes and to decrease TNFα production by activated macrophages. Btk, on the other hand, does not regulate TNFα mRNA association with polysomes, suggesting that Btk regulates TNFα production through a non-translational mechanism. The role of Btk in LPS-induced TNFα production in macrophages has also been further clarified with the finding that Btk is dispensable for TNFα production in early LPS-TLR4 signalling. Finally, it has also been found that the small RNA binding protein TIA-1 is not involved in the IL-10 AIR. Taken together these results help to clarify how IL-10 functions in activated macrophages and identifies SHIP1 and Btk as protein targets for inhibiting TNFα production in the body. Btk and SHIP1 activators might also be used to mimic the anti-inflammatory effects of IL-10, an application that would aide in the treatment of many inflammatory diseases and disorders.

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