AUTOPHAGY REGULATES CELL SURFACE EXPRESSION OF CLASS II INVARIANT CHAIN PEPTIDE (CLIP) AND PEPTIDE PRESENTATION IN B CELLS

A Dissertation

by

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ABSTRACT

The mechanisms by which the immune system distinguishes self from non-self are essential in providing protection from pathogenic invaders and to prevent autoimmunity. Over the last few decades, the discovery of regulatory cells that can suppress specific immune responses has introduced a paradigm shift in the study of self versus non-self discrimination. Combined with the discovery of pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patters (DAMPS), and their respective Toll Like Receptors (TLRs) that trigger an immediate inflammatory response to dangerous pathogens or to damaged self, recognizing the pathogens specifically, and maintain self-tolerance simultaneously, has led to the requirement for re-assessing both the innate and the adaptive immune response to either. We have examined the contribution of a highly conserved invariant protein, called CD74, to this conundrum.

Our experiments involved both activation with TLR agonists or with antigen-specific receptors to determine the contribution of CD74 in regulating the balance. TLR activation and antigen receptor engagement both result in the proteolysis of CD74 into peptides that get loaded into the peptide-binding groove of MHC-II. We reasoned that CD74 and its products might provide a molecular contribution that would prevent self-antigen presentation until a response to specific exogenous antigens is required. While we observed that both TLR activation and antigen receptor engagement rapidly result in moderate to high levels of cell surface CLIP, respectively, treatment with rapamycin, a well-established inhibitor of mTOR that induces autophagy, resulted in loss of cell

surface CLIP under either activation stimulus. Furthermore, by using a synthetic peptide designed to have a high binding constant for the peptide-binding groove of MHC-II, rapamycin rapidly enabled exogenous peptide presentation. The implications of this observation in terms of self versus non-self discrimination are supported by our experiments using autoimmune prone animals. In these studies antigen receptor engagement as well as LPS-induced CLIP expression was reduced by rapamycin treatment, enabling exogenous antigenic peptide presentation. These results suggest that at least for endosomal TLR activation, preferential expression of endogenous self-antigens may contribute to the development of autoimmune responses and reciprocally to self-tolerance that may prevent an anti-tumoral response.

DEDICATION

This work is dedicated to my parents for their personal sacrifices, the love they gave and valuable lessons they taught to make me a better person, to my brother who stood by me and supported me through good and bad times and who never doubted my potential, and to my sisters who have always cared for me and advised me on every important decision that I have taken in my life so far.

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This Work was supervised by my dissertation committee, consisting of Dr. M. Karen Newell-Rogers (Advisor), Dr. Travis Hein of the Department of Surgery, Texas A&M University College of Medicine, by Dr. Cynthia Meininger of the Department of Medical Physiology, Texas A&M University College of Medicine and Dr. Daniel Jupiter of Department of Preventive Medicine and Community Health from University of Texas Medical Branch, Galveston, Texas.

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NOMENCLATURE

ACK	Ammonium-Chloride-Potassium
APC	Antigen Presenting Cell
Bcl-2	B-cell Lymphoma 2
BCR	B Cell Receptor
BFA	Brefeldin A
BLNK	B-Cell Linker
CD	Cluster of Differentiation
CIITA	Class II Major Histocompatibility Complex Transactivator
CLIP	Class II-Associated Invariant Chain Peptide
CMA	Chaperone Mediated Autophagy
COX	Cyclooxygenase
CpG ODN	CpG Oligodeoxynucleotides
DAG	Diacylglycerol
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ETS	E-Twenty Six
FBS	Fetal Bovine Serum
FO	Follicular
FOXO3	Forkhead Box Protein O3
HCQ	Hydroxychloroquine

HDAC	Histone Deacetylase
HGFR	Hepatocyte Growth Factor Receptor
HIF1α	Hypoxia Inducible Factor 1 Alpha
HLA	Human Leukocyte Antigen
ICD	Intracellular Domain
IFNγ	Interferon Gamma
Ig	Immunoglobulin
Ii	Invariant Chain
IκB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B Cells Inhibitor
IKK	IκB Kinase
iNKT	Invariant Natural Killer T
IP3	Inositol Trisphosphate
IRAK4	Interleukin-1 Receptor-Associated Kinase 4
LPS	Lipopolysaccharides
LRR	Leucine Rich Region
MARCH-1	Membrane Associated RING-CH Protein-1
MAP	Mitogen-Activated Protein
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
MIIC	MHC Class II Compartment

mTORC1	Mammalian Target of Rapamycin Complex 1
MyD88	Myeloid Differentiation Primary Response Gene 88
MZ	Marginal Zone
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NFAT	Nuclear Factor of Activated T-cells
NKT	Natural Killer T
NTF	N-Terminal Fragment
PAMPs	Pathogen-Associated Molecular Patterns
PerC	Peritoneal Cavity
PI3K	Phosphatidylinositide 3-Kinase
PKB	Protein Kinase B
PLCγ	Phospholipase C Gamma
PTK	Protein Tyrosin Kinase
RIP1	Receptor-Interacting Protein Kinase 1
Syk	Spleen Tyrosin Kinase
TAB	TAK1-Binding Protein
TAK1	TGF-β-Activated Kinase 1
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor Beta
TGN	Trans Golgi Network
TNF	Tumor Necrosis Factor
TIRAP	Toll/Interleukin-1 Receptor Domain-Containing Adapter Protein

TIR	Toll/IL-1R
TLR	Toll-Like Receptor
TRAF6	TNF Receptor Associated Factor 6
TRAM	Translocating Chain-Associating Membrane Protein
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-β
Treg	T Regulatory Cells
TSA	Trichostatin A
UPS	Ubiquitin-Proteasome System
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

TABLE OF CONTENTS

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

B cells

Over the last few years, B cells have become centrally important with respect to their potential contribution(s) to a variety of inflammatory and autoimmune diseases. B cell depletion therapies, using Rituxan™ and/or Rituximab™, have shown promise in multiple diseases, including multiple sclerosis, type I diabetes, and rheumatoid arthritis amongst others, which were conventionally attributed to the activity of autoreactive T cells (Cross et al., 2006; Mok, 2013; Pescovitz et al., 2009).

In mice, B cells have been characterized as members of two major subsets, B-1 and B-2 B cells as shown in figure 1. These subcategories of B cells can be distinguished based on their anatomical locations and origins, phenotypes and functions (Kantor and Herzenberg, 1993). B2 cells continually arise from bone marrow precursors and are continually renewing (Kantor, 1991). These cells circulate through the blood and occupy lymphoid organs, such as lymph nodes and the spleen. B2 cells respond to antigens that are both T cell dependent and T cell independent (Martin et al., 2001). These cells, once stimulated, are capable of immunoglobulin isotype class switching, are known to become long-lived memory cells, and are capable of responding to antigens long after the initial exposure to the antigen (Martin and Kearney, 2000).

B-lymphocytes in humans and mice are classified either into B-1 or B-2 B cells based on their ontogeny and anatomical location; B-1 B cells include B-1a, and B-1b

cells, while marginal zone (MZ) and Follicular (FO) B cells are considered B-2 lineage (Figure 1). B-1 B lymphocytes arise in fetal liver and are self-renewing, while B2 lymphocytes develop from transitional 2 (T2) B cells from bone marrow precursors with continued replacement from the bone morrow (Montecino-Rodriguez and Dorshkind, 2012; Pillai and Cariappa, 2009).

MZ B cells produce polyreactive IgM antibodies to remove microorganisms and dead cells; they express polyreactive BCR on the surface; CD21 and CD35 complement receptors; and CD1d a nonpolymorphic MHC I like molecule (Cerutti et al., 2013). MZ B cells develop from transitional B cells and are localized in the marginal sinus of the spleen; they recognize T cell independent antigens and T cell dependent protein antigens through generation of high-affinity class switched isotypes of antibodies and transport complement-bound opsonins onto splenic follicular dendritic cells helping in germinal center immune activation (Arnon et al., 2013).

B-1 and MZ B cells respond to pathogen-associated molecular patterns (PAMPs) or endogenous TLR ligands via TLR expression and through BCR, with or without antigen recognition (Qian et al., 2006). By initiating a rapid IgM antibody response to sustain immunity against infections MZ and B-1 B cells act as innate immune cells until the FO B cell derived IgG antibody takes over. FO B cells are part of the adaptive immune system and are the most common of all B cells present in the spleen and lymph nodes. In the spleen, they develop from transitional B cells and with the help of T cell interactions produce IgG a long-lasting antibody important in the humoral immune response (Otero et al., 2003).

B-1 B cells, often considered "innate" immune cells, are the predominant B cell type during fetal and neonatal development (Montecino-Rodriguez and Dorshkind, 2012). Throughout life, however, the B-1 B cells can self-renew from stem cell precursors and, once formed, B-1 B cells localize to the peritoneal and pleural cavities where they produce "natural" IgM and IgA antibodies, which are the front line defense against pathogens that often express multiple repeating subunits (Martin et al., 2001; Yang et al., 2007). Subsets of B-1 B cells are defined by differential expression of a variety of cell surface molecules, including CD5. $CD5^{+}IgD^{-}B220^{+}$ B-1a B cells produce widely reactive IgM antibodies. By contrast $CD5^{-}IgD^{-}B220^{+}$ B-1b B cells can produce long lasting, T cell independent, memory type IgM antibodies to some pathogens (Baumgarth, 2011). These B cells express B cell receptors (BCRs) with a repertoire that is enriched for cells reactive to highly polymeric antigens, including both microbial and self-antigens. Surprisingly, the autoreactive B-1 B cells appear to contribute to tissue homeostasis, and potentially self-tolerance (Macpherson and Slack, 2007).

The repertoire of antigen receptors and the potential for production of "natural antibodies" by B-1 B cells result during development, but in the complete absence of exposure to foreign antigens (Gronwall et al., 2012). In fact, autoantibody-producing B-1 B cells have been shown to undergo a "positive selection" event, where the cells are actually selected for their self-reactivity (Choi and Baumgarth, 2008). This positive selection process thus explains the existence of a natural antibody repertoire that includes a high frequency of auto-reactive antigen receptors. The existence of a subset of B cells with autoreactive antigen receptors appears to contradict the established

paradigm that B cells that are self-reactive are deleted during development to prevent autoimmunity. Emerging data reveal that it may be the self-reactive component of B-1 B cells that allow these cells to maintain tissue homeostasis as a result of the "self recognition" (Ferry et al., 2007). However, this also requires a regulatory mechanism to control unwanted activation. We suggest that this function may be provided by selective activation of a regulatory category of T cells, known as T regulatory cells (Tregs) (Hsu et al., 2015).

Following activation in the peritoneum or pleural cavities by stimuli including LPS, or other Toll-like receptor (TLR) ligands, or some cytokine signals, B-1 B cells are rapidly mobilized to the spleen, lymph nodes, and other distal sites from the peritoneum (Ha et al., 2006; Itakura et al., 2005). The function of these quickly mobilized cells has yet to be fully elucidated, but evidence is mounting that B-1 B cells can serve as both effectors, producing large amounts of natural antibodies perhaps aided by their interactions with innate-like T cells, natural killer T cells (NKT) and invariant natural killer T cells (iNKT) and γδ T cells, and as regulators of adaptive immune responses (Campos et al., 2006; Watanabe et al., 2000).

The B-2 and B-1 subsets of B cells are defined by variable expression of specific differentiation antigen, CD5 which is expressed on B-1 B cells but not on B-2 B cells (Hardy, 2006). B-1 B cells are one of the main sources of IL-10 production making them unlikely candidates for promoting autoimmunity (O'Garra et al., 1992; Popi et al., 2004). Reconstitution of B-1 B cells in models of autoimmune diabetes in mice increased infiltrating T cells, and there appeared to be limited islet cell destruction (Ryan et al.,

2010). Protection from diabetes was associated with the entry of Tregs into the pancreas following B-1 B cell injection (Ryan et al., 2010).

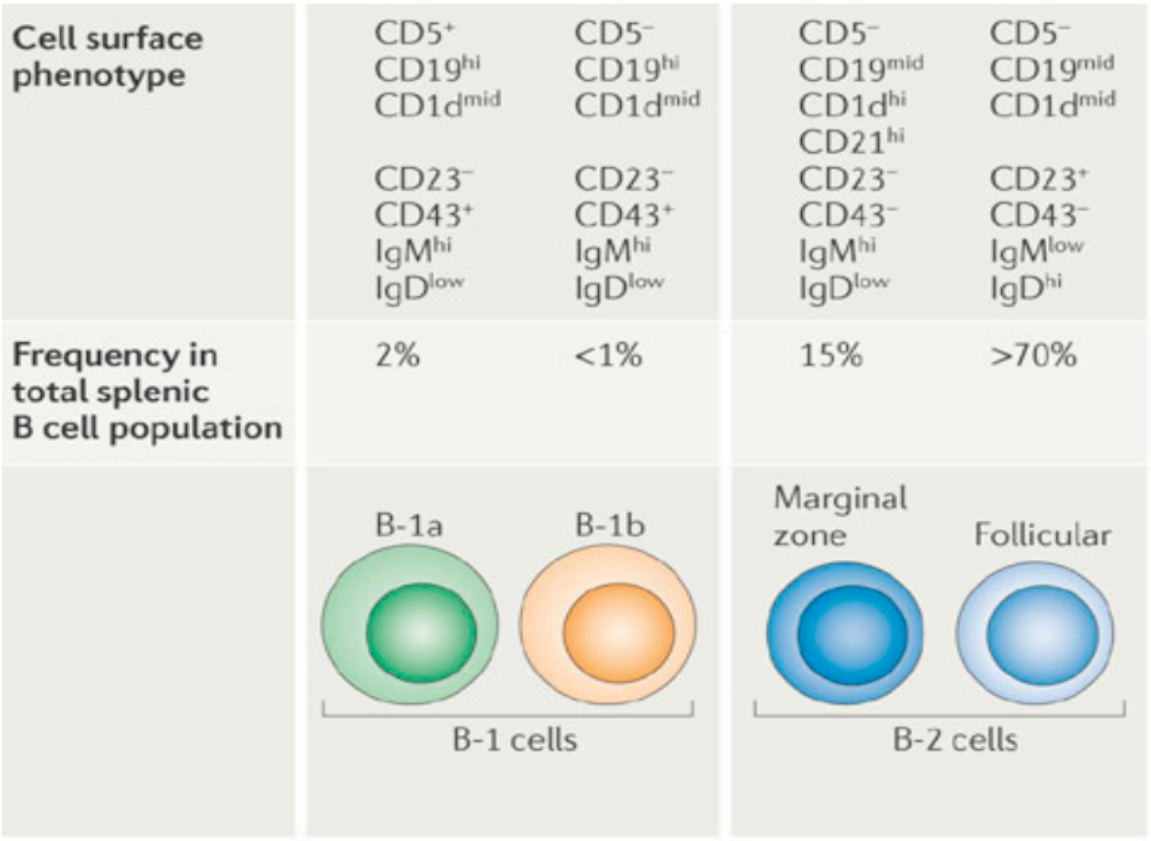


Figure 1: B cell subsets. This picture shows the frequency of B-1 and B-2 B cells in the spleen and the surface markers of each cell phenotype (Baumgarth, 2011). Figure reproduced with permission from Nature Publishing Group.

B cell receptor (BCR)

B cells are highly efficient at antigen presentation to T cells, followed in efficiency by macrophages or dendritic cells. The earliest step in B cell antigen processing and presentation requires binding of an antigen to the B cell receptor (BCR) and internalization resulting in B cell activation, along with the synthesis and transportation of new MHC-II molecules associated with CD74 to the late endosomes. The antigen is brought into the endosome with BCR and is exchanged onto the MHC-II molecule, which is then transported to the surface for presentation to, and recognition by, CD4+ T cells, the initiating event in an adaptive immune response. BCR engagement is important for B cell development and clonal selection, for selective removal of non-specific B cells and for insuring an antigen-specific immune response (Kraus et al., 2004). The membrane bound BCR complex is made up of two immunoglobulin heavy chains (IgH), and two light chains (IgL) covalently linked to the heavy chains and accompanied by Igα and Igβ as signaling components (Niiro and Clark, 2002; Wienands and Engels, 2001).

Once the antigen binds to the BCR, cell proliferation and antigen processing are regulated by a downstream signaling cascade (Figure 2), involving a number of activated protein tyrosine kinases (PTK). Protein tyrosine kinases SYK and LYN, which are activated upon antigen binding initiate signal transduction by phosphorylating the activation sites in the cytoplasmic tail of Igα and Igβ. This results in recruitment of adapter protein BLNK, which gets activated by its interaction with SYK and helps recruit further adaptor proteins BLK and PLCγ2 (Kim et al., 2004). PLCγ2 generates IP3

which helps in calcium mobilization from the endoplasmic reticulum and activation of nuclear factor of activated T cells (NFAT). In addition, PLCγ2 generates DAG along with calcium mobilization leading to induction of PLCβ and activation of NF-κB transcription factor, which translocates to the nucleus and initiates transcription of genes important for B cell proliferation (Kim et al., 2004). Antigen engagement with BCR also results in activation of class I PI3K, which via the Akt pathway inhibits FOXO3 activity, leading to B cell proliferation and maintenance (Yusuf et al., 2004).

The BCR bound antigen undergoes endocytosis and is engulfed by an endosome that fuses with a lysosome (forming late endosome), containing acidic hydrolases, which help to break down antigens into peptides (Figure 3). The late endosome fuses with the MHC-II compartment (MIIC) where antigenic peptides are loaded onto MHC-II (Neefjes et al., 2011). The antigen engagement by BCR not only results in intracellular organelle rearrangement but also increases the synthesis of MHC-II bound with invariant chain (Ii) (CD74) in the ER (Hao and August, 2005). The nonameric MHC-II – Ii protein complex translocates to the MIIC where the Ii bound to the MHC-II undergoes proteolytic cleavage by some of the cathepsins (Cathepsin S in B cells) leaving behind MHC-II associated invariant peptide (CLIP) in the groove of the MHC-II binding pocket. Under acidic conditions in the MIIC HLA-DO (H-2O in mice) is inactivated allowing HLA-DM (H-2M in mice) to catalyze the replacement of CLIP with the antigenic peptide and the MHC-II/antigen complex is finally trafficked to the cell surface for CD4+ T cell recognition via its T cell receptor (TCR) (Neefjes et al., 2011).

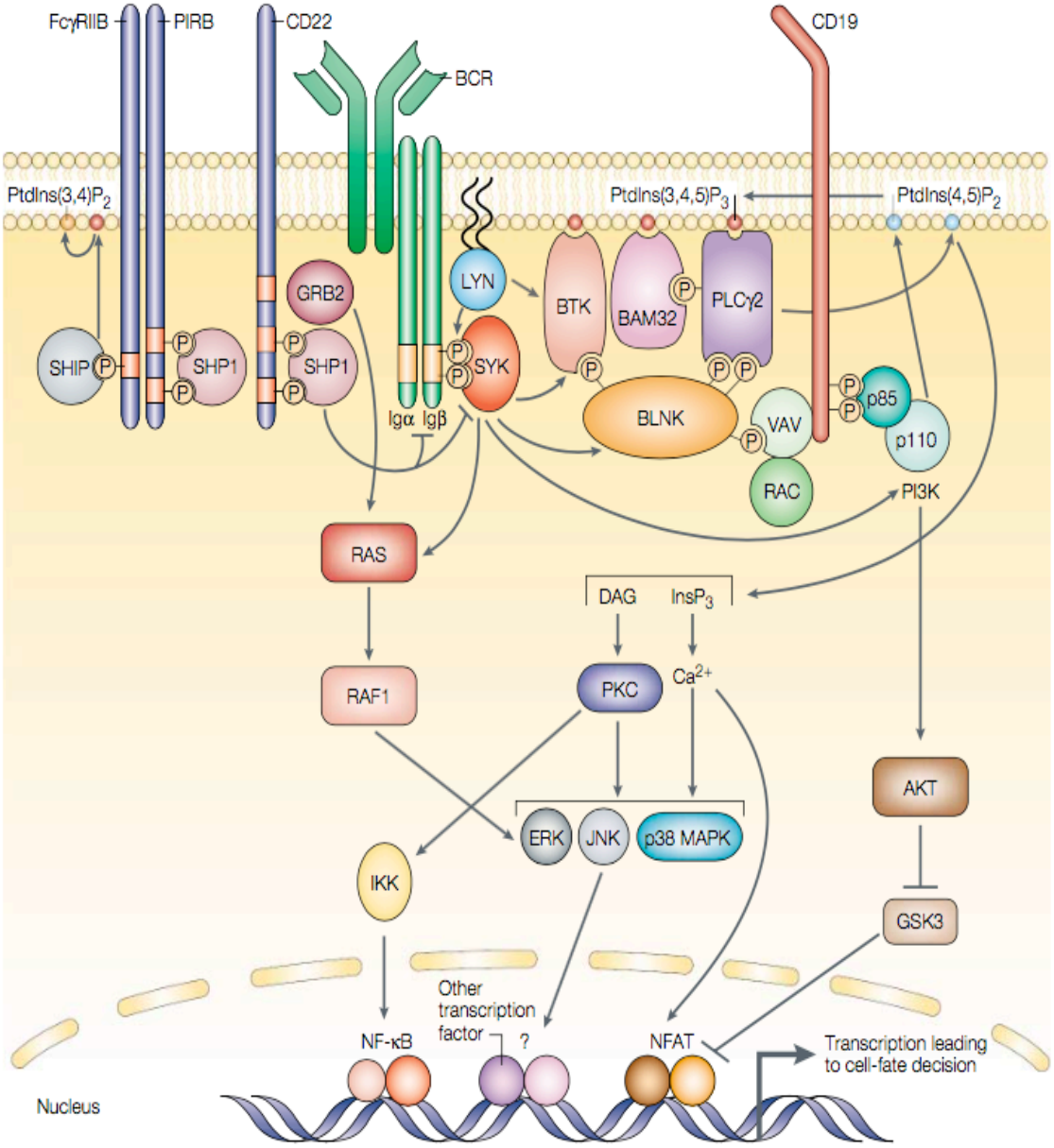


Figure 2: B cell receptor signaling. Schematic presentation of B cell receptor activation and down stream signaling (Niiro and Clark, 2002). Figure reproduced with permission from Nature Publishing Group.

Major histocompatibility complex class-II (MHC-II) and invariant chain (Ii)

The major histocompatibility complex class II (MHC-II) presents antigens to CD4+ T cells. These glycoprotein complexes are present on the surface of antigen-presenting cells (APCs) including macrophages, dendritic cells and B cells. The MHC-II heterodimer complex is made up of one α and one β chain (Neefjes et al., 2011). Non-APCs like endothelial cells, intestinal epithelial cells, fibroblasts and glial cells can express MHC-II under conditions of inflammation or by exposure to interferon gamma (IFN-γ) (Bland, 1988; Geppert and Lipsky, 1985). The MHC-II encoding genes are present on chromosome 6 in humans and on chromosome 17 in mice. In humans, MHC-II encoding genes are called human leukocyte antigen (HLA) genes and are encoded by HLA-DR, -DP, -DQ genes; in mice H-2 genes encode MHC-I genes, H-2K and H-2D, and the MHC-II complex I region gene products, I-A and I-E proteins (Shiina et al., 2009). Class II transactivator (CIITA) is called the "master regulator of MHC-II gene expression", CIITA interacts with various transcription factors at the proximal promoter region also known as the locus control region (LCR) of the MHC-II gene and initiates mRNA transcription (Lochamy et al., 2007).

Histone modifications regulate MHC-II transcription as IFN-γ has been shown to increase acetylation of H3 and H4 in the HLA-DR promoter region. Histone deacetylase inhibitors (HDACs), like trichostatin A (TSA), can also induce MHC-II transcription independent of CIITA (Beresford and Boss, 2001; Chou et al., 2005). MHC-II gene expression is also regulated at the epigenetic level by suppressing CIITA-PIII promoter activity by over expressing the Blimp-1 protein resulting in a lower surface expression of

MHC-II (Piskurich et al., 2000). Post-translational modifications are also known to regulate MHC-II protein expression; for example, MARCH-1 (membrane associated ring-CH-type finger 1) protein acts as an E3 ubiquitin ligase for MHC-II (De Gassart et al., 2008).

MHC-II molecules are assembled in the endoplasmic reticulum (ER) where the three MHC-II dimer complexes are assembled on the trimeric invariant chain (Ii), also know as CD74 molecule. CD74 is a type II trans-membrane protein. The nonamer complex of MHC-II – CD74 moves from ER to the trans-golgi network (TGN) and directly to the endocytic system compartment or to the plasma membrane before moving into the endocytic compartments (Cresswell, 1994). CD74 acts as a chaperone for MHC-II enabling its proper folding and transportation from the ER. Post-translational phosphorylation of CD74 is essential for the transport of the nonameric complex from the ER (Anderson and Roche, 1998). CD74 also prevents binding of self-antigenic peptides in the ER by blocking the MHC-II antigen-binding groove through its association with CLIP, which sits in the groove of the MHC-II peptide binding pocket (Ghosh et al., 1995).

Movement of the MHC-II – CD74 complex from the ER or cell surface to the endocytic compartment for antigen loading is regulated by the “di-leucine like” motifs in the N-terminal cytoplasmic tail of the CD74 (Pond et al., 1995). Once the MHC-II – CD74 is internalized into the late endosome the CD74 bound to MHC-II undergoes various proteolytic cleavages by proteases (such as cathepsin S in B cells) (Figure 3), leaving behind only CLIP in the MHC-II peptide binding pocket, which is replaced by

the ability of HLD-DM (H-2M in mice) to catalyze its exchange (Sloan et al., 1995). Under highly acidic conditions in the late endosome, CLIP in the MHC-II binding groove is replaced with exogenous antigenic peptide and the complex is transported to the cell surface for antigen presentation to CD4+ T cells.

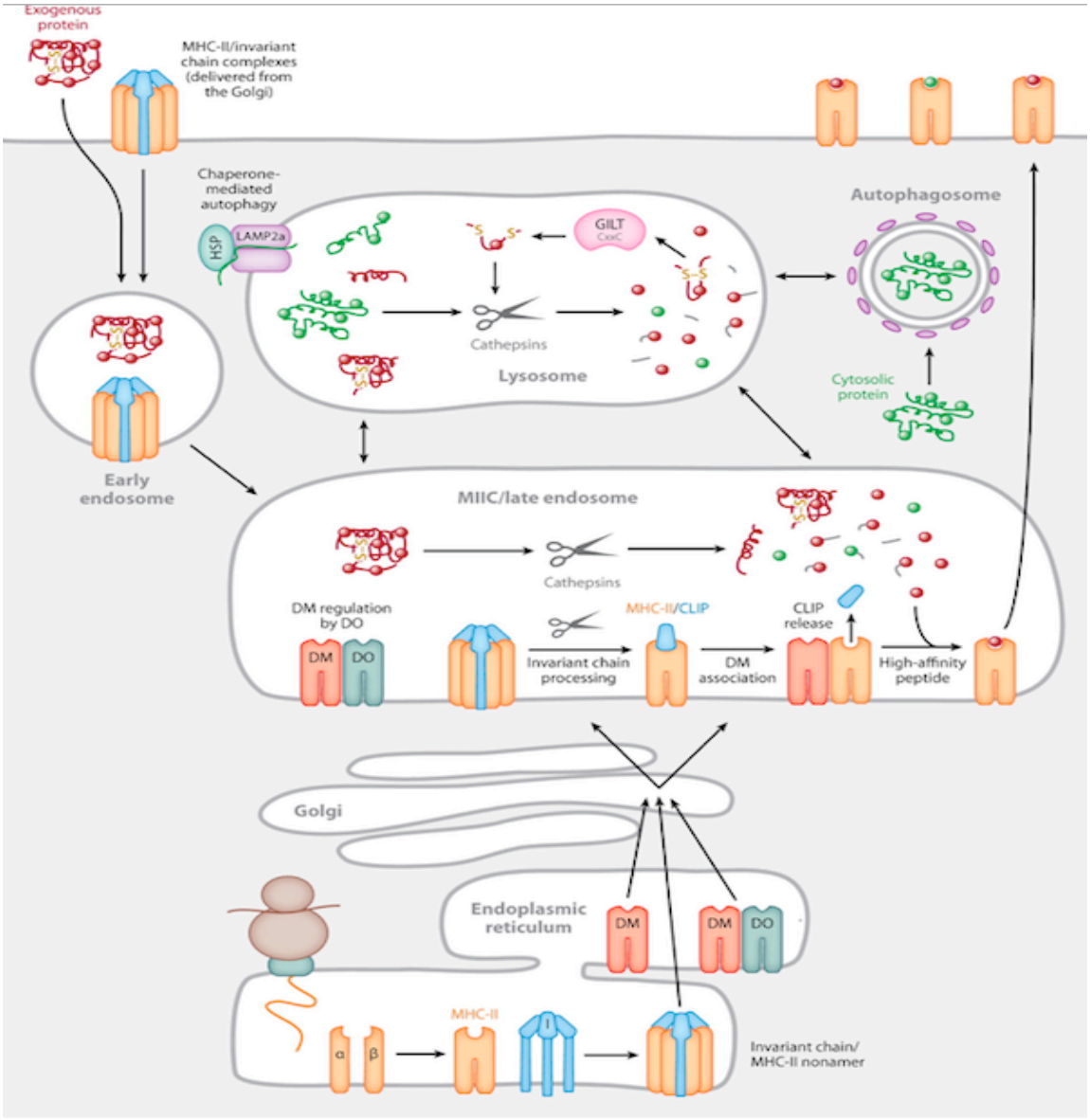


Figure 3: MHC-II and antigen processing and presentation. This figure shows a brief overview of the MHC-II – CD74 complex formation and its translocation from the ER, processing of CD74, and replacement of CLIP with the antigenic peptide presented on the surface of the B cells (Blum et al., 2013).

CD74 exists in more than one isoform due to alternative splicing, (Figure 4). In mice, CD74 has two forms, p31 (p31Ii) and p41 (p41Ii), which are 215 and 279 amino acids long. In humans, CD74 exists in four isoforms p31 (p31Ii), p41 (p41Ii), p35 (p35Ii) and p43 (p43Ii) with the latter two containing a longer N-terminal (O'Sullivan et al., 1987; Shachar et al., 1995). There is no difference in binding affinity of any splice variants of CD74 to the MHC-II dimer. In humans, p33Ii is the most common isoform accounting for about 80% of the total CD74 in immune cells and the CD74 trimer can contain more than one isoform with at least one p35Ii in the trimer complex (Lamb and Cresswell, 1992). The p35Ii has an ER retention motif in the N-terminus which acts as a quality check to retain the misfolded or improper MHC-II – CD74 complex in ER. The p35Ii ER retention motif is phosphorylated and inhibited once it properly associates with the MHC-II complex, thereby resulting in transportation of the nonamer complex (Anderson et al., 1999).

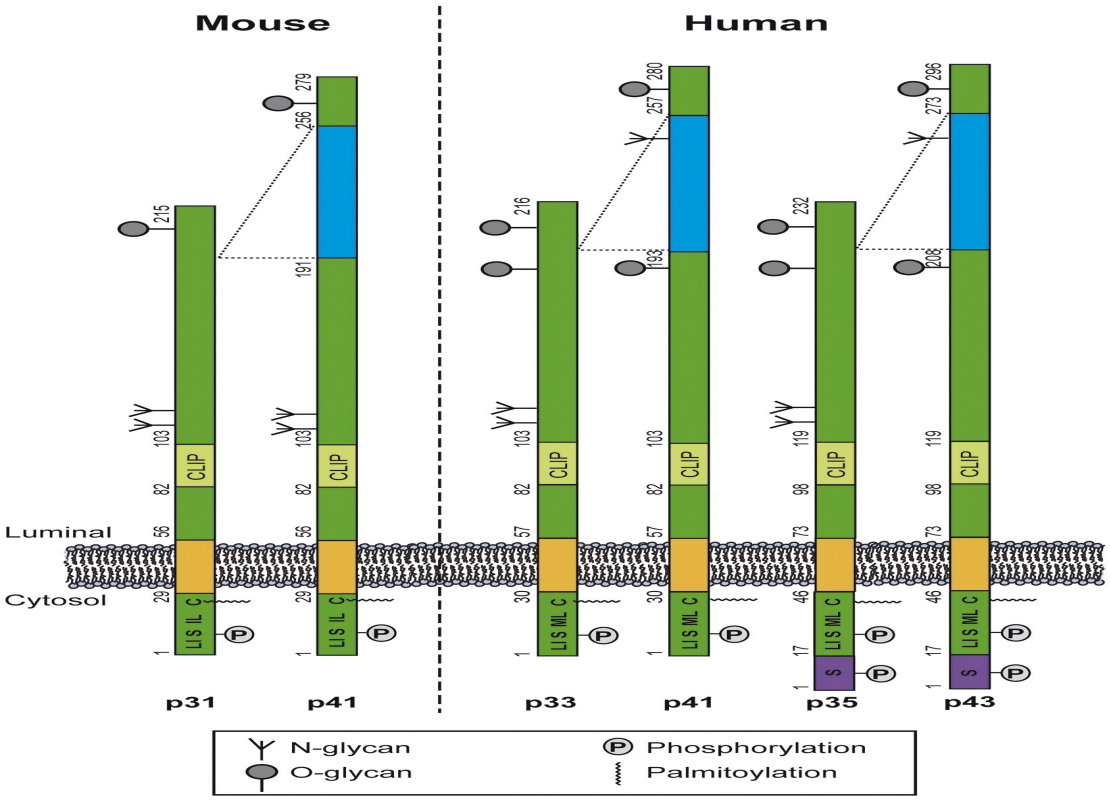


Figure 4: CD74 isoforms in mice and humans. This diagram shows the differences in the various isoforms of CD74 both in mice and humans (Schroder, 2016). Figure reproduced with permission from Elsevier Publishing Group.

In B cells and other immune cells like macrophages, a small percentage of modified CD74 (~ 3 – 5 %) is known to translocate to the cell surface independent of the MHC-II complex. This chondroitin sulfate modified CD74 acts as a receptor for macrophage migration inhibitory factor (MIF), which binds to the CD74 along with the co-receptor CD44 (Figure 5), thus initiating a downstream inflammatory signaling pathway that results in activation of NF-κB and CD74-dependent B cell proliferation and

survival (Gore et al., 2008). CD74 is important not only for proper MHC-II assembly but it also plays an important role in B cell survival, maturation, and proliferation and in proper functioning of the adaptive immune system. Loss of CD74 results in reduction in MHC-II expression and poor B cell differentiation, and a significant reduction in CD4+ T cells in the thymus and periphery, likely due to the lack of a diverse MHC-II peptide repertoire required for thymic selection of CD4+ T cells (Bodmer et al., 1994).

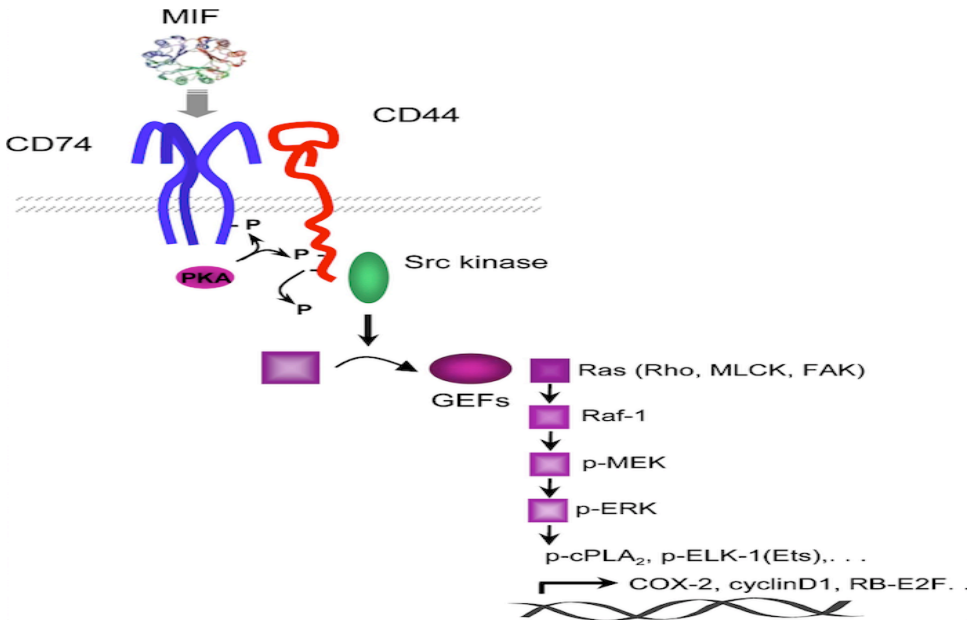


Figure 5: CD74 mediated downstream signaling. This figures shows the signaling transduction downstream of CD74-MIF interaction leading to activation of the ERK/MAPK pathway, which plays an important role in cell proliferation and survival (Shi et al., 2006). Figure reproduced with permission from Elsevier Publishing Group.

Macrophage migration inhibitory factor (MIF)

Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, is one of the earliest discovered soluble factors that has an important role in regulation of innate, adaptive immunity and an inflammatory response. Located on chromosome 22 (22q11.2) in humans and on chromosome 10 in mice, the molecules are highly homologous (Kozak et al., 1995). MIF is conserved across different species, as a mammalian homolog of MIF has been detected in fish, insects and bacteria (Jaworski et al., 2001; Sato et al., 2003). Initially MIF was thought to be released only by T cells, as it was identified as a T cell-derived soluble factor that inhibits migration of macrophages, but over the next years, many studies have shown strong evidence that MIF expression is ubiquitous. It has a broad tissue distribution, including the immune system, with even higher levels of expression in endocrine glands (Baugh and Bucala, 2002). Innate immune cells when exposed to exotoxins upregulate the expression of TLR4 and release MIF along with other cytokines to clear the infection. This release of MIF is regulated in an autocrine fashion as it is a positive regulator of TLR4 expression (Roger et al., 2001).

MIF, by binding to CD74 and the chemokine receptors CXCR2 and CXCR4 plays an important role in inflammation, immune cell proliferation, activation and migration (Bernhagen et al., 2007; Leng et al., 2003). MIF binding to CD74 initiates activation of extracellular regulated kinases 1/2 (ERK1/2), which in turn activate down stream proteins such as PLA2, which is known to inhibit the anti-inflammatory effects of steroids (glucocorticoids) and neutralize their immune suppressive activity (Leng et al.,

2003). MIF has been shown to bind to the chemokine receptors CXCR2 and CXCR4 alone or along with CD74 and can cause migration of monocytes and T cells resulting in progression of atherosclerosis (Bernhagen et al., 2007).

MIF also plays an important role in B cell migration and maturation as studies have shown that MIF binding to CD74 or CD74 along with CXCR4 receptors initiates these processes by recruiting surface receptors and activating alternate protein tyrosine kinases (Klasen et al., 2014). MIF binding to CD74 and CXCR4 has been shown to induce B cell recruitment via activation of ZAP-70 protein tyrosine kinase in B cell immune disorders (Klasen et al., 2014). In mature B cells MIF recruits c-Met, a ligand of the hepatocyte growth factor receptor (HGFR), to the MIF-CD74/CD44 complex. This stimulates the production of hepatocyte growth factor (HGF) and induces activation of ETS-2 family transcription factors, which are known to activate anti-apoptotic Bcl-2 family proteins resulting in cell survival (Gordin et al., 2010).

MIF is highly expressed in different primary tumors, as it is a negative regulator of the tumor suppressor gene p53, an important player in cell cycle regulation and induction of apoptosis. MIF inhibits the function of p53 via activation of JAB1 and PLA2 proteins. JAB1 inhibits the cell cycle inhibitor KIP1, along with p53, while PLA2 activates COX2 production which inhibits p53 activity (Conroy et al., 2010). MIF helps maintain tumor growth and survival by inducing angiogenesis under hypoxic conditions by stabilizing HIF1α expression and by inducing Bcl-2 expression upon binding to CD74 (Binsky et al., 2007).

Toll-like receptor (TLR)

The discovery of TLRs changed the perception of innate immunity from being a non-specific unsophisticated subunit of the immune system to being a very important and focused player in mounting an initial immune defense. TLRs help in recognition and clearance of pathogens and damaged organelles. Products of pathogen-associated molecular patterns (PAMPs) and products of damaged organelles, the danger-associated molecular patterns (DAMPs), bind to receptors known as pattern recognition receptors (PRR). The Medzhitov and Janeway groups were the earliest investigators to clone TLRs; they showed that transfection of hToll chimeric protein into macrophages resulted in NF-κB mediated activation of CD80, a co-stimulator of T cells, thus linking innate to adaptive immunity (Medzhitov et al., 1997). The protein hToll was later renamed TLR4. Since the first cloning of TLR4 there have been 9 more TLRs genes discovered in humans and 12 in mice (Kawai and Akira, 2009).

The N-terminal domain of the TLRs plays an important role in pathogen recognition via the leucine rich region (LRR) while the intracellular Toll/IL-1R (ITR) domain initiates downstream signaling. TLRs are expressed both at the cell surface and intracellularly. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface, while TLR3, TLR7, TLR8 and TLR9 are expressed in the endosome or in the lysosome (Figure 6). TLRs can form homodimers or heterodimers, and recognize various microbial products like proteins, lipids, and/or the nucleic acids (dsDNA, ssRNA) (Akira et al., 2006; Kawai and Akira, 2009).

TLR signaling involves activation of the cytosolic adaptor proteins MyD88 (Myeloid differentiation primary response gene 88), TIRAP (Toll/interleukin-1 receptor domain-containing adapter protein), TRAM (translocating chain-associating membrane protein) and TRIF (TIR-domain-containing adapter-inducing interferon-β), which are involved in both MyD88-dependent and MyD88-independent pathways. The downstream signaling from both pathways results in activation of NF-κB and MAP (Mitogen-activated protein) kinase that regulate inflammation. All TLRs except TLR3 signal through the MyD88-dependent pathway (Kawai and Akira, 2007). In the MyD88-dependent pathway, TLR interaction with downstream MyD88 activates IRAK4 (interleukin-1 receptor-associated kinase 4) recruitment, which interacts with and activates TRAF6 (TNF receptor associated factor 6), an E3 ligase that forms a complex with Ubc13 initiating the ubiquitylation process that activates TAK1 (TGF-β-activated kinase 1). TAK1 along with TAB1 (TAK1-binding protein 1), TAB2 (TAK1-binding protein 2) and TAB3 (TAK1-binding protein 3) activates the IKK (IκB kinase) complex that phosphorylates and degrades IκB (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor), resulting in NF-κB activation and translocation to the nucleus (Chen, 2005; Kawai and Akira, 2007).

In the MyD88-independent pathway, TRIF plays an important adaptor protein role in initiating downstream signal activation. TRIF binds to the cytoplasmic tail of TLR3 activating TRAF3 and initiating TBK1 and IKKi complex formation. The TBK1-IKKi complex phosphorylates IRF3 resulting in its dimerization and translocation to the nucleus where it activates IFNβ. The N-terminal region of TRIF induces the NF-κB

activation pathway by activating TRAF6 via the RIP1 (receptor-interacting protein kinase 1).

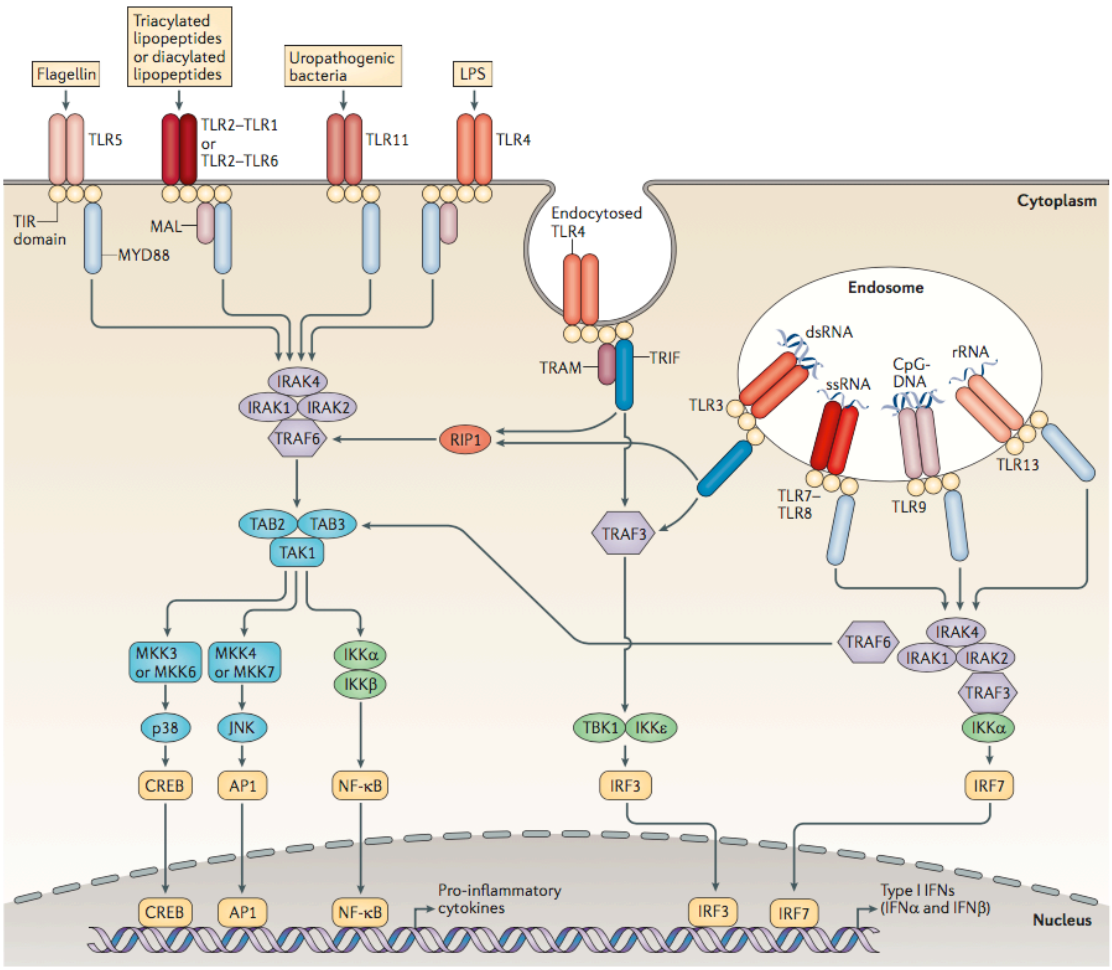


Figure 6: TLR ligands and TLR signaling. This figure shows various TLR activators and their downstream signaling pathways (O'Neill et al., 2013). Figure reproduced with permission from Nature Publishing Group.

Ubiquitin-proteasome system (UPS)

The ubiquitin-proteasome system (UPS) plays an important role in post-translational regulation of protein quality control in the cells. The UPS system regulates ubiquitination, a process of tagging misfolded or improperly assembled proteins with multiple ubiquitin (Ub) molecules, which contain 76 amino acids, as 8.5kDa (kilodalton) proteins, and targeting them for ATP dependent hydrolyses via the 26S proteasome system (Ciechanover and Iwai, 2004). Post-translational modification of proteins by Ub molecules occurs on the lysine site of the target protein where the glycine near the carboxy terminus of Ub binds to the amino group in lysine on the target protein (Nandi et al., 2006). The binding of Ub to the protein targets can regulate their degradation and translocation; there are usually multiple Ub molecules binding at Lys-48 of a protein resulting in proteasomal degradation, while mono/single Ub binding or classical Lys-63 binding helps in protein translocation, signal transduction and DNA repair (Weissman, 2001).

The Ub tagging of the protein involves a multi-step process before the protein can be delivered for proteasomal degradation. The protein-Ub binding is initiated by activation of Ub through an ATP-dependent mechanism by enzyme E1. Activated Ub is transferred by enzyme E2 from the E1 to the protein substrate that is bound to Ub-protein ligase family E3. E3 catalyzes the final step in covalent conjugation of Ub to the target protein. Successive Ub molecules are added to the previously conjugated Ub on the target protein resulting in the polyUb chain that is later recognized by 26S proteasome, a multi-catalytic protease complex, for degradation (Mani and Gelmann,

2005; Pickart and Eddins, 2004). The 26S proteasome is made up of a 20S catalytic core that carries out the degradation activity and a 19S regulatory component at both ends that regulates substrate entry into the catalytic core (Zwickl et al., 2001).

Autophagy

The word autophagy means self-eating. This cellular process was observed about 50 years ago in rat liver cells in which the mitochondria and other intracellular organs were observed to undergo lysosomal degradation (Deter and De Duve, 1967). Autophagy is a selective recycling pathway for clearing up damaged intracellular organelles and protein aggregates, where the intracellular components are delivered to lysosomes to enable cell survival during starvation conditions. Cells use autophagy to breakdown lipids, carbohydrates and amino acids to synthesize new proteins or to meet the energy demands for normal cellular function.

There is a basal level of autophagy in every cell that is upregulated when the cells are stressed. The stress stimulus can be either nutrient deprivation, exposure to radiation, and/or oxidative stress. All these conditions result in a systemic imbalance in cells and the autophagy process can help restore intracellular homeostasis (Mizushima et al., 2008). The similarity between autophagy and proteasomal degradation ends at clearing of misfolded proteins. Autophagy not only clears long-lived proteins, but it also is involved in the clearance of intracellular organelles, while proteasomal degradation targets removal of short-lived proteins (Wu et al., 2010). In the last 10 years many groups have shown that there is an important crosstalk between these two degradation

pathways as it was shown that inhibition of UPS resulted in a compensatory upregulation of the autophagy process to clear the ubiquitinated proteins and inhibition of autophagy enhanced the proteasomal degradation pathway (Wang et al., 2013; Wu et al., 2010; Wu et al., 2008).

Based on the mode of delivery of cargo, in the lysosomes there are three types of autophagy processes that have been identified (Figure 7): microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy. Microautophagy is not involved in the cell survival process as it plays a role in maintaining cell size and membrane integrity under starvation conditions. This process is the simplest of all of the autophagic processes as proteins are directly transported to the lysosome for degradation without the requirement of any adapter proteins or membrane vacuoles, as occurs in CMA and macroautophagy. Like macroautophagy, rapamycin can induce this process, thus indicating that the target of rapamycin (TOR) regulates the microautophagy process (Uttenweiler et al., 2007).

CMA is a very selective process and is involved in the delivery of soluble cytosolic proteins only. It does not move cellular organelles for degradation nor does it require any special membrane structures for delivery of the substrate proteins. The CMA process involves binding of chaperone proteins to the target substrate containing a conserved peptide motif KFERQ, which is based on this amino acid sequence being present in over 25% of the cytosolic proteins (Dice, 1990). Heat shock protein family member Hsc70 acts as the chaperone in CMA by binding to the substrate protein at the KFERQ amino acid sequence site and transporting them to the lysosome (Chiang et al.,

1989; Dice, 1990). Substrate protein unfolding, which is an essential step for the translocation into the lysosome, is a poorly understood process. LAMP-2A, a single-span membrane protein on the lysosome acts as receptor protein in the CMA process. The substrate binds to the monomeric LAMP-2A resulting in formation of a multimeric complex that aids in translocation of the substrate protein into the lysosome for degradation (Cuervo and Dice, 1996).

Macroautophagy is the most widely studied of all the autophagic processes and it is the most complex process of the three types of autophagy. Macroautophagy can deliver not only proteins but also cellular organelles like damaged mitochondria, ER, and Golgi to the lysosome for acidic hydrolysis. The process of macroautophagy involves engulfment of the substrate protein/organelle into a double membrane vesicle, called the autophagosome, which moves along the microtubules to fuse with the lysosome for delivery of the cargo for degradation (Gallagher and Chan, 2013). Akt regulates macroautophagy via the mammalian target of rapamycin (mTOR) protein. Under nutrient replete conditions Akt is active and protects the mTOR phosphorylation by downregulating TSC1/2, a negative regulator of mTOR. By contrast under stress conditions Akt is inactivated, leading to AMPK activation, which stabilizes the TSC1/2 complex resulting in the phosphorylation and inactivation of mTOR as well as causing "induction of autophagy " (Huang and Manning, 2008, 2009).

The process of macroautophagy involves sequestration (Figure 8) of proteins and damaged organelles that includes initiation, elongation and closure of the autophagosome, as well as transportation and degradation of the autophagosome

(Gallagher and Chan, 2013). The process of formation of the double layered isolation membrane, called the autophagosome, is an essential step in the initiation of the autophagic process. The isolation membrane can originate from anywhere in the cell though there is strong evidence that it is assembled near the ER (Fujita et al., 2008). There are over 30 autophagy-associated genes (*atg*) that are involved in the process of macroautophagy, with the ULK1 complex initiating the process (Mizushima et al., 2011).

The process of autophagosome formation and the targeting of the substrate to the autophagosome occur simultaneously. Processing of the target substrate for the autophagosome involves ubiquitination of the substrate by several E3 ligases and binding of these modified substrates to the adaptor proteins, like NBR1 and SQSTM1/p62, which translocate them to the autophagosome. The mature autophagosome, containing the substrate, moves along the microtubules, fuses with the lysosome, and the autophagosome/lysosome contents undergo acidic hydrolysis and recycled molecules are exported back into the cytosol via the lysosomal efflux transporter. These recycled molecules are used for biosynthesis of new organelles or for cell metabolism (Johansen and Lamark, 2011; Kuang et al., 2013; Yang et al., 2006).

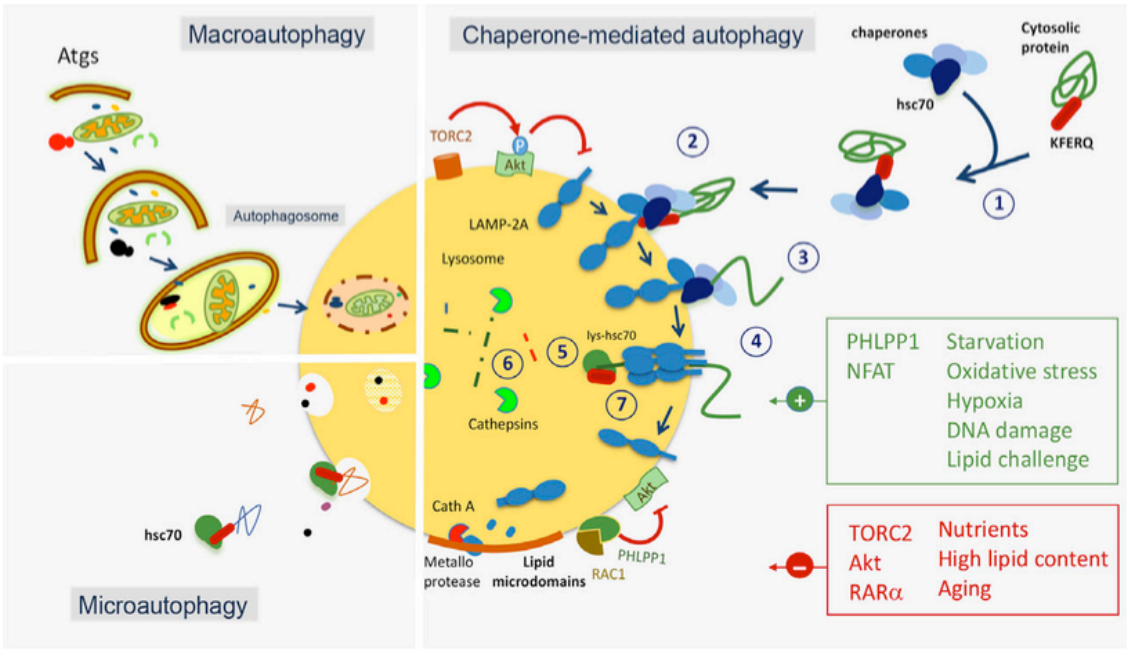


Figure 7: Types of autophagy. This figure shows different types of the autophagy processes and the adaptor proteins and secondary structures that are involved in the delivery of the substrate to the lysosome (Tasset and Cuervo, 2016). Figure reproduced with permission from John Wiley and Sons Publishers.

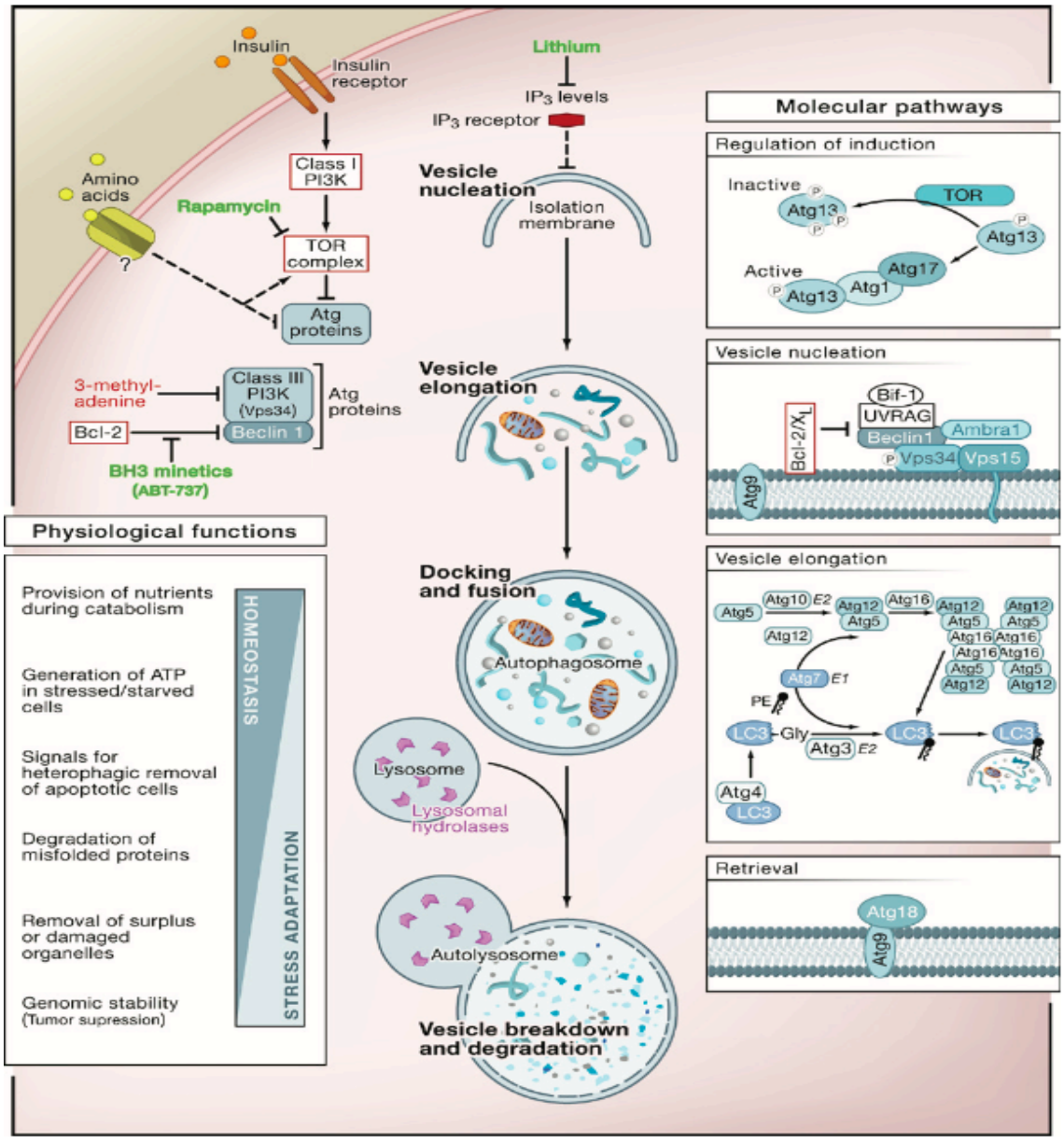


Figure 8: Molecular pathway of macroautophagy. This figure shows the most important autophagy-associated proteins that are involved in the macroautophagy process along with the physiological importance of macroautophagy (Levine and Kroemer, 2008). Figure reproduced with permission from Elsevier Publishing Group.

Macroautophagy and CMA are important player in many human diseases and in immunity. Cancer cells utilize the autophagy process for their survival, as many studies of tumors have shown a positive association between autophagy and multi-drug resistance to chemotherapeutic agents (Sui et al., 2013). Autophagy has a complex interaction with the inflammasomes in cells, as studies have shown that inhibition of autophagy by chemical inhibitors or gene silencing results in reduced pro-inflammatory cytokine production by macrophages (Harris et al., 2011). The autophagy process is a major regulator of chronic inflammation, as studies of Crohn's disease have shown that mutations in autophagy protein ATG16L an important regulator of autophagy genes, results in diminished bacterial clearance by autophagy (xenophagy) leading to bacterial overload and inflammation (Murthy et al., 2014).

Autophagy is indispensible in B and T cell development and function. $Atg5^{-/-}$ mice have shown a significant reduction in the peritoneal B-1a B cell population and impaired pre- and mature B2 cell development (Miller et al., 2008). $Atg5^{-/-}$ thymus graft transplantation into a recipient mouse results in a significant decrease in the negative selection of T cells in these animals indicating that autophagy is essential for negative selection of T cells (Klein et al., 2010). One third of all the antigens presented by B cells in the MHC-II are intracellular antigens and autophagy plays an important role in the processing and presentation of intracellular proteins, especially mitochondrial proteins, to T cells. While autophagy is essential for intracellular antigen presentation it is not a significant player in membrane-bound antigen presentation (Aichinger et al., 2013; Dengjel et al., 2005).

Rationale

The aim of this work was to determine the mechanism(s) by which the processing of CD74 and subsequent cell surface expression of CLIP in the groove of MHC-II on activated B cells contributes to, or regulates, endogenous versus exogenous antigenic peptide presentation by B cells. We also explore the contribution of CD74 processing to B cell activation, survival, proliferation, expansion, and inflammation. We propose that cell surface CLIP expression is differentially regulated by macroautophagy and CMA in B cells. The central hypothesis of this proposal is that macroautophagy and CMA are important in differentiating "self versus non-self" antigen presentation and that these processes regulate the expansion and survival of distinct B cell subsets. The rationale for our hypothesis is that if we determine how autophagic processes affect the ability of B cells to present endogenous versus exogenous antigenic peptides, this knowledge can help improve therapeutic approaches in the treatment of a wide range of diseases, including autoimmune diseases, cardiovascular disease, chronic inflammatory diseases, and cancers in which B cell presentation of endogenous versus exogenous antigens may be major contributors.

CHAPTER II

MATERIALS AND METHODS

Reagents

RPMI 1640 cell culture media, heat inactivated fetal bovine serum (FBS), 200mM L-glutamine, 1M HEPES buffer, ACK lysis buffer, gentamicin sulfate, 100mM sodium pyruvate, phosphate buffered saline (PBS), LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit and CellTrace™ Violet Cell Proliferation Kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA); penicillin – streptomycin (concentration of 10,000 units/mL - 10 mg/mL, respectively) was purchased from Sigma–Aldrich Inc. (St. Louis, MO); and 40μm nylon cell strainers were purchased from Fisher Scientific Inc. (Hampton, NH).

TLR4 agonist lipopolysaccharide (LPS) was purchased from Sigma – Aldrich Inc., TLR9 ligand CpG – oligodeoxynucleotide CpG – ODN 2006 (CpG) was purchased from InvivoGen Inc. (San Diego, CA), F(ab')2 fragment of anti-mouse IgM was purchased from Jackson ImmunoResearch Laboratories Inc.(West Grove, PA), and interleukin-4 (IL-4) was purchased from Affymetrix Inc. (Santa Clara, CA).

Small molecular inhibitors and antibodies

For *in vitro* treatment of splenocytes we purchased rapamycin (Sirolimus) and bortezomib (PS-341) from Selleckchem Inc. (Houston, TX), and Hydroxychloroquine sulfate (HCQ) from Sigma – Aldrich Inc. Cathepsin Inhibitor 1, an inhibitor of

cathepsins was purchased from ApexBio Inc. (Houston, TX), brefeldin A (BFA) a fungal metabolite was purchased from Sigma–Aldrich Inc., and the MIF inhibitor ISO-1 was purchased from TOCRIS Bioscience Inc. (Bristol, UK). For B and T cell (including CD4+ and CD8+ T cells) staining for flow cytometry, Pacific Blue™-conjugated hamster anti-mouse CD3e, APC-Cy™7-conjugated rat anti-mouse CD19 (Clone 1D3), and PE-Cy™7™-conjugated rat anti-mouse CD8 antibodies were purchased from BD Biosciences Inc. (San Jose, CA), while APC-conjugated anti-mouse MHC Class II (I-A/I-E) antibody was purchased from Affymetrix Inc. (Santa Clara, CA). PerCP/Cy5.5-conjugated rat anti-mouse CD4 was purchased from BioLegend Inc. (San Diego, CA), and FITC-conjugated mouse anti-mouse CLIP (clone 15G4), which can only detect the CLIP in the binding pocket of MHC-II I-A^b (of H-2^b haplotype) mice, was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Dr. Richard Bucala from Yale University, School of Medicine (New Haven, CT) generously provided MIF-098, a potent inhibitor of MIF.

For staining of the B-1 B cells and B-2 B cells from the peritoneal cavity the following antibodies were purchased for B-1/B-2 B cell panel staining. APC-Cy™7-conjugated rat anti-mouse CD19 and PE-conjugated rat anti-mouse CD45R/B220 were purchased from BD Biosciences Inc., while PE-Cy™7-conjugated rat anti-mouse CD5 and Pacific Blue™-conjugated rat anti-mouse IgD were purchased from BioLegend Inc.

For competitive peptide binding studies, the target biotinylated peptide APi1702 with higher binding affinity for the MHC alleles than CLIP, was synthesized using an algorithm that detects peptides with high binding affinity for MHC-II (Newell et al.,

2010). The sequence of the biotinylated peptide, which is a 9-mer of SGG GAN SGF RIM AVL ASG GQY, was synthesized by Elim Biopharma Inc. (Hayward, CA)

Mice

C57BL/6 mice and B6.*lpr* mice, with a spontaneous mutation (Fas^{lpr}) making them prone to autoimmune diseases, were purchased from Jackson Laboratory Inc. (Bar Harbor, ME). CD74-deficient ($CD74^{def}$) mice were generously provided by Dr. Scott Zamvil (University of California, San Francisco, in San Francisco CA), All animals were housed in the Baylor Scott and White Hospital vivarium facility according to the Institutional Animal Care and Use Committee guidelines.

Splenocyte isolation

Mice were anaesthetized using isoflurane, followed by cervical dislocation and dissection of spleens from the animals. Spleens were collected, washed in PBS containing 3% FBS (final vol/vol %) (FBS/PBS) and passed through 40μm nylon cell strainers to make single cell suspensions. The red blood cells were lysed by incubating the cell pellet in ACK buffer for 6 minutes at room temperature (25^{o}C) in the dark and washed twice with FBS/PBS to remove traces of ACK buffer. The splenocytes were collected as a pellet and left on ice.

Peritoneal cavity lymphocyte isolation

Mice were anesthetized using isoflurane and then euthanized in a CO_2 chamber. Five mL of ice cold FBS/PBS was injected into the peritoneum of the animal and after 90 seconds the solution was collected using a Pasteur pipette. The collected FBS/PBS from the animal contains a mixture of B-1 and B-2 B cells and other immune cells. Since we euthanize the animals in a CO_2 chamber, there is very little contamination with red blood cells and so there is no need to do an ACK lysis step. Collected cells were washed once and were prepared for experimental use.

Splenocyte activation and treatment

Isolated splenocytes were plated at a concentration of 1 x 10^6 cells/mL in a Corning® Costar® 24-well flat bottom cell culture plate in RPMI 1640 cell culture medium supplemented with heat inactivated FBS at 5% final concentration (vol/vol %), 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 100 units of penicillin and 50 milligrams of streptomycin. Splenocytes were activated with TLR4 agonist LPS, TLR9 agonist CpG or F(ab')2 fragment of anti-mouse IgM + IL-4 (anti-IgM + IL-4) along with various chemical activators and inhibitors of autophagy and proteasomal degradation. The cells were incubated in a water jacketed CO_2 incubator (Thermo Fisher Scientific Inc., Waltham, MA) with 5% CO_2 concentration and 95% humidity.

Proliferation assay

The B cell proliferation assay was performed using CellTrace™ Violet Cell Proliferation Kit. The basic principle of this proliferation assay is dye dilution and reduction in fluorescence with each generation (i.e. division). For this experiment, we prepared a 5 mM stock solution of CellTrace™ Violet Cell Proliferation dye in dimethyl sulfoxide (DMSO). Isolated splenocytes from the mice were cleared of red blood cells by incubating with the ACK buffer followed by washing with FBS/PBS solution. For staining, a pre-calculated number of splenocytes were resuspended in prewarmed PBS. The dye was added for a final concentration of 5μM concentration per 1 million cells per mL and cells were incubated at 37^{o}C for 20 minutes in the dark. Cells were washed twice with FBS/PBS to quench unreacted dye. The stained cells were plated in a 24-well plate and stimulated with TLR agonist with and without a macroautophagy activator.

Competitive peptide binding studies

For the *in vitro* competitive peptide binding studies, the APi1702 was dissolved in DMSO at a concentration of 5mg/mL as a stock. For the assay, splenocytes isolated from the C57BL/6 mouse spleens were plated at a concentration of 1 million cells per well of a 24-well plate in 1 mL RPMI1640 growth medium supplemented with 5% FBS containing all the standard amino acids. The cells were then activated with the TLR stimulants (CpG or LPS) or treated for BCR activation (with anti-IgM + IL-4) and were treated either with rapamycin or HCQ at a concentration of 10μM along with APi1702 peptide at a final concentration of 5μg/mL. After 48 hours of incubation in the CO_2

incubator, the cells were washed and first stained for the surface markers CD19, CD3, CLIP, and MHC-II utilizing antibodies conjugated with different fluorochromes and incubated on ice for 20 minutes. Cells were washed twice with FBS/PBS and later stained with streptavidin to stain for the surface expression of APi1702. A negative control was included in experiment where few non-treated but activated wells were stained to get the background signal of the biotin – avidin system. Once the cells were analyzed, the geometrical mean fluorescence intensities of CLIP and APi1702 were normalized and the ratio of APi1702 to CLIP was calculated for each sample and plotted.

Cell surface staining and flow cytometry

B and T cell subsets in the cultured splenocytes were evaluated by surface staining of the splenocytes with Pacific Blue™ rat anti-mouse CD3e, APC-Cy™7 rat anti-mouse CD19, PE-Cy™7 rat anti-mouse CD8, APC rat anti-mouse MHC Class II (I-A/I-E), PerCP/Cy5.5 rat anti-mouse CD4, and FITC mouse anti-mouse CLIP (15G4) along with LIVE/DEAD® Fixable Aqua Dead Cell Stain. The cells were analyzed on a Becton Dickson FACSCanto II flow cytometer (BD Biosciences Inc., San Jose, CA), consisting of a 3 laser 10 parameter system with FACSDiva software (BD Biosciences Inc., San Jose, CA). The flow data was analyzed using FlowJo® software (FlowJo, LLC, Ashland, OR)

Statistical analysis

The acquired data from the FlowJo® software was saved in Microsoft Excel (Redmond, WA) files. The data plotting was done and statistical significance was analyzed using GraphPad Prism 6 software (La Jolla, CA).

CHAPTER III

RESULTS

Differential cell surface CLIP expression in response to TLR agonists and BCR engagement

To characterize the extent of CLIP expression resulting from various stimulators of B cell activation, we conducted *in vitro* experiments using the splenocytes from C57BL/6 mice. Splenocytes stimulated with TLR9 agonist CpG-ODN 2006 (CpG) at a concentration of 5μg/mL, TLR4 ligand LPS at a concentration of 5μg/mL, or F (ab)'2 fragment of anti-mouse IgM + IL-4 (anti-IgM + IL-4) at a concentration of 10μg/ml and 5ng/mL, respectively. We used a fragment of the antibody against the receptor instead of intact antibody against the BCR because intact antibody binding results in B cell inactivation and cell death (Phee et al., 2001). After 48 hours of *in vitro* culture, the cultured splenocytes were harvested and stained with fluorochromes-conjugated CD19, CD3, CLIP, and MHC-II antibodies.

We observed a statistically significant increase in the number of CLIP positive (CLIP+) B cells with both LPS and CpG stimulation as well as with anti-IgM + IL-4 stimulation (Figure 9A; $n = 3$; $P < 0.05$). The geometric mean of fluorescence intensity (MFI), used as an indicator of the level of expression of the molecule per B cell increased with statistical significance when compared to the level of expression on resting B cells (figure 9B; $n= 3$; $P<0.05$). The highest level of CLIP per B cell resulted from stimulation with anti-IgM + IL-4 CLIP. The geometric mean fluorescence intensity

of MHC-II per B cell (MHC-II (MFI)) also increased with CpG activation and anti-IgM + IL-4 stimulation (P<0.05), while LPS did not change the expression of MHC-II (Figure 9C; n= 3;). The frequency of B cells increased with all B cell stimulators when compared to resting B cells (Figure 9D; n= 3; P<0.05)

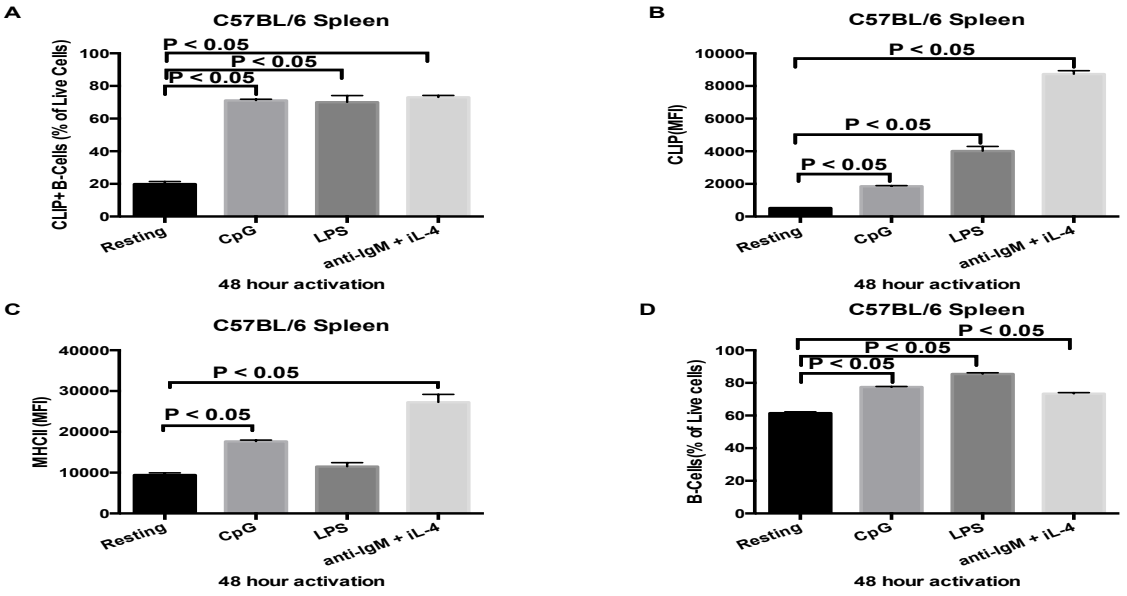


Figure 9: CLIP expression with TLR agonists and BCR activation. Panel A shows that the frequency of CLIP+ B cells increase with the TLR4 and TLR 9 agonists, LPS and CpG, respectively, and with anti-IgM + IL-4 (n = 3; P<0.05). Panel B shows a significant increase in geometric mean fluorescence intensity (MFI) of CLIP per B cell when activated with different ligands of TLR and anti-IgM + IL-4 (n = 3; P<0.05), as indicated. Panel C shows geometric mean fluorescence intensity (MFI) of MHC-II surface expression per B cell stimulated with CpG (n = 3; P<0.05), LPS (n = 3) and anti-IgM + IL-4 for 48 hours (n = 3; P<0.05). Panel D shows the change in number of B cells resulting from treatment with LPS, CpG or anti-IgM + IL-4 (n = 3; P<0.05). Statistical significance was evaluated by one-way ANOVA using GraphPad Prism.

Rapamycin inhibits B cell proliferation and CLIP expression on B cells and reduces B cell viability and CLIP expression

Autophagy is a process that helps degrade cellular material and organelles under conditions of stress or nutrient deprivation. Rapamycin is an established inhibitor of mTOR that promotes autophagy. To address the possibility that autophagy contributes to the increase in the level of CLIP or the frequency of CLIP+ B cells resulting from TLR or antigen receptor engagement, we studied the effect of rapamycin, using *in vitro* experiments, on B lymphocytes from the spleen of C57BL/6 mice. Splenocytes isolated from the C57BL/6 were stimulated with either CpG at a concentration of 5μg/mL or with LPS at a concentration of 5μg/mL or with anti-IgM + IL-4 at concentrations of 10μg/ml and 5ng/mL respectively, in a 24 well plate with and without rapamycin at a concentration of 10μM for 48 hours.

We observed that *in vitro* rapamycin treatment reduced the frequency of total B cells in all the three stimulation groups (Figure 10A; $n = 3$; $P < 0.05$). This drop in the population of B cells was significant. Rapamycin treatment reduced the number of viable CLIP+ B cells in the TLR4 and TLR9 agonist activated groups as well as in the BCR activation group (Figure 10B; $n = 3$; $P < 0.05$). Next we looked at the level of expression of CLIP per cell (MFI) and observed a significant reduction in all the groups (Figure 10C; $n = 3$; $P < 0.05$). Because the CLIP antibody used in our staining can only detect the CLIP in the peptide-binding groove of MHC-II we wanted to see if rapamycin-induced reduction in CLIP was the result of reduced expression of MHC-II. In fact, rapamycin significantly increased the level of expression of MHC-II (MFI) (Figure 10D;

P < 0.05) in all treatment groups, indicating that induction of autophagy can reduce the expression of CLIP on B cells, while maintaining or even increasing the level of expression of MHC-II.

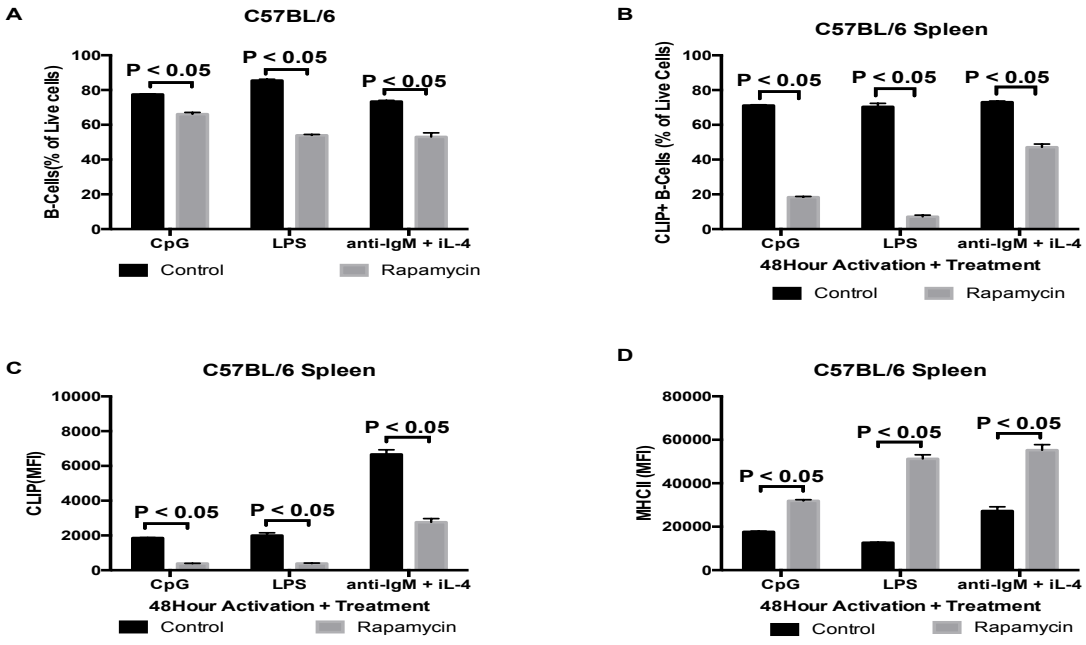


Figure 10: Rapamycin-induced inhibition of CLIP expression. Panel A shows that rapamycin treatment results in increases in B cell death in LPS, CpG, and anti-IgM + IL-4 activated groups when treated *in vitro* at a concentration of 10μM (n = 3; P < 0.05). Panel B shows that rapamycin strongly suppresses the increase in CLIP+ B cell expression (n = 3; P < 0.05) in all the treatment groups. Panel C shows that rapamycin reduces the level of CLIP (MFI) per cell in all the activation groups (n = 3; P < 0.05). Panel D shows that rapamycin significantly increases the level of expression of MHC-II in the cells in all the three stimulant groups (n = 3; P < 0.05). P values were calculated using Student's t-test on GraphPad Prism.

Rapamycin inhibits B cell proliferation

We observed that rapamycin inhibits the B cell proliferation in both the CpG-activated splenocyte population and the anti-IgM + IL-4 activated group (Figure 11A, Figure 11B; n = 3; $P < 0.05$). We analyzed the number of B cell divisions that the splenocytes had undergone. Under the "no rapamycin" conditions the splenocytes underwent five doubling generations in the 48-hour time period. When treated with rapamycin the cells underwent fewer divisions in both CpG activated (Figure 11C; n = 3; #: $P < 0.05$) and anti-IgM + IL-4 stimulated groups (Figure 11D; n = 3; #: $P < 0.05$), indicating that rapamycin inhibits the proliferation of CLIP+ B cells.

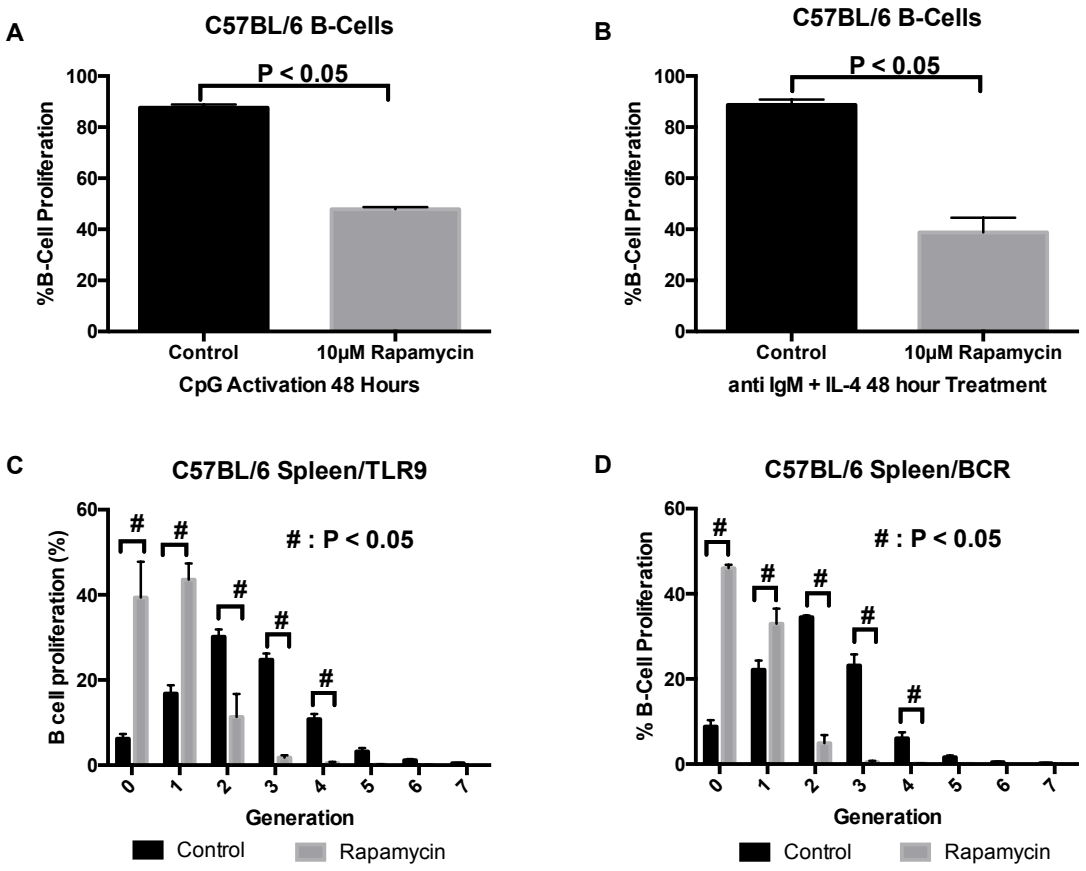


Figure 11: Rapamycin inhibits B cell proliferation. Panel A shows the reduction in the total percentage of B-cell proliferation with rapamycin when the cells were treated with CpG ($n = 3$; $P < 0.05$). Panel B shows treatment of IgM + IL-4 activated splenocytes with rapamycin inhibits B cell proliferation and the reduction is significant ($n = 3$; $P < 0.05$). Panel C shows that rapamycin treated B cells under conditions of TLR9 (CpG) activation undergo cell cycle arrest in the second generation ($P < 0.05$). Panel D shows the rapamycin-mediated inhibition of B cell proliferation in anti-IgM + IL-4 stimulated splenocytes ($n = 3$; $P < 0.05$). P values were calculated using Student's t-test on GraphPad Prism.

Hydroxychloroquine, as an autophagy inhibitor, increases cell surface CLIP expression

Our data has shown that rapamycin reduces the level of CLIP (MFI) and the frequency of CLIP+ B cells. Thus, we predicted that hydroxychloroquine (HCQ), reported to inhibit macroautophagy, would increase the level of expression of CLIP (MFI) and the frequency of CLIP+ B cells. As predicted, the levels of expression of cell surface CLIP (MFI) increased when macroautophagy was inhibited via HCQ treatment (Figure 12C; $n = 3$; $P < 0.05$). After 48 hours of *in vitro* culture, with 10μM HCQ, we observed that splenocytes from C57BL/6 cultured in the presence of HCQ and stimulated with LPS, or with anti-IgM+IL-4, showed no reduction in the frequency of CLIP+ B cells (Figure 12B; $n = 3$), while the frequency was reduced following CpG activation in the presence of HCQ (Figure 12B; $n = 3$; $P < 0.05$). The TLR9 receptor is present on the endosomal/lysosomal membrane and we postulated that the reduction in the CLIP+ B cells with HCQ in CpG activated cells could be due to HCQ-induced neutralization of the endosome/lysosomal compartment.

HCQ treatment reduced B cell viability in CpG-activated splenocytes and in anti-IgM + IL-4 (Figure 12A; $P < 0.05$; $n = 3$), but not in LPS-activated splenocytes (Figure 12A; $n = 3$; $P > 0.05$). HCQ did not induce changes in the surface expression of MHC-II in LPS activated or anti-IgM+IL-4 stimulated cells. However HCQ increased the surface expression of MHC-II in CpG-activated B cells (Figure 12D; $P < 0.05$; $n = 3$). These data suggest that CpG-induced increases in the number of CLIP+ B cells depends upon

the ability of CpG to signal via TLR9 and thus may be a function of lysosomal/endosomal pH.

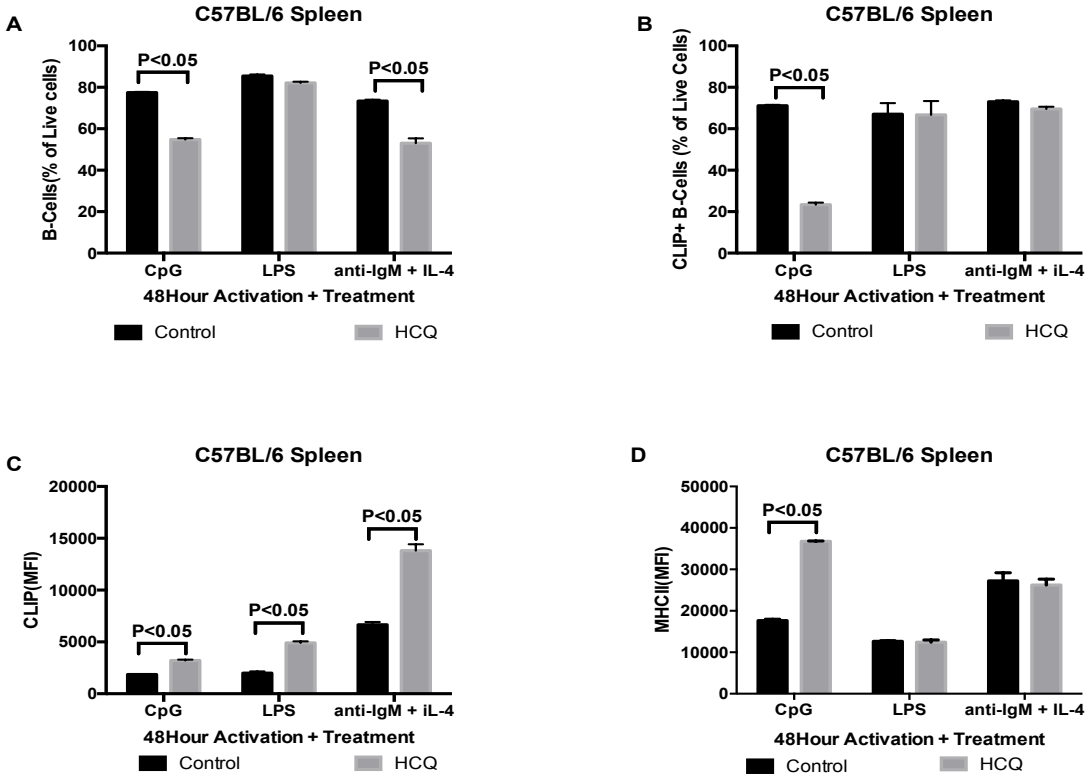


Figure 12: HCQ induces CLIP expression on B cells. In Panel A HCQ reduces the viability of B cells in CpG activated and anti-IgM+IL-4 activated splenocytes ($P < 0.05$) ($n = 3$). Panel B shows that HCQ only inhibited CLIP+ B cells in CpG activated splenocytes but not in the other two stimulations ($P < 0.05$) ($n = 3$). Panel C shows that HCQ significantly increased the expression of CLIP in MHC-II under all three conditions of stimulation ($P < 0.05$) ($n=3$). Panel D shows that HCQ treatment of CpG activated splenocytes resulted in higher expression of MHC-II on B cells ($P < 0.05$), while there was no change in MHC-II expression following LPS or anti-IgM+IL-4 activation ($n = 3$). P values were calculated using Student's t-test on GraphPad Prism.

Rapamycin alters B-1/B-2 B cell ratio

Because our previous results have shown that rapamycin reduces CLIP (MFI) on the surface of the B cells, we sought to determine if the effect of rapamycin was equivalent or different on sub-populations of B cells, i.e. on the B-1 versus the B-2 B cell subsets. The peritoneum has a relatively high frequency of B-1 B cells when compared to spleen. We isolated the peritoneal cavity-derived lymphocytes (PerC) from C57BL/6 mice and activated the cells *in vitro* in a 24 well plate with CpG at a concentration of 5μg/mL with or without rapamycin (10μM) and cultured the cells for 48 hours.

Rapamycin treatment reduced the frequency of $IgD^{-}B220^{+}CD19^{+}$ B-1 B cells subsets (Figure 13A; $n = 3$; $P < 0.05$) from the peritoneum, resulting in an increase in the ratio of $IgD^{+}B220^{-}CD19^{+}$ B-2 to $IgD^{-}B220^{+}CD19^{+}$ B-1 B cells (Figure 13B; $n = 3$; $P < 0.05$). We explored the possibility that rapamycin might selectively affect either B-1a or B-1b subsets of B-1 B cells. We also observed that rapamycin reduced the frequency of both B-1 $CD5^{+}IgD^{-}B220^{+}CD19^{+}$ B-1a and $CD5^{-}IgD^{-}B220^{+}CD19^{+}$ B-1b B cell subsets (Figure 13C, 13D; $n = 3$; $P < 0.05$). These results suggest that autophagy, via inhibition of mTOR, selects for an increase in the relative number of B-2 B cells, increasing the likelihood for T-dependent B cell activation of B-2 cells and the production of T-dependent B cell antibody production.

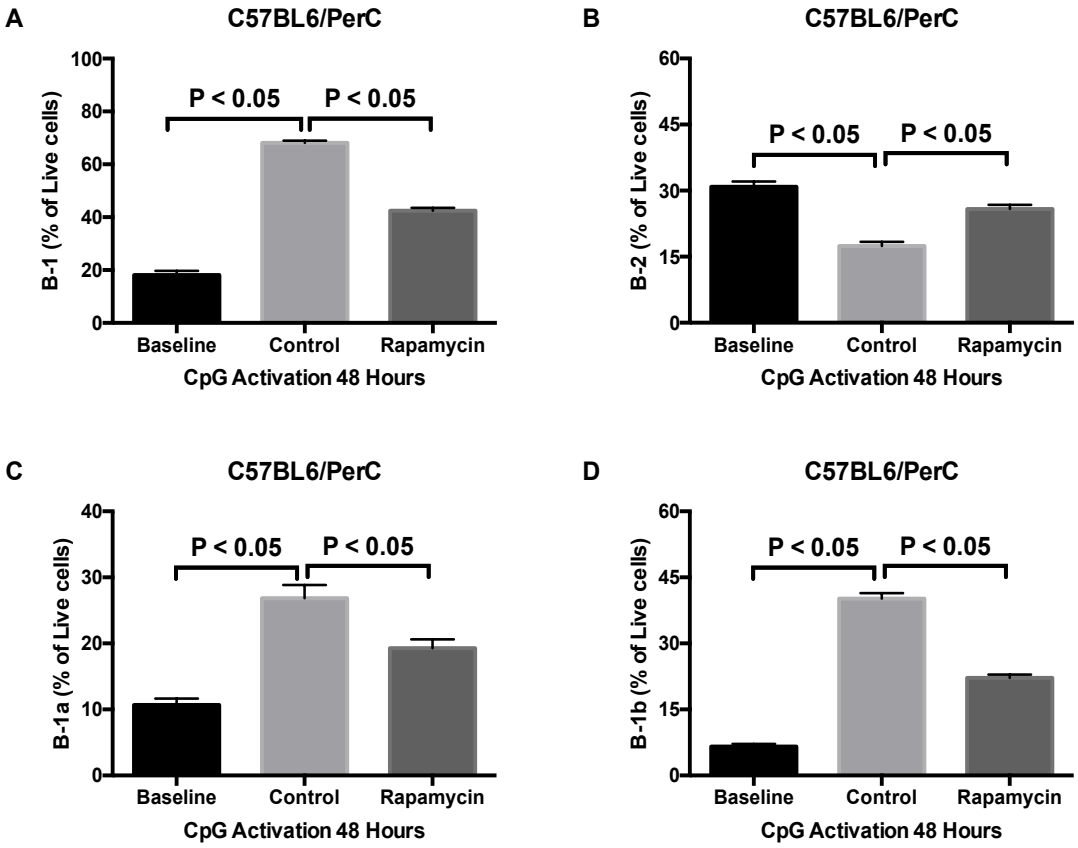


Figure 13: Rapamycin alters the B-1/B-2 B cell ratio. Peritoneal cavity cells were stimulated with CpG and cultured *in vitro* for 48 hours with and without 10μM rapamycin, Panel A shows that CpG stimulation activated $IgD^{-}B220^{+}CD19^{+}$ B-1 B cells (n = 3; P<0.05) and rapamycin treatment reduced the percentage of viable $IgD^{-}B220^{+}CD19^{+}$ B-1 B cells (n = 3; P<0.05). Panel B also shows that the $IgD^{+}B220^{-}CD19^{+}$ B-2 cell frequency was increased. The percentage of B-1 B cells upon exposure to CpG and rapamycin was reduced and we observed an increase in the total number of B-2 B-cells (n = 3; P<0.05). Panel C shows that the $CD5^{+}IgD^{-}B220^{+}CD19^{+}$ B-1 B cell subset, known as B-1a B cells, which act as natural IgM producing cells are reduced in the presence of rapamycin (n=3; P<0.05). Panel D shows that $CD5^{-}IgD^{-}B220^{+}CD19^{+}$ B-1 cells known as B-1b B cells and which act as memory B cells, are also reduced in response to rapamycin treatment (n=3; P<0.05). P values were calculated using Student's t-test on GraphPad Prism.

Rapamycin increases, and HCQ reduces, antigenic peptide loading

Because our data shows that rapamycin causes a reduction in the surface expression of CLIP, we hypothesized that loss of CLIP should be compensated for by an increase in antigenic peptide presentation by the B cells. We used a biotinylated peptide that has a higher binding affinity for the peptide-binding groove of MHC-II than for CLIP. The peptide APi1702 was reconstituted in DMSO and was added at a final concentration of 5μg/mL to splenocytes activated with CpG, LPS, or anti-IgM + IL-4, along with either 10μM of rapamycin or 10μM of HCQ.

The cells were stained 48 hours later, using a two-step method. First, the cells were stained with fluorochrome-conjugated antibodies to the cell surface markers CD19, CD3, CLIP, or MHC-II. Then streptavidin was added to detect the surface bound biotinylated APi1702 in the groove of MHC-II. The normalized ratio of the geometric mean fluorescence intensities of Api1702 to CLIP is plotted for rapamycin treatment and HCQ, giving an estimate for the ratio of Api1702 to CLIP in the groove of MHC-II. We found that the APi1702/CLIP ratio went up with rapamycin treatment, compared to control, while it went down in response to HCQ treatment, as was the case for all the activators (Figure 14; $n = 3$). This indicated that macroautophagy aids in the replacement of CLIP with antigenic peptide. Rapamycin increased the APi1702/CLIP ratio by about two-fold in CpG, LPS, and anti-IgM + IL-4 stimulations, while HCQ reduced the APi1702/CLIP MFI in all the three activation groups (Figure 14A, 14B, 14C; $n = 3$; $P < 0.05$).

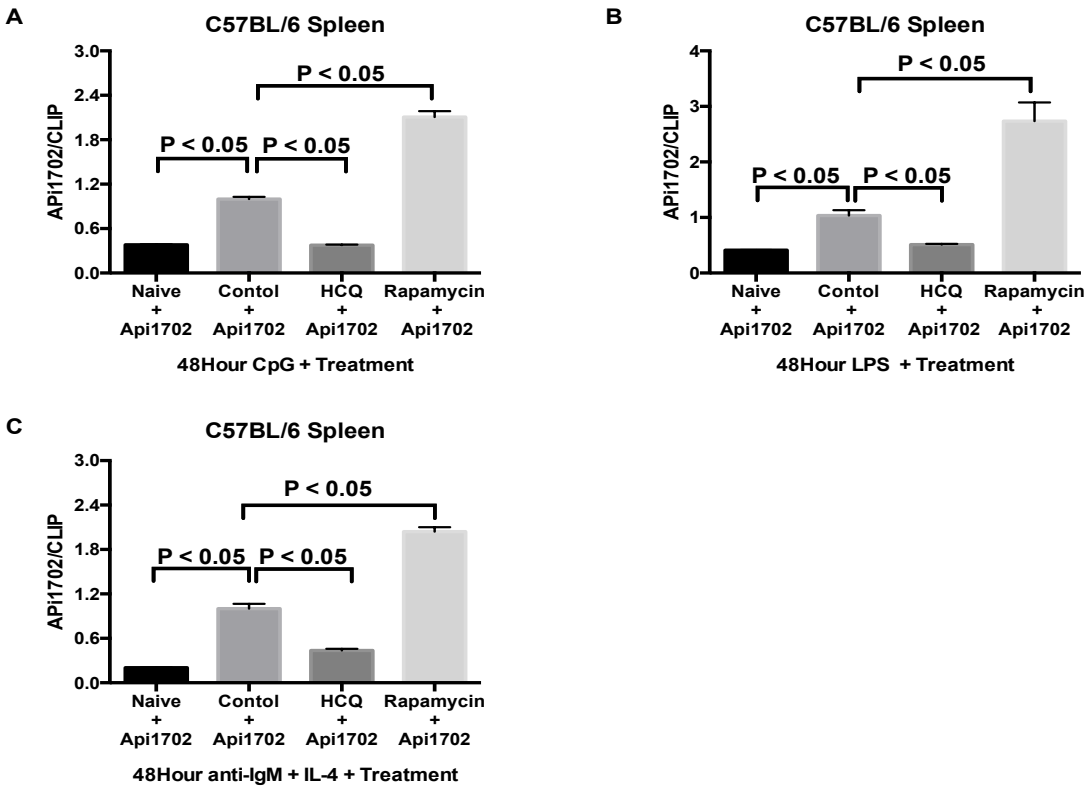


Figure 14: Macroautophagy regulates antigenic peptide loading. Panel A shows that with CpG stimulation and the ratio of synthetic peptide (APi1702) to CLIP in the groove of MHC-II went up with rapamycin treatment ($P < 0.05$; $n = 3$), while the ratio is reduced with HCQ treatment ($P < 0.05$; $n = 3$). Panel B shows that under conditions of LPS activation, the APi1702/CLIP ratio increased in B cells with rapamycin treatment ($P < 0.05$; $n = 3$), while the ratio was reduced significantly with HCQ ($P < 0.05$; $n = 3$). Panel C shows that, analogous to CpG and LPS, treatment with anti-IgM + IL-4 along with rapamycin or HCQ differentially regulates the ratio of APi1702/CLIP. Treatment with rapamycin significantly increased the ratio ($P < 0.05$; $n = 3$), while treatment with HCQ significantly reduced the APi1702/CLIP ratio ($P < 0.05$; $n = 3$). Statistical significance was evaluated by one-way ANOVA using GraphPad Prism.

Chaperone mediated autophagy (CMA) regulates the expression of CLIP per B cell *in vitro*

Based on our preliminary data, and on data from the laboratory of Janis Blum, and colleagues (Perez et al., 2016), we see that TLR activation increases the level of the lysosomal protein LAMP-2C and inhibits MHC Class II Presentation of cytoplasmic antigens by disrupting CMA. We addressed the possibility that stimulation of B cells with antigen receptor engagement (i.e., anti-IgM+IL-4) would stimulate CMA resulting in increased cell surface CLIP expression, while TLR activation that promotes at least some degree or some aspects of macroautophagy, would result in lower levels of cell surface CLIP than anti-IgM + IL-4 treated cells that utilize CMA via LAMP2A induction.

We performed experiments to determine if activation of CMA affects CLIP expression and frequency of CLIP+ B cells under two conditions of activation. For these experiments, we isolated splenocytes from C57BL/6 mice and activated them with either CpG at a concentration of 5μg/mL, LPS at a concentration of 5μg/mL or anti-IgM + IL-4 at a concentrations of 10 μg/ml and 5 ng/mL respectively, for 24 hours with and without 140 μM 6-amino-nicotinamide (6-AN), a well-established inhibitor of 6-phosphogluconate dehydrogenase, the NADP+-dependent enzyme that activates CMA.We noted a significant increase in the frequency of B cells (Figure 15A; $n = 3$; $P < 0.05$) with no change in CLIP+ B cell frequency (Figure 15B; $n = 3$), in CpG, LPS and anti-IgM + IL-4 activated splenocytes, when treated with 6-AN.Treatment with 6-AN resulted in reduction of cell surface CLIP (MFI) on B cells in all three activation groups

(Figure 15C; n = 3; $P < 0.05$). While 6-AN treatment did not affect the surface level of MHC-II expression on B cells in the CpG-activated group, it significantly reduced MHC-II (MFI) on the B cell surface in LPS and anti-IgM + IL-4 activated groups (Figure 15D; n = 3). These data suggest that even though CMA reduces the surface CLIP expression, it might not contribute to antigenic peptide presentation, because it also reduces the expression of MHC-II, at least in TLR4 activation and BCR engagement.

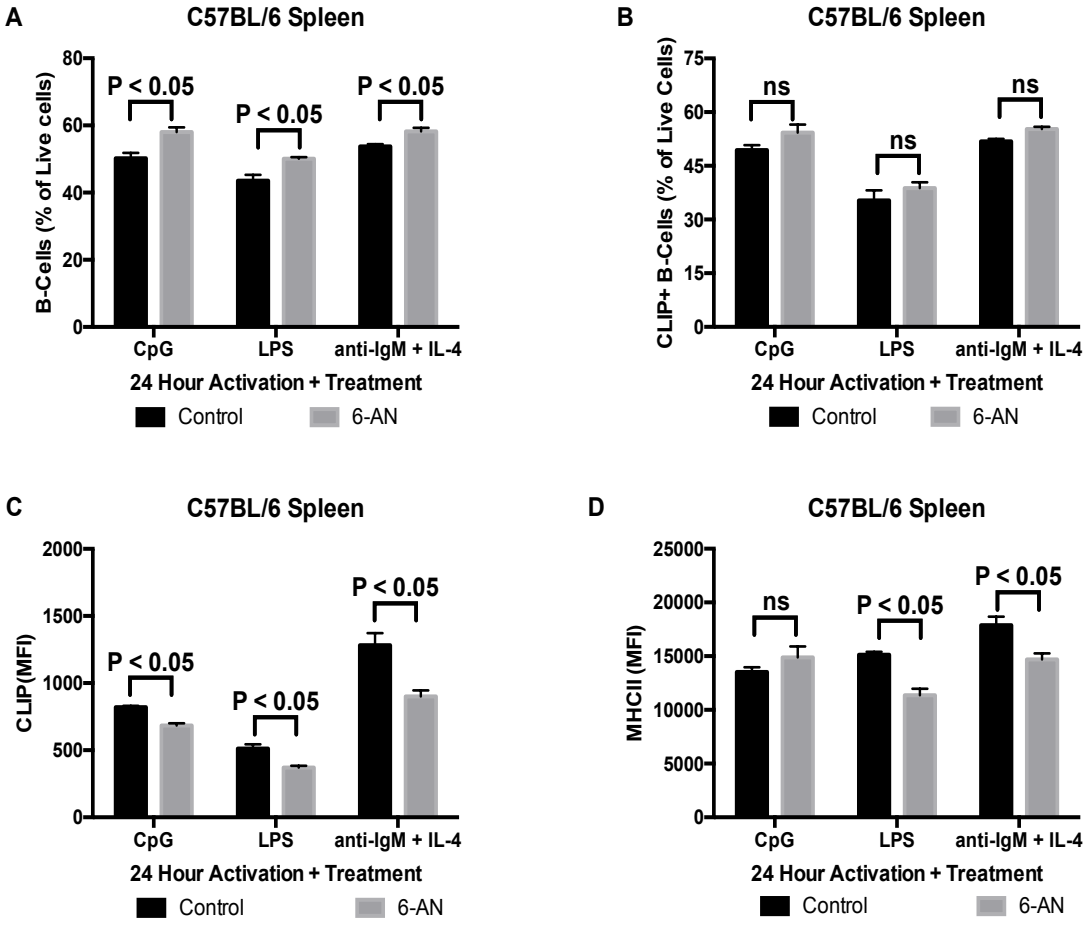


Figure 15: 6-AN reduces the surface expression of CLIP on B cells. Panel A shows that, in 24 hours of *in vitro* stimulation with CpG, LPS or anti-IgM + IL-4 activation along with 6-AN treatment, significantly increases the frequency of B cells (n = 3; $P < 0.05$). Panel B shows that 6-AN treatment concomitant with CpG or LPS or anti-IgM + IL-4 activation for 24 hours *in vitro* does not affect the frequency of CLIP+ B cells (n = 3). Panel C shows that 6-AN strongly suppresses cell surface CLIP expression on B cells. Panel D shows that the effect of 6-AN on surface expression of MHC-II on the B cells. With the exception of the CpG-activated group, 6-AN significantly reduced MHC-II (MFI) in both LPS-activated and anti-IgM + IL-4 activated splenocytes (n = 3; $P < 0.05$). P values were calculated using Student's t-test on GraphPad Prism.

CD74 deficient ($CD74^{def}$) mice have an altered immune cell profile

CLIP is the proteolytic product of CD74, also known as invariant chain (Ii). CLIP is loaded in the peptide-binding groove of MHC-II until a peptide replaces it. We performed some of the same studies described above using CD74 deficient ($CD74^{def}$) mice. These mice were generated on a C57BL/6 background. We first analyzed the B and T cell profiles of $CD74^{def}$ mice and observed that the immune cell profile in these animals is different than in C57BL/6 wild type (BL6 WT). We stained for B cells, MHC-II expression on B cells, CD4+ T cells, and CD8+ T cells from the spleen of the $CD74^{def}$ mice and compared their frequency to that of BL6 WT mice (Figure 16; n = 3).

$CD74^{def}$ mice have significantly fewer B cells (Figure 16A; n = 3; $P < 0.05$). Because CD74 plays an important role in MHC-II assembly, the absence of CD74 likely explains the significantly lower expression of MHC-II on B cells in the $CD74^{def}$ mice compared to WT (Figure 16B; n = 3; $P < 0.05$). This result is consistent with reports by other groups (Bikoff et al., 1993). Lower levels of MHC-II inhibit the expansion of CD4+ T cells in the thymus. Our results show that $CD74^{def}$ animals have significantly fewer CD4+ T cells in spleen than the BL6 WT (Figure 16C; n = 3; $P < 0.05$), but have a higher frequency of CD8+ T cells compared to the WT animals (Figure 16D; n = 3; $P < 0.05$).

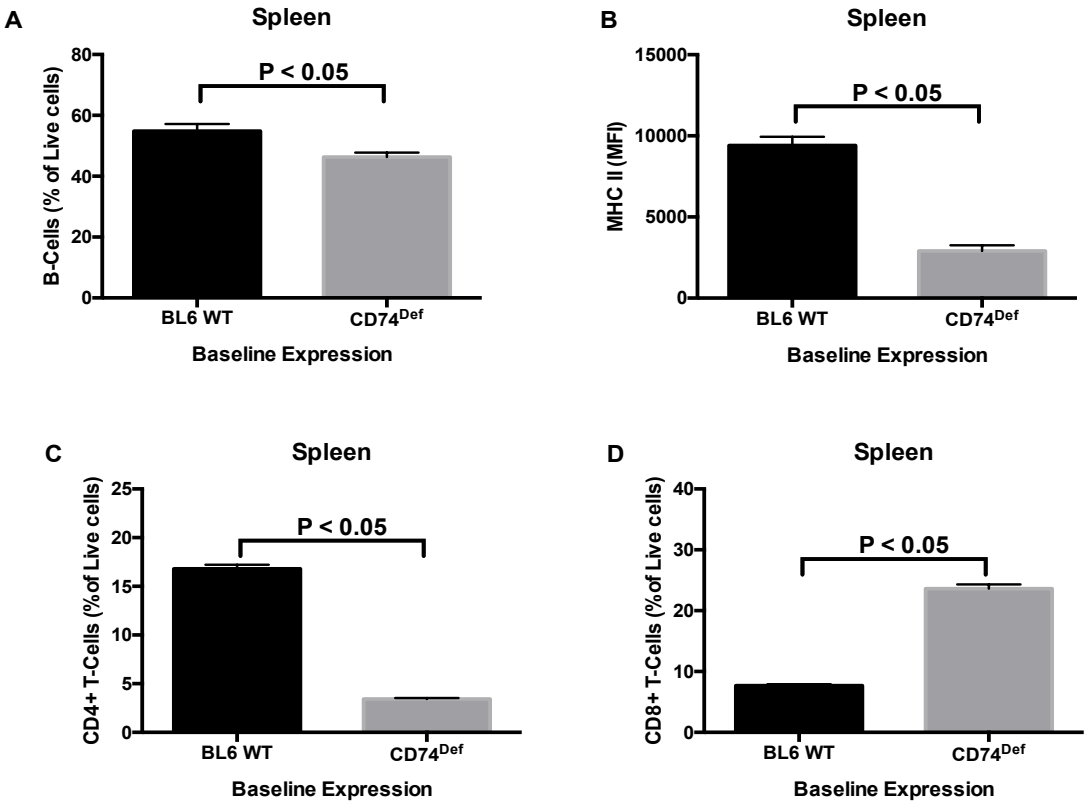


Figure 16: CD74^{def} mice B and T cell profiles in the spleen. Panel A shows that the frequency of B cells in the spleen of the CD74^{def} mice was significantly lower than in BL6 WT mice (n = 3; P < 0.05). Panel B shows surface MHC-II expression on B cells in CD74^{def} mice was significantly lower than in the BL6 WT mice (n = 3; P < 0.05). Panel C shows that lack of MHC-II repertoire potentially resulted in fewer CD4+ T cells in CD74^{def} than in the BL6 WT mice (n = 3; P < 0.05). Panel D shows there was a higher frequency of CD8+ T cells in the CD74^{def} mice than in the BL6 WT mice (n = 3; P < 0.05). P values were calculated using Student's t-test on GraphPad Prism.

We stained the lymphocytes from the peritoneal cavity of the CD74^{def} animals. The peritoneal cavity of these animals had a lower frequency of B-1 and B-2 B cells in their peritoneum (Figure 17; n = 3). The frequency of B-1 (Figure 17A; n = 3; P < 0.05),

B-2 (Figure 17B; n = 3; P < 0.05), and B-1a (Figure 17C; n = 3; P < 0.05) B cells was low compared to the BL6 WT mice, while the B-1b B cell (Figure 17D; P < 0.05) frequency was higher.

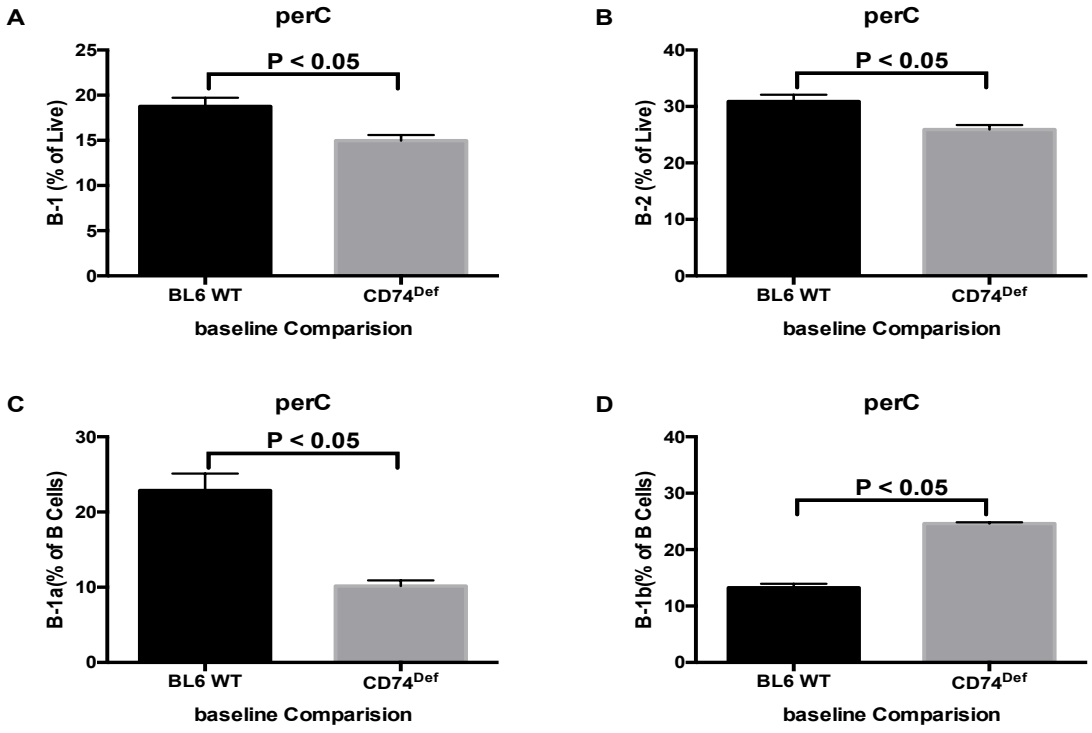


Figure 17: CD74^{def} mice B-1 and B-2 B cell subset profile. Panel A shows that the frequency of B-1 B cells in the peritoneum of the CD74^{def} mice was significantly lower than in BL6 WT mice (n = 3; P < 0.05). Panel B shows that frequency of B-2 B cells in CD74^{def} mice was lower than in the BL6 WT mice (n = 3; P < 0.05). Panel C shows that BL6 WT mice had a higher frequency of the B-1a B cell subset than CD74^{def} animals (n = 3; P < 0.05). Panel D shows that there was a higher frequency of B-1b B cells in the CD74^{def} mice than in the BL6 WT mice (n = 3; P < 0.05). Student's t-test was used to evaluate significance with GraphPad Prism.

CD74^{def} splenocyte response to HCQ, rapamycin, and 6-AN

Splenocytes isolated from CD74^{def} mice were activated *in vitro* with CpG, LPS, or anti-IgM + IL-4 for 48 hours, along with either 10μM HCQ, 10μM rapamcyin (Rapa), or with 6-AN at a concentration of 140μM. As CD74^{def} B cells do not express CLIP, we stained for MHC-II expression on the B cells and found that compared to naïve splenocytes, fewer B cells survive with the TLR stimulants or with anti-IgM + IL-4 activation (Figure 18; #: $P < 0.05$; $n = 3$). The effect of rapamycin varied with stimulation, as rapamycin upregulated MHC-II surface expression on B cells after CpG stimulation, without affecting the B cell frequency (Figure 18A; #: $P < 0.05$; $n = 3$) and reduced MHC-II expression on B cells along with B cell frequency during anti-IgM + IL-4 stimulation (Figure 18C; #: $P < 0.05$; $n = 3$). While LPS/TLR4 activation along with rapamycin had no effect on MHC-II surface expression, this treatment caused a strong reduction in the frequency of activated B cells (Figure 18B; #: $P < 0.05$; $n = 3$).

HCQ did not affect the B cell frequency, while it reduced B cell surface MHC-II expression during CpG activation (Figure 18A; #: $P < 0.05$; $n = 3$). MHC-II (MFI) was upregulated in LPS-activated splenocytes that were treated with HCQ (Figure 18B; #: $P < 0.05$; $n = 3$) with no change in the frequency of CLIP+ B cells. 6-AN had a strong inhibitory effect on the CpG-activated B cell levels of cell surface MHC-II (Figure 18A; #: $P < 0.05$; $n = 3$) with no effect on B cell frequency. In contrast, LPS in the presence of 6-AN reduced the B cell frequency and increased MHC-II surface expression (Figure 18B; #: $P < 0.05$; $n = 3$) in LPS-activated splenocytes. In anti-IgM + IL-4 activated

splenocytes 6-AN reduced MHC-II surface expression along with the frequency of B cells (Figure 18C; #: $P < 0.05$; n = 3).

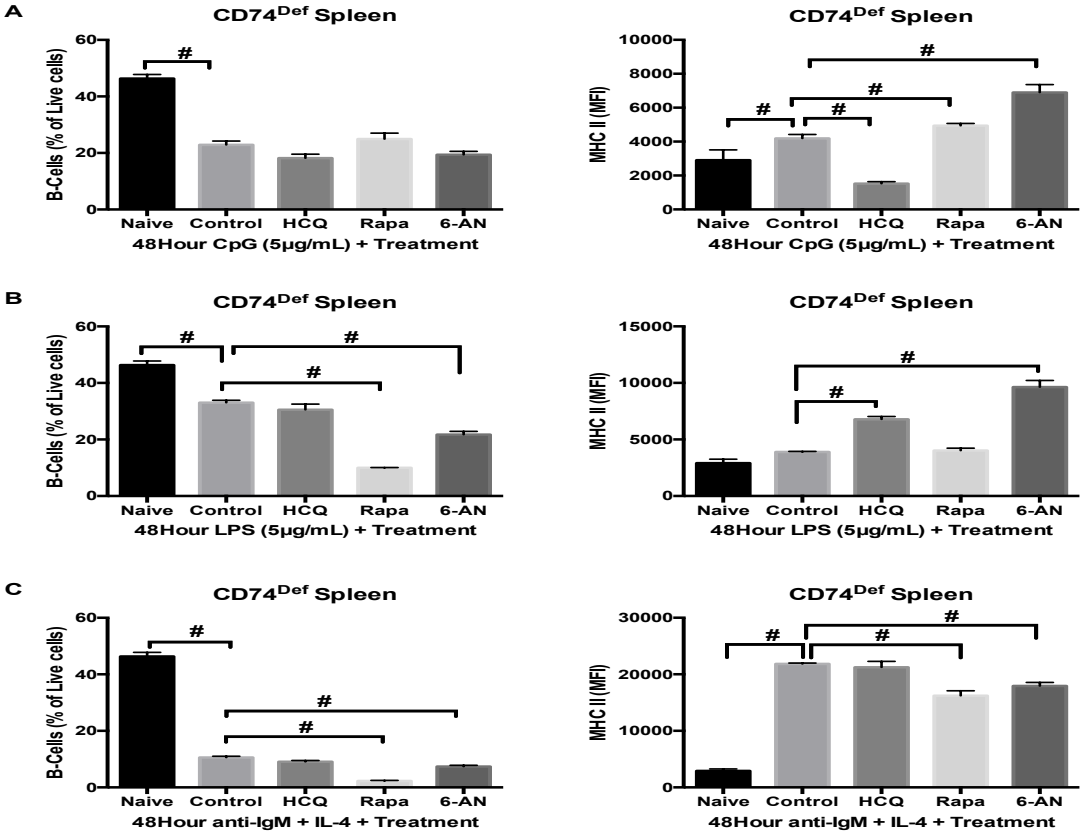


Figure 18: The response of CD74^{def} splenocytes to HCQ, Rapamycin, or 6-AN *in vitro*. Panel A shows that rapamycin (Rapa) or 6-AN increased the B cell surface expression of MHC-II, while HCQ reduced the MHC-II expression (n = 3; #: $P < 0.05$) in CpG activated splenocytes from CD74^{def} animals. Neither rapamycin nor 6-AN had any affect on B cell frequency. Panel B shows that in LPS-activated splenocytes, rapamycin reduced the B cell frequency (n = 3; #: $P < 0.05$) with no effect on MHC-II (MFI), while 6-AN reduced the B cell frequency and significantly increased the level of MHC-II (MFI) (n = 3; #: $P < 0.05$). HCQ increased the surface expression of MHC-II on the

$CD74^{def}$ B cells without affecting the frequency of the B cells. Panel C shows that in anti-IgM + IL-4 activated cells, induction of macroautophagy and CMA results in B cell loss and a reduction in the level of cell surface of MHC-II on the B cells from $CD74^{def}$ splenocytes ($n = 3$; #: $P < 0.05$), while inhibition of macroautophagy had no effect on the B cell frequency nor on the level of MHC-II (MFI) on the B cells. Statistical significance was calculated by one-way ANOVA method using GraphPad Prism.

Bortezomib (PS-341) reduces CLIP expression in B cells

PS-341 is an inhibitor of proteasomal degradation. To determine if cell surface CLIP results from proteasomal degradation of CD74, C57BL/6 splenocytes were activated for 24 hours *in vitro* with CpG at a concentration of 5 μg/mL, with LPS at a concentration of 5 μg/mL, or anti-IgM + IL-4 at concentrations of 10 μg/ml and 5 ng/mL, respectively, in a 24 well plate for 24 hours with or without 100 nM PS-341.

We observed that the treatment with PS-341 significantly reduced the frequency of B cells (Figure 19A; $n = 3$; $P < 0.05$), CLIP+ B cells (Figure 19B; $n = 3$; $P < 0.05$), and cell surface CLIP expression on B cells (Figure 19C; $n = 3$; $P < 0.05$). While PS-341 also significantly reduced the mean surface MHC-II fluorescence intensity on the B cells in LPS activated and anti-IgM + IL-4 activated groups, it upregulated the B cell surface expression of MHC-II (MFI) in CpG activated cells (Figure 19D; $n = 3$; $P < 0.05$).

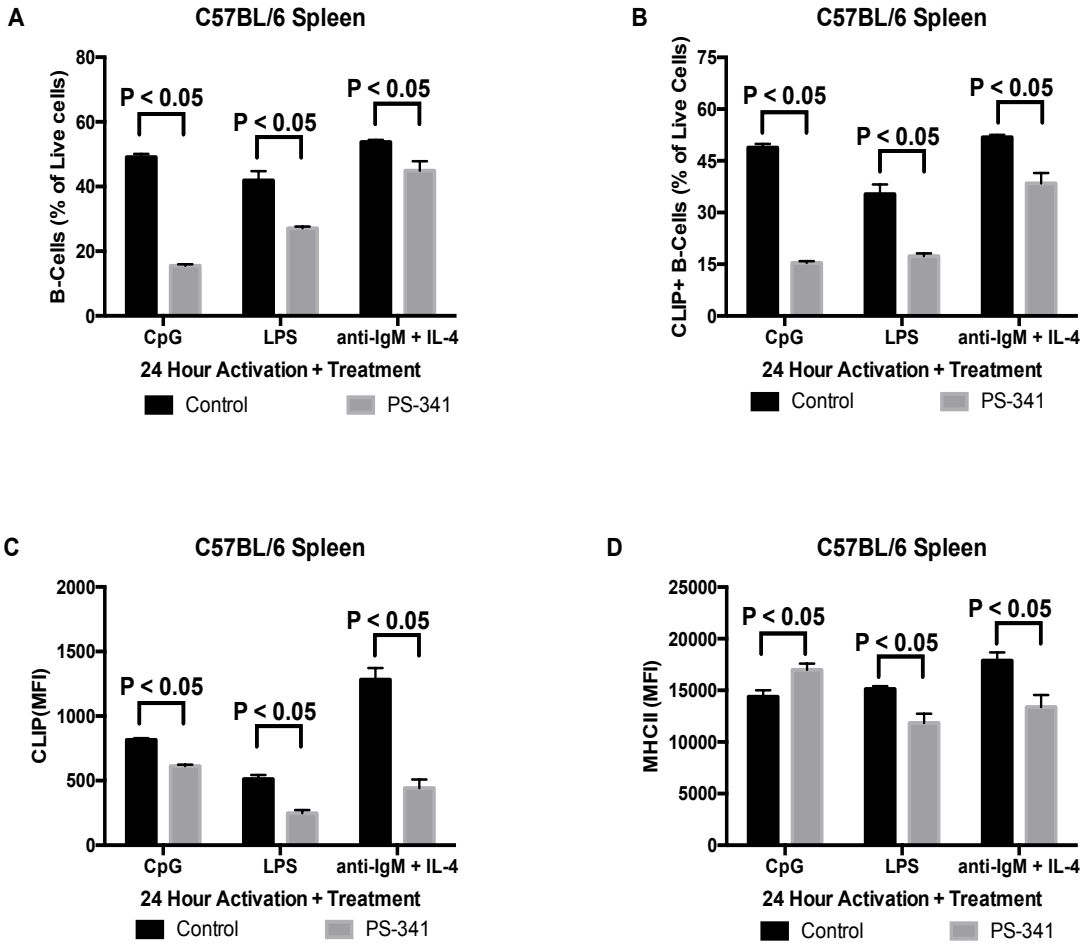


Figure 19: Proteasomal inhibitor reduces CLIP per B cell. Figure A shows that *in vitro* treatment of CpG, LPS and anti-IgM + IL-4 activated splenocytes with PS-341 significantly reduced the frequency of B cells ($n = 3$; $P < 0.05$). Figure B shows that PS-341 blocked the expansion of CLIP+ B cells in all three activation groups ($n = 3$; $P < 0.05$). Figure C shows that in all three activation groups, PS-341 treatment suppressed the CLIP surface expression on the B cells ($n = 3$; $P < 0.05$). Figure D shows that the effect of PS-341 on the MHC-II surface expression on B cells is stimulation-dependent, as CpG-activated groups showed a significant increase in MHC-II (MFI) on B cells while in LPS and anti-IgM + IL-4 activated groups, PS-341 reduced the B cell surface expression of MHC-II ($n = 3$; $P < 0.05$). P values were calculated using Student's t-test on GraphPad Prism.

Cathepsin inhibitor 1 regulation of surface CLIP

Cathepsins are cysteine proteases that play an important lysosomal role in proteolytic cleavage of antigenic peptides and of the MHC-II-bound CD74. Cathepsin cleavage of CD74 is known to generate the CLIP peptide that gets loaded into the MHC-II peptide binding groove, until it is replaced by antigenic peptides. We explored the possibility that blocking cathepsin activity will affect CLIP expression. For this experiment, we isolated splenocytes, activated them with either CpG or anti-IgM + IL-4 and treated them for 48 hours with cathepsin inhibitor 1 (Cystatin-1, 10μM), which inhibits cathepsin S, K, L and B.

Cystatin-1 reduced the frequency of B cells in the CpG-activated group (Figure 20A; $n = 3$; $P < 0.05$) but not in anti-IgM + IL-4 activated cells (Figure 20A; $n = 3$;). Cystatin-1 also reduced the frequency of CLIP+ B cells in CpG-activated cells (Figure 20B; $n = 3$; $P < 0.05$), while it had no effect on BCR activation (Figure 20B; $n = 3$). Cystatin-1 inhibited the cell surface expression of CLIP on B cells in anti-IgM + IL-4 activated cells, but not in CpG-activated splenocytes (Figure 20C; $n = 3$; $P < 0.05$). The surface expression of MHC-II on the B cells is unaltered in either of the activation groups with Cystatin-1 treatment (Figure 20D; $n = 3$). Cystatin-1 increased B cell surface expression of CD74 (MFI) in the anti-IgM + IL-4 activated group (Figure 20E: $n = 3$; $P < 0.05$), while it had no effect on B cell surface expression of CD74 (MFI) in the CpG-activated group (Figure 20E: $n = 3$).

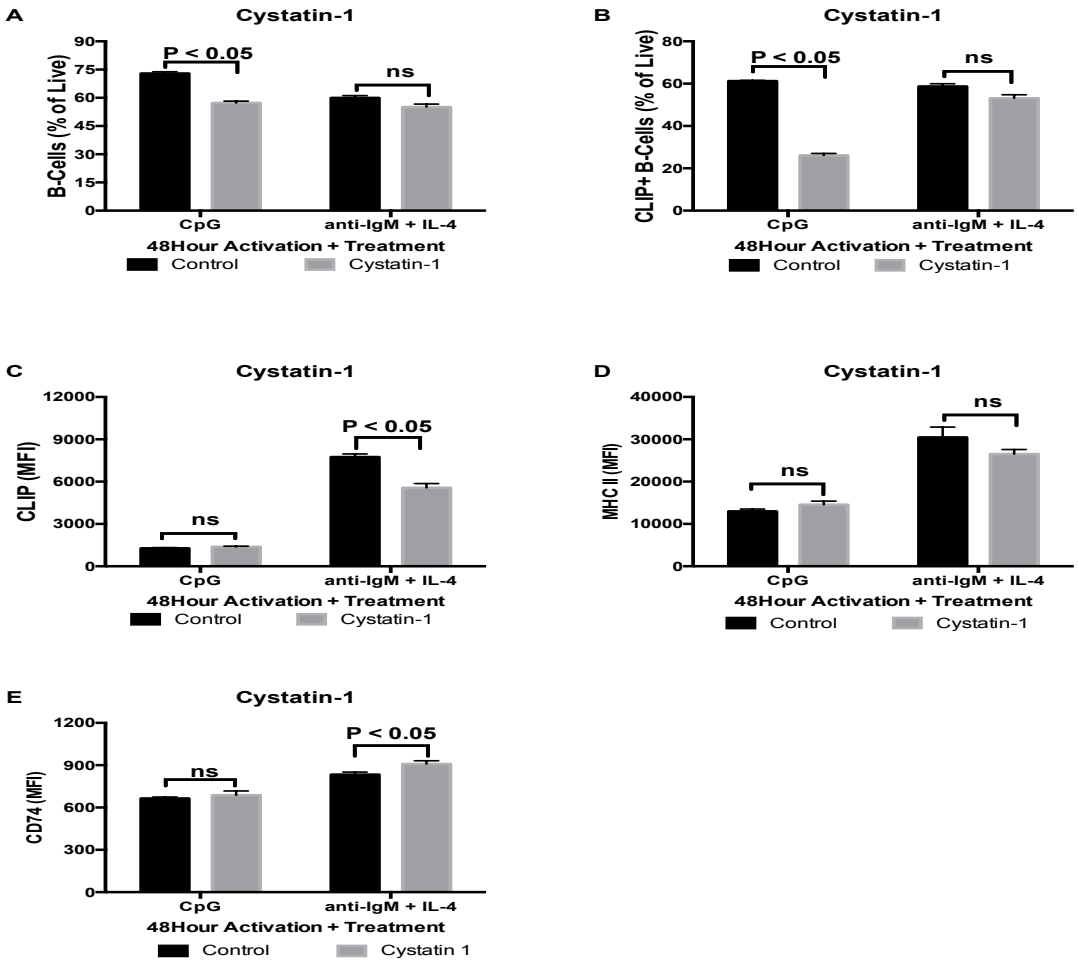


Figure 20: Cathepsin Inhibitor 1 (Cystatin-1) reduces CLIP+ B cells. Panel A shows that Cystatin-1 reduces the frequency of B cells in the CpG-activated group, but not in anti-IgM + IL-4 stimulated group ($n = 3$; $P < 0.05$). Panel B shows that Cystatin-1 has a significant effect on the number of CLIP+ B cells in CpG stimulation, but not in BCR activation ($n = 3$; $P < 0.05$). Panel C shows that cell surface CLIP expression upon BCR activation is reduced with Cystatin-1 ($n = 3$; $P < 0.05$), while cystatin-1 had no effect on the level of cell surface CLIP in CpG-activated B cells. Panel D shows that Cystatin-1 did not inhibit the surface MHC-II expression in either of the stimulations ($n = 3$). Figure E shows that Cystatin-1 significantly increases CD74 surface expression on B cells in anti-IgM + IL-4 activated splenocytes ($n = 3$; $P < 0.05$), while it had no effect on the CpG-activated group ($n = 3$). P values were calculated using Student's t-test on GraphPad Prism.

Brefeldin A inhibits CLIP expression *in vitro*

Cell surface CD74, when bound by MIF, is proteolytically cleaved by the signal peptide peptidase-like 2a (SPPL2a) protease (Schroder and Saftig, 2016). The intracellular domain of CD74 (ICD) that gets cleaved at the membrane plays an important role in B cell differentiation and activation of NF-κB and activation of an inflammation pathway via its translocation to the nucleus (Matza et al., 2002a). In this experiment we used brefeldin A (BFA) an antibiotic. Splenocytes were activated with either CpG or with anti-IgM + IL-4 along with BFA at a concentration of 2.5μg/mL and incubated for 48 hours. We stained to assess markers for B cells, surface expression of CLIP, and MHC-II on the B cell subset. We observed that CpG-activated B cells were very sensitive to BFA treatment, with over half of B cells undergoing BFA-mediated cell death (Figure 21A; $n = 3$; $P < 0.05$), while in the anti-IgM + IL-4 activated group, the frequency of B cells did not change with BFA treatment (Figure 21A; $n = 3$). BFA strongly reduced the frequency of CLIP+ B cells in both the CpG and anti-IgM + IL-4 activated cell cultures, (Figure 21B; $n = 3$; $P < 0.05$). The mean surface expression of CLIP per B cell went down in both stimulation groups (Figure 21C; $n = 3$; $P < 0.05$). The MHC-II surface expression in CpG-activated cells was reduced significantly while in anti-IgM + IL-4 activated cells it increased when treated with BFA (Figure 21D; $n = 3$; $P < 0.05$). BFA significantly increased B cell surface CD74 (MFI) in anti-IgM + IL-4 activated and CpG-activated splenocytes (Figure 21E: $n = 3$; $P < 0.05$).

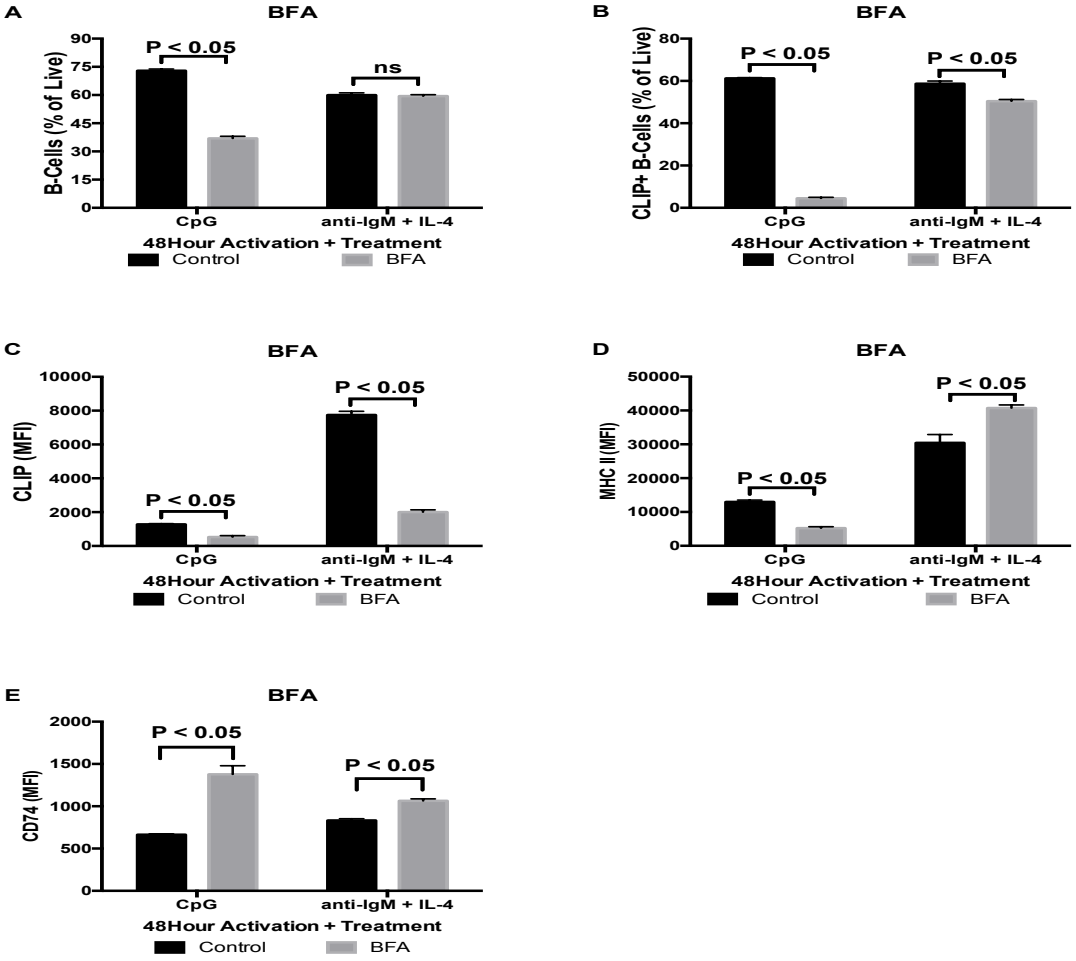


Figure 21: BFA reduces CLIP+ B cells and surface CLIP. Panel A shows that the frequency of CpG-activated cells was reduced by half ($n = 3$; $P < 0.05$), when exposed to BFA, while there was no change in the frequency of anti-IgM + IL-4 activated splenocytes ($n = 3$). Panel B shows that the frequency of CLIP+ B cells was reduced in both CpG activated and anti-IgM+IL-4 stimulated cells ($n = 3$; $P < 0.05$). Panel C shows that CLIP (MFI) was significantly reduced in both CpG-activated as well as in anti-IgM + IL-4 stimulated B cells with BFA treatment ($n = 3$; $P < 0.05$). Panel D shows that BFA reduced MHC-II expression on CpG-activated B cells, while it upregulated MHC-II on anti-IgM + IL-4 activated B cells ($n = 3$; $P < 0.05$). Panel E shows that BFA significantly increased CD74 surface expression on B cells in anti-IgM + IL-4 activated splenocytes and also in the CpG-activated group ($n = 3$; $P < 0.05$). P values were calculated using Student's t-test on GraphPad Prism.

CLIP expression in MIF-KO B cells *in vivo*

In our preliminary experiment we compared the expression of CLIP on the surface of B cells in spleens of MIF-knockout (KO) mice that were generated on the C57BL/6 background to that of the C57BL/6 wild type. This experiment was done with the help of our colleagues in Dr. Richard Bucala's group at Yale University, School of Medicine. We injected CpG dissolved in saline via the intraperitoneal route at 1 mg/kg of body mass of the animals into MIF-KO animals (n=2) and age matched C57BL/6 wild type control mice (n=3). At 48 hours post injection, the animals were sacrificed and the spleens were processed into single cell suspensions, free of red blood cells, and were stained for markers of B cells, MHC-II and CLIP.

The data shows that MIF-KO animals had less B cells than wild type and CpG increased the frequency of B cells in wild type but not in MIF-KO animals (Figure 22A). CpG increased the frequency of CLIP+ B cells in wild type but not in MIF-KO (Figure 22B). CpG increased the expression of CLIP (MFI) on the B cell surface in wild type but not in MIF-KO mice (Figure 22C). MIF-KO animals had a higher level of MHC-II surface expression on the B cells than in the wild type animals and CpG did not affect the expression of MHC-II in either of the mouse strains (Figure 22D). Even though we did not have enough animals to calculate statistical significance, these data show a trend that MIF is an important negative regulator of antigenic peptide presentation.

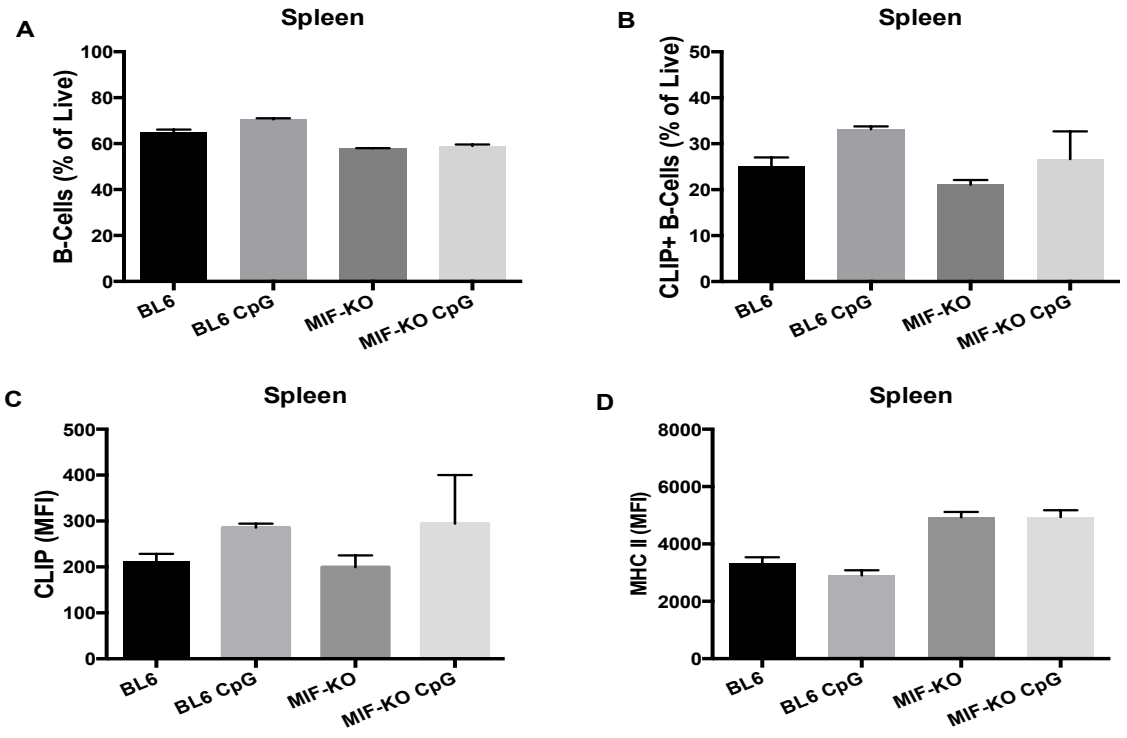


Figure 22: MIF-KO spleens have higher CLIP *in vivo*. Panel A preliminary data show that intraperitoneal injection of CpG into MIF-KO (n=2) animals did not increase the frequency of B cells as observed in C57BL/6 (BL6) wild type mice (n=3). In addition, the frequency of B cells in MIF-KO mice started off lower than in wild type animals. Panel B shows that MIF-KO mice started off with a high frequency of CLIP+ B cells, comparable to the wild type mice, and did not increase with CpG, unlike in the wild type animals. Panel C shows that the surface expression of CLIP on B cells in the MIF-KO animals was similar to the expression on the B cells from BL6 wild type mice, and while BL6 B cell surface CLIP expression went up with CpG there was no change in the CLIP expression on the B cells from MIF-KO animals. Panel D shows that MIF-KO animals started off with higher MHC-II expression when compared to that of BL6 wild type and it does not increase with CpG treatment. This was a preliminary experiment with fewer animals in each group and statistical analysis was not performed due to lower animal numbers.

The impact of MIF on B cell numbers, cell surface CLIP and cell surface MHC-II

CD74 is the cell surface receptor for MIF. ISO-1 (4,5-Dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester), is a cell permeable MIF antagonist that binds to the catalytic domain of MIF, thereby inhibiting its activity. In this experiment, splenocytes isolated from C57BL/6 mice were cultured for 48 hours stimulated with either CpG or anti-IgM+IL-4 along with ISO-1 (concentration of 40μg/mL) and the cells were later stained for B cell markers and CLIP, along with MHC-II expression and CD74. We predicted that ISO-1 would block the autocrine release of MIF from B cells upon CpG activation or anti-IgM + IL-4 stimulation.

ISO-1 reduced the number of B cells in anti-IgM+IL-4 activated splenocytes (Figure 23A; $n = 3$; $P < 0.05$), while it did not change the total B cell frequency in the CpG-activated group. ISO-1 strongly inhibited the CLIP+ B cell frequency in CpG-activated as well as in anti-IgM+IL-4 activated cells (Figure 23B; $n = 3$; $P < 0.05$). ISO-1 strongly inhibited the expression of CLIP on the B cell surface in both CpG-activated and anti-IgM + IL-4 activated cells (Figure 23C; $n = 3$; $P < 0.05$). ISO-1 also reduced MHC-II expression in CpG-activated cells, likely indicating that the loss of CLIP in these cells was due to reduction in MHC-II translocation to the cell surface; while in anti-IgM + IL-4 activated cells, ISO-1 did not affect MHC-II expression (Figure 23D; $n = 3$; $P < 0.05$). ISO-1 increased B cell surface CD74 (MFI) in the anti-IgM + IL-4 activated group (Figure 23E: $n = 3$; $P < 0.05$), while it had no effect on B cell surface CD74 (MFI) in the CpG-activated group (Figure 23E: $n = 3$).

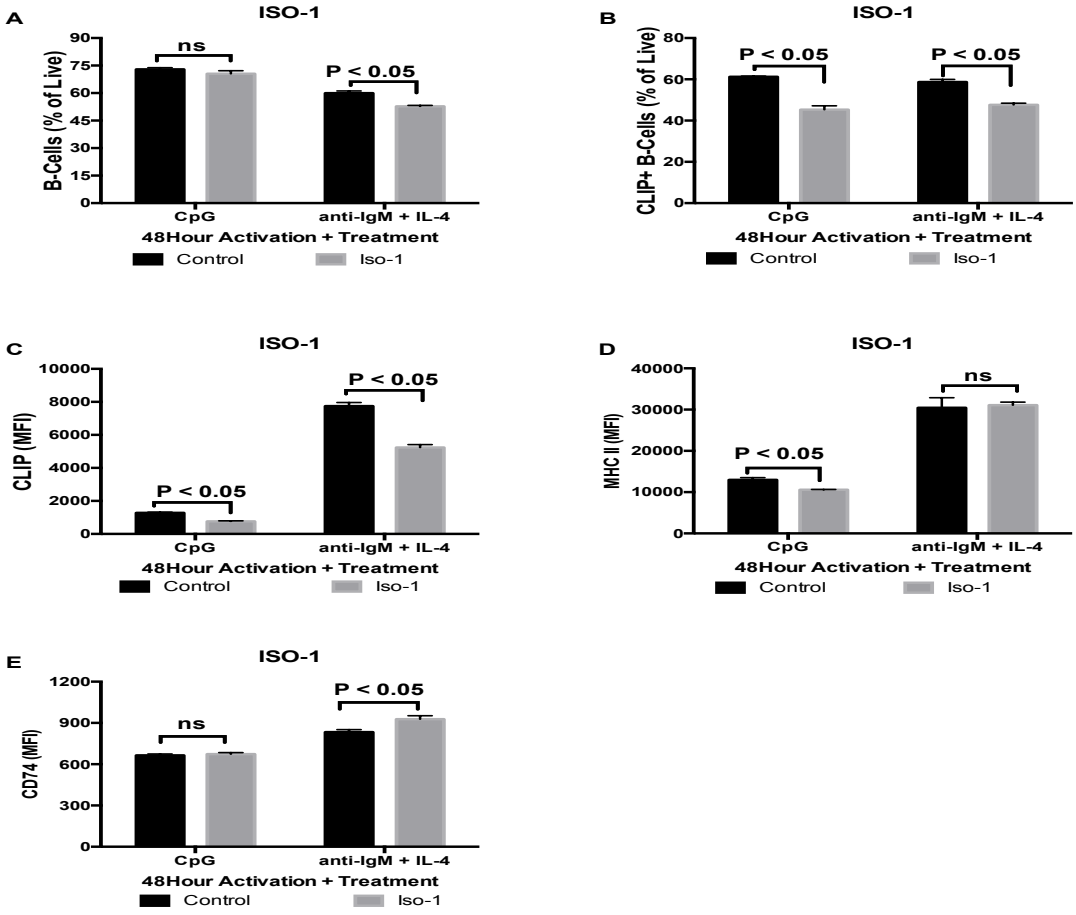


Figure 23: ISO-1 regulates CLIP expression *in vitro*. Panel A shows that anti-IgM + IL-4 activated splenocytes treated with ISO-1 display a reduced B cell frequency (n = 3; P < 0.05), while there was no change in the frequency of B cells in CpG-activated cells. Panel B shows that ISO-1 reduced the number of CLIP+ B cells in both CpG and anti-IgM + IL-4 activated splenocytes. Panel C shows that splenocytes activated with CpG or anti-IgM + IL-4 when treated with ISO-1 had reduced cell surface CLIP expression on B cells (n = 3; P < 0.05). Panel D shows that ISO-1 reduced MHC-II on B cells in CpG-activated splenocytes, while there was no change in the anti-IgM + IL-4 activated group (n = 3; P <0.05). Panel E shows that ISO-1 significantly increased CD74 surface expression on B cells in anti-IgM + IL-4 activated splenocytes (n = 3; P < 0.05), while it had no effect on the CpG-activated group (n = 3). P values were calculated using Student's t-test on GraphPad Prism.

MIF-098 inhibitor reduces CLIP expression

ISO-1 is a weak inhibitor of MIF so we repeated the same experiment with a strong antagonist of MIF, called MIF-098, generously provided by Dr. Richard Bucala. In this *in vitro* experiment, we activated C57BL/6 splenocytes with CpG, LPS, or anti-IgM+IL-4 along with the MIF antagonist MIF-098 added at a concentration of 40 μg/mL for 48 hours, and then stained for CLIP, CD19, and MCH-II. Splenocytes have been found to be very sensitive to MIF-098. MIF-098 strongly reduced the frequency of CLIP+ B cells (Figure 24; $n = 3$; $P < 0.05$) and CLIP expression on the surface of B cells (Figure 24; $n = 3$; $P < 0.05$) under all the three stimulation conditions (CpG, LPS, anti-IgM + IL-4). MIF-098 significantly increased the MHC-II surface expression (Figure 24A, 24B, 24C; $n = 3$; $P < 0.05$) on the remaining B cells.

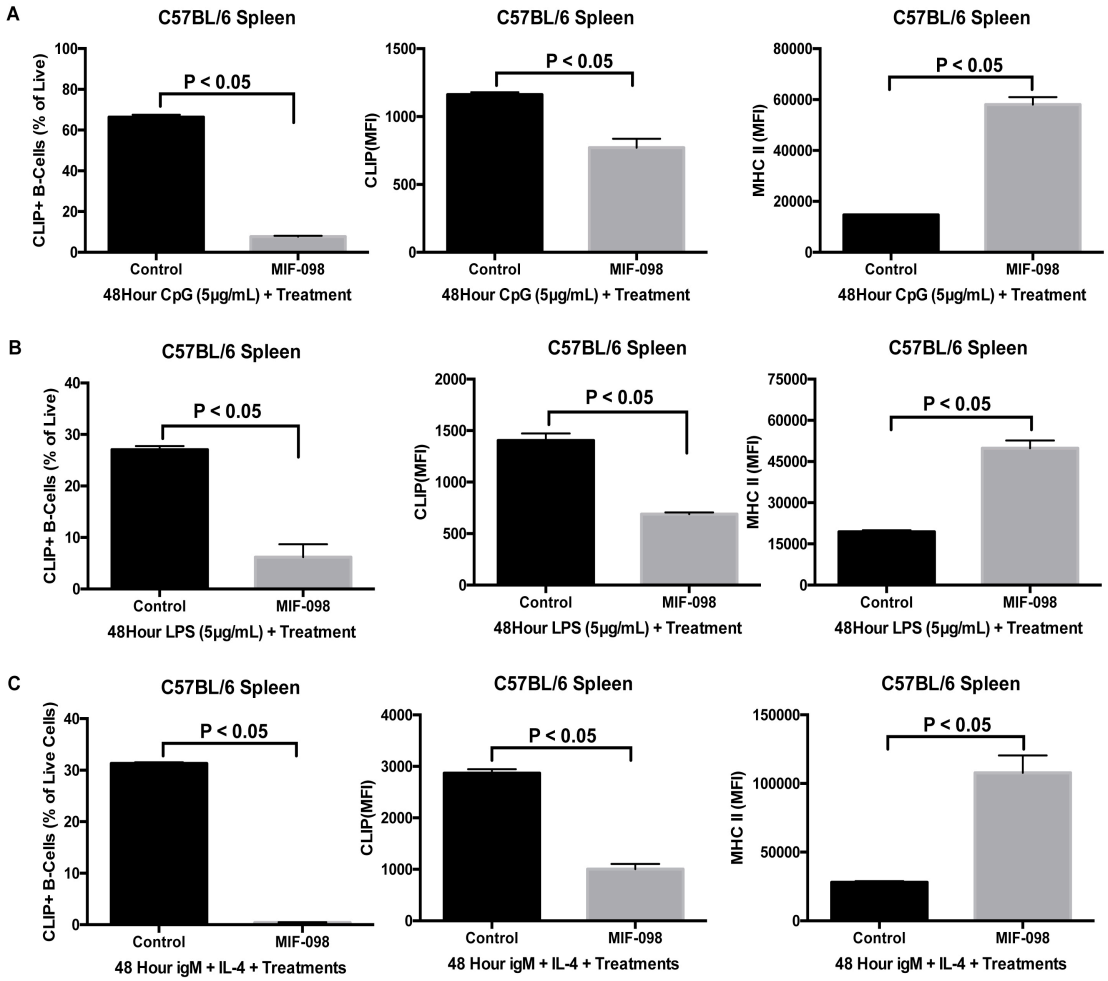


Figure 24: MIF-098 inhibits surface CLIP expression and CLIP+ B cell frequency. In Panel A, CpG-activated splenocytes when exposed to MIF-098 for 48 hours, had significantly reduced in CLIP+ B cells and CLIP (MFI), and increased MHC-II (MFI) ($n = 3$; $P < 0.05$). Panel B shows CLIP+ B cell suppression in the LPS-activated splenocytes treated with MIF-098. In addition, there was a significant reduction in CLIP (MFI) on B cells along with an increase in MHC-II (MFI) ($n = 3$; $P < 0.05$). Panel C shows that the 48 hour treatment with MIF-098 *in vitro* obliterated CLIP+ B cells and reduced the CLIP per B cell in the surviving cells, while increasing the MHC-II (MFI) ($n = 3$; $P < 0.05$) in anti-IgM + IL-4 activated splenocytes. P values were calculated using Student's t-test on GraphPad Prism.

MIF-098 facilitates antigenic peptide loading

As MIF-098 inhibits surface expression of CLIP on the B cell and CLIP+ B cell frequency, we addressed the possibility that MIF-098 treatment would facilitate antigenic peptide loading into the groove of MHC-II. For this experiment we activated splenocytes from C57BL/6 mice *in vitro* with either TLR9 agonist, TLR4 agonist or with BCR activation by anti-IgM + IL-4 treatment and incubated them for 48 hours with MIF-098 and biotinylated APi1702 peptide, a peptide with high affinity for MHC-II. The cells were stained for CLIP, and with fluorescein-conjugated streptavidin to detect the surface expression of APi1702. MIF-098 increased the ratio of APi1702 peptide loading to CLIP by nine-fold in CpG-activated cells (Figure 25A; $n = 3$; $P < 0.05$). LPS-activated B cells treated with MIF-098 showed a seven-fold increase in the level of APi1702 peptide relative to the amount of CLIP loaded into the groove of MHC-II on the B cells (Figure 25B; $n = 3$; $P < 0.05$). In anti-IgM + IL-4 stimulated splenocytes, the MIF-098 facilitated an increase in the peptide loading into the MHC-II groove by over fifteen fold, when compared to the control group (Figure 25C; $n = 3$; $P < 0.05$).

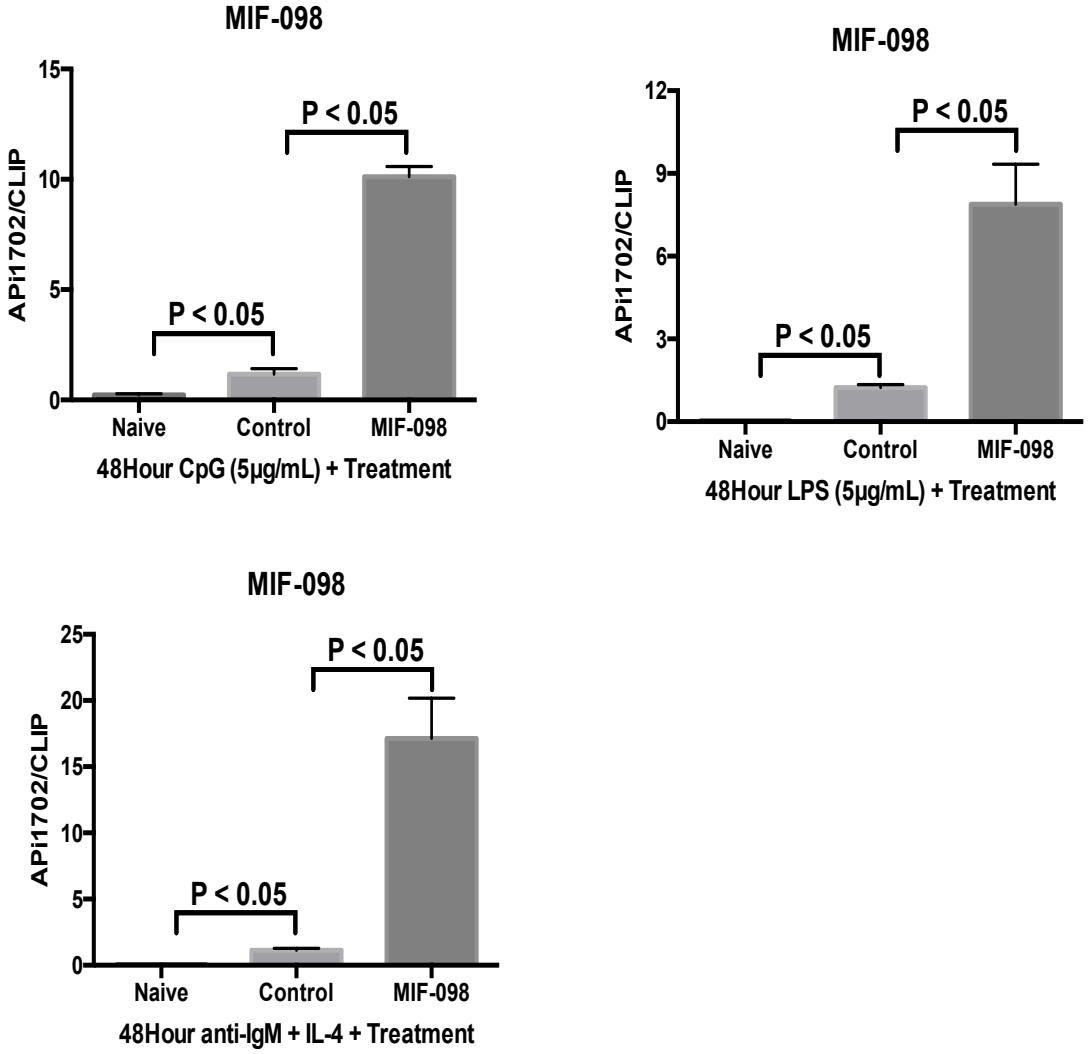


Figure 25: MIF-098 increases antigenic peptide loading. Panel A shows that in CpG-activated splenocytes incubated with APi1702, this competitive binding peptide was loaded into the MHC-II at a higher efficiency when treated with MIF-098 than under control conditions ($n = 3$; $P < 0.05$). Panel B shows that in LPS-activated splenocytes incubated with APi1702 and MIF-098 there was a seven-fold increase in peptide loading ($n = 3$; $P < 0.05$). Panel C shows that in BCR activation, MIF-098 treatment significantly increased the APi1702 peptide loading efficiency into the MHC-II by fifteen-fold compared to control ($n = 3$; $P < 0.05$). P values were calculated using Student's t-test on GraphPad Prism.

In vivo B6.*lpr* mice have high CLIP expression

B6.*lpr* mice have a mutation in the gene that encodes the Fas molecule and these mice exhibit the symptoms of systemic autoimmunity. In this experiment, we examined the level of CLIP per B cell and the frequency of CLIP+ B cells, compared to BL6 wild type mice. For these *in vivo* experiments, B6.*lpr* mice were injected, via the intraperitoneal route, with CpG (n = 3) at 1mg/kg body weight. We also injected C57BL/6 mice (BL6) (n = 3) with CpG at the same concentration. At 72 hours post-injection, the animals were sacrificed along with respective age-matched controls (n = 3). The spleens were processed into single cell suspensions and stained for B cell, CLIP, and MHC-II expression.

B6.*lpr* mice had fewer B cells when compared to the BL6 wild type mice and CpG activation did not increase the B cell frequency in these animals (Figure 26A; n = 3). There was no significant increase in CLIP+ B cells in B6.*lpr* mice when compared to BL6 wild types (Figure 26B; n = 3). B6.*lpr* mice started off with higher CLIP (MFI) per B cell than the BL6 wild type mice (not statistically significant (ns)) and unlike BL6 mice where the CLIP (MFI) increased with CpG injection, B6.*lpr* mice did not show any significant increase in the surface CLIP expression (Figure 26C; n = 3). There was no change in the surface expression of MHC-II on the B cells following CpG injection in both B6.*lpr* and wild type mice (Figure 26D; n = 3).

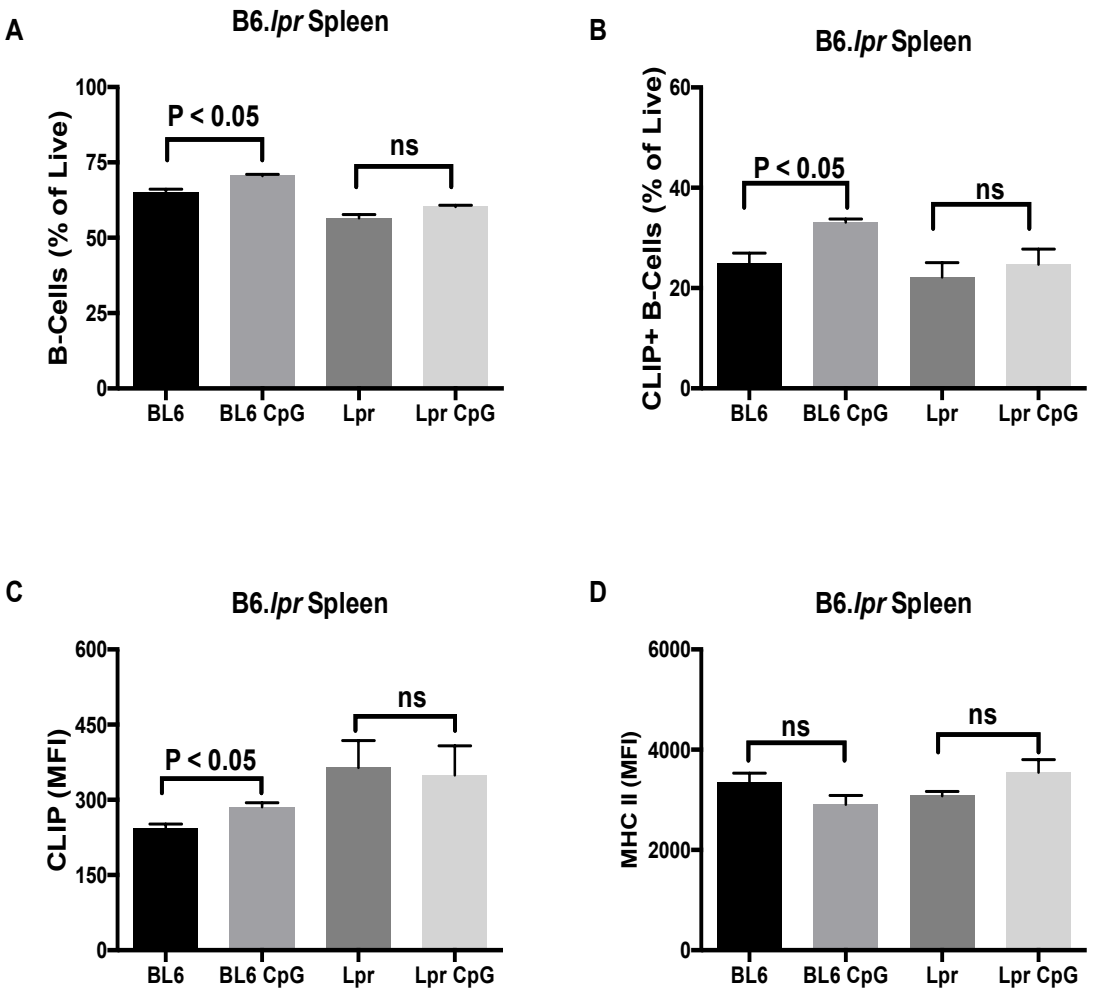


Figure 26: B6.*lpr* B cell surface CLIP expression *in vivo*. Panel A shows that *in vivo* CpG injection does not increase B cell frequency in B6.*lpr*, while in BL6 there was a significant increase in B cells (n = 3; $P < 0.05$). Panel B shows that CpG induced increase in CLIP+ B cells in BL (n = 3; $P < 0.05$), but not in B6.*lpr* mutant mice (n = 3). Panel C shows that B6.*lpr* mice tend to start off with higher CLIP (MFI) in B cells and CpG does not change (n = 3) the expression but in BL6 mice CpG significantly increased CLIP (MFI) on B cells (n = 3; $P < 0.05$). Panel D shows there was no change in MHC-II (MFI) in either of the mice strains injected with CpG (n = 3). P values were calculated using Student's t-test on GraphPad Prism.

Antigenic peptide presentation by B cells from B6.*lpr* mice *in vitro*

Because B6.*lpr* mice constitutively express higher levels of B cell surface CLIP than BL6 mice, we performed an *in vitro* experiment to determine the antigenic peptide loading ability and capability of peptide antigen presentation by MHC-II in B cells from these animals. For this experiment splenocytes from B6.*lpr* mice were activated with either CpG or LPS, at a concentration of 5μg/mL, or with anti-IgM + IL-4, at a concentration of 10μg/mL and 5ng/mL, respectively. Cells were treated for 48 hours with HCQ or rapamycin at a concentration of 10μM, along, with the biotinylated competitive binding peptide APi1702 at a final concentration of 5μg/mL. The cells were then stained for B cells, CLIP, and MHC-II on the B cell surface, followed by a second incubation with streptavidin to identify the surface bound APi1702 peptide in the groove of MHC-II.

In CpG-activated splenocytes, HCQ significantly reduced the ratio of APi1702 to CLIP on the B cell surface ($n = 3$; $P < 0.05$), while rapamycin treatment did not change the ratio of APi1702 to CLIP on the B cell surface (Figure 27A; $n = 3$). Rapamycin increased the ratio of LPS-activated *lpr* cell surface APi1702 to CLIP by over fifteen-fold, while HCQ reduced the APi1702 surface expression on B cells (Figure 27B; $n = 3$; $P < 0.05$). In anti-IgM + IL-4 activated *lpr* splenocytes, rapamycin treatment resulted in a fifteen-fold increase in the APi1702 peptide presentation by B cells when compared to the control, while HCQ significantly reduced peptide presentation by the B cells (Figure 27C; $n = 3$; $P < 0.05$).

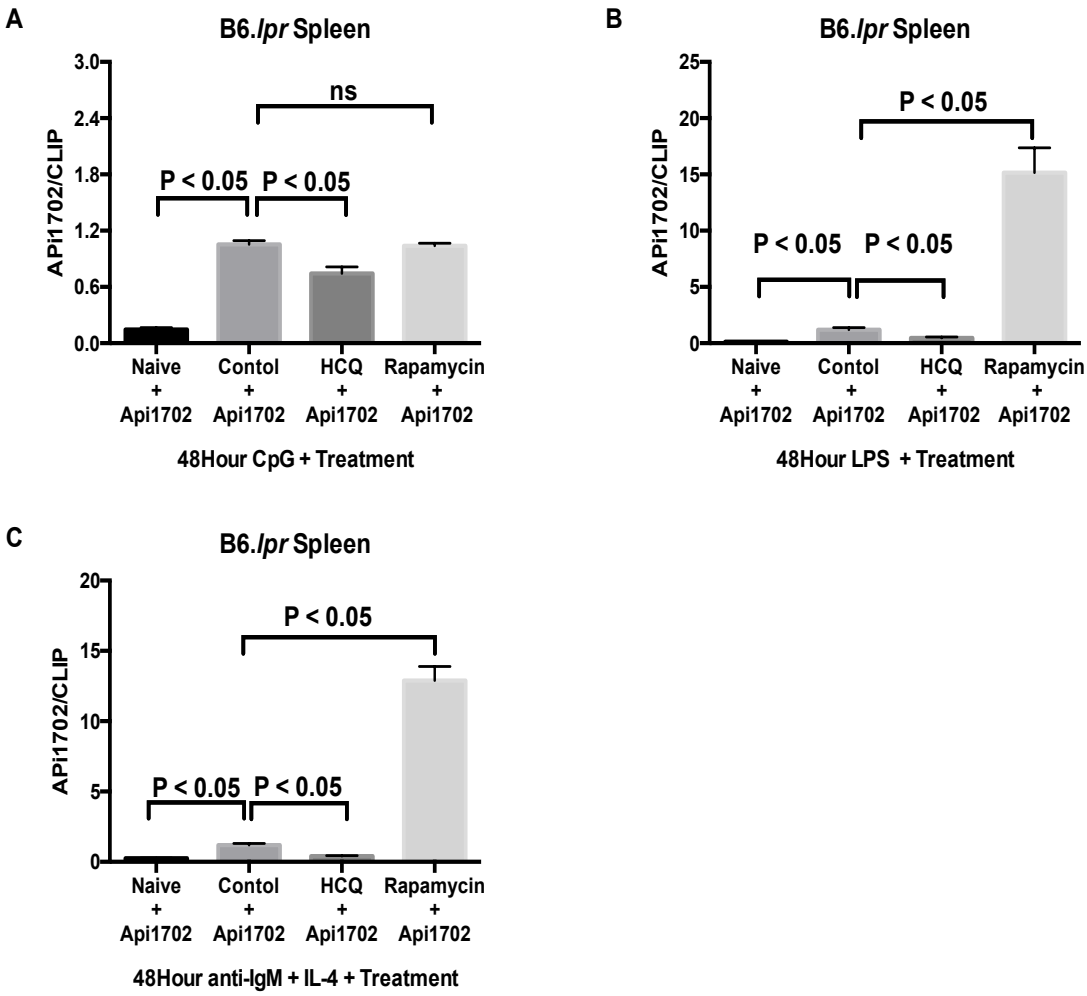


Figure 27: B6.*lpr in vitro* antigenic peptide presentation by B cells. Panel A shows that in B6.*lpr* splenocytes activated with CpG and treated with HCQ or rapamycin along with APi1702, HCQ reduces the APi1702/CLIP ratio ($n = 3$; $P < 0.05$) while rapamycin has no effect. Panel B shows that rapamycin increases the surface expression of APi1702/CLIP ratio by fifteen fold compared to control while HCQ reduces the competitive peptide surface expression ($n = 3$; $P < 0.05$). Panel C indicates that in anti-IgM + IL-4 activated splenocytes rapamycin treatment increases B cell surface expression of APi1702 just as with LPS activation and HCQ significantly reduces APi1702 by inhibiting autophagy ($n = 3$; $P < 0.05$). Statistical significance was evaluated by one-way ANOVA using GraphPad Prism.

6-AN and PS-341 reduce CLIP expression on cancer cells

CLIP in the groove of the MHC-II peptide-binding site inhibits cells death (Tobin, 2013). In this *in vitro* experiment we evaluated the possibility that induction of CMA will cause the loss of CLIP on the surface of cancer cells. Daudi and Raji human Burkitt lymphoma cells were treated with either 6-AN, at a concentration of 140 μM, or with PS-341, at 100 nM for 48 hours and then stained for CLIP on the cell surface. 6-AN significantly reduced the expression of CLIP in both Raji and Daudi cell lines without affecting the MHC-II surface expression (Figure 28; $n = 3$; $P < 0.05$). PS-341 was toxic to Raji cells (data not shown) and it reduced the level of cell surface CLIP in both Raji and Daudi cells, without affecting the MHC-II cell surface expression (Figure 28; $n = 3$; $P < 0.05$), indicating that these two drugs may increase the immunogenicity of the two cell lines for recognition by CD4+ T cells.

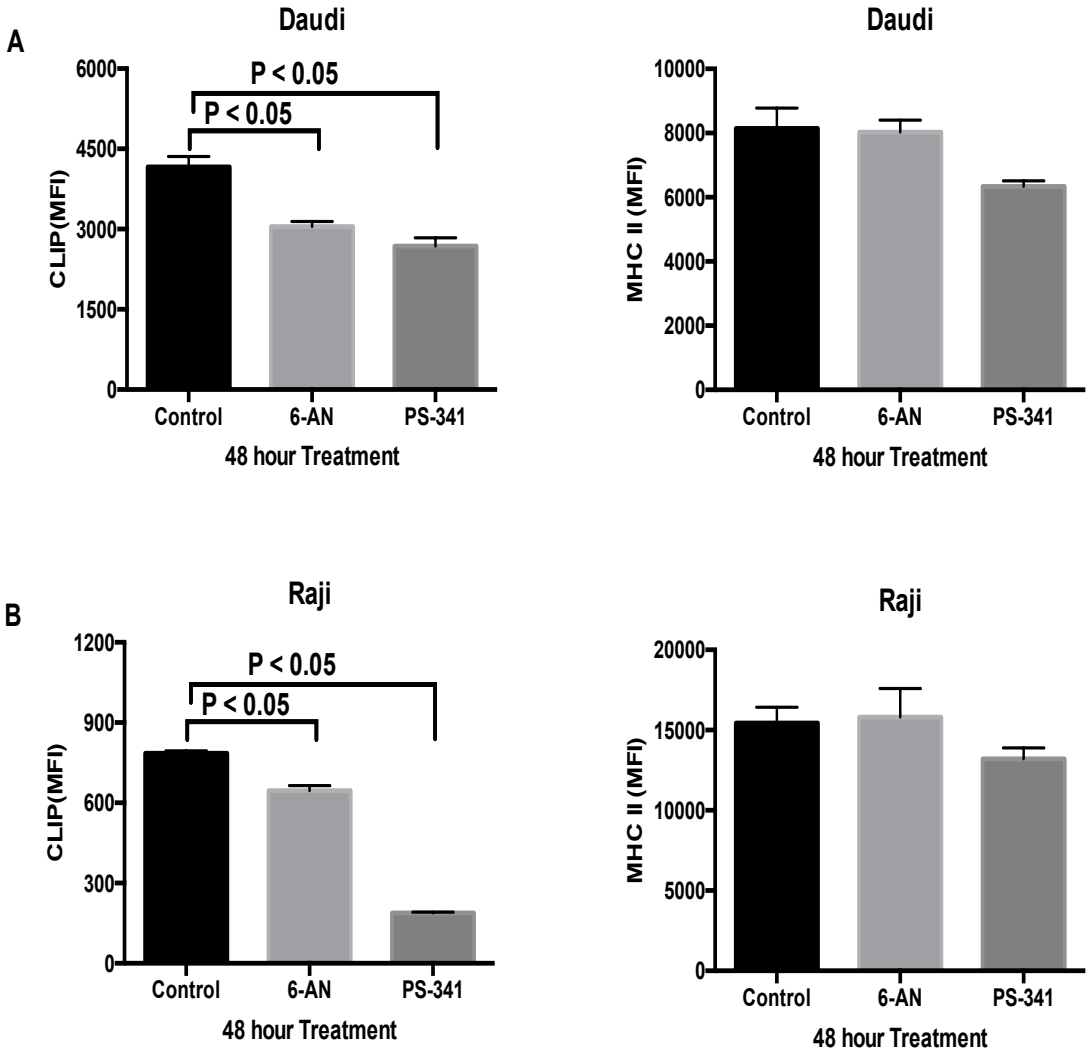


Figure 28: 6-AN and PS-341 reduce CLIP on surface of cancer cells *in vitro*. Panel A shows that 6-AN and PS-341 inhibited the expression of cell surface CLIP on the Daudi cell lines when incubated with these drugs for 48 hours ($n = 3$; $P < 0.05$), without reducing MHC-II surface expression. Panel B shows that 48 hours of treatment with 6-AN or PS-341 reduced cell surface CLIP expression on Raji cells without affecting the level of MHC-II expression ($n = 3$; $P < 0.05$). Statistical significance was evaluated by one-way ANOVA using GraphPad Prism.

CHAPTER IV

CONCLUSIONS

The immune system functions to protect us from harm, and as such, is required to distinguish pathogenic invaders or harmful injury from harmless microbes or self-antigens. However, with every exposure to pathogens, the battle to control infectious disease is accompanied by the risk of autoimmunity, without strict controls to distinguish what is "self" from what is not. Although the immune system has historically been considered a system of "self versus non-self discrimination," the recent discovery of cells that actively suppress antigen-specific responses, the regulatory networks (including myeloid suppressor cells as well as T and B regulatory cells that can help maintain self tolerance) suggest that, in fact, the immune system readily recognizes self and thus needs to be highly capable of suppressing self reactivity to avoid blatant autoimmunity.

In the present thesis, our hypothesis was that CD74, and control over its processing into CLIP and cell surface CLIP presentation, are regulated by macroautophagy and CMA, and as such, are important in discriminating between "self" and "non-self" antigen presentation by B cells. Furthermore, these processes regulate the expansion and survival of distinct B cell subsets that, in turn, may promote regulatory versus non-regulatory T cells. Toward that end, we examined (1) the contributions of macroautophagy to B cell survival, proliferation, cell surface CLIP expression, B cell subset selection, and antigenic peptide presentation; (2) the

mechanisms required to process CD74 and generate CLIP on activated B cells, including those mechanisms involving MIF binding to CD74 and the regulation that facilitates antigenic peptides loading into MHC-II on B cells; and (3) how the regulation of cell surface CLIP expression affects the potential for autoimmune disease and cancers.

Macroautophagy: regulation of B cell survival, proliferation, cell surface CLIP expression, B cell subset selection, and antigenic peptide presentation

Our previous work demonstrated that TLR activation, resulting in the proliferative expansion of naïve B cells, results in increased cell surface CLIP loaded into MHC-II on the responding, TLR-activated B cells (Newell et al., 2010). We reasoned that the TLR-driven response that results in proteolytic cleavage of CD74 into the CLIP peptide resulted from the absence of antigen-specific, BCR-driven activation of H-2M that would normally facilitate peptide exchange of CLIP for antigenic peptide. Thus, TLR activation would not activate H-2M and CLIP would remain in the groove of MHC-II in TLR-activated cells. However, to our surprise, we discovered that BCR engagement, along with IL4, resulted in even higher levels of cell surface CLIP (Tobin, 2013), thereby likely limiting B cell MHC-II/antigen presentation to antigens recognized by the BCR as well as those processed and presented to CD4 T cells, with the remaining MHC-II molecules protected by the "placeholder" CLIP. Tobin et al. (Tobin, 2013) went on to show that while MHC-II engagement can result in the death of naïve B cells, CLIP in the groove of MHC-II is sufficient to prevent MHC-II-mediated cell death. Taken

together, these results illustrate that processing of CD74 and presentation of CLIP peptides may regulate presentation of antigenic peptides by B cells.

Autophagy is a process whereby cytoplasmic contents are delivered for lysosomal degradation. However, this pathway can also be used to deliver intracellular antigens for presentation to T cells and more recent evidence shows that autophagy also facilitates the processing of extracellular antigens via both MHC-II and MHC-I (Roche and Furuta, 2015). Furthermore, some antigens can be presented via MHC-II after intracellular processing, resulting in MHC-II presentation of the autophagic cargo (Schmid et al., 2007). Because TLR activation results in autophagy, we reasoned that cleavage of full length CD74 into CLIP might result from TLR-mediated autophagic events. To test the hypothesis that TLR-mediated increases in cell surface CLIP expression on B cells results from macroautophagy, we performed experiments in which we activated splenocytes with well-established TLR agonists - LPS or CpG, agonists for TLR 4, a cell surface receptor, and TLR 9, a lysosomal receptor, respectively. We also included stimulation with anti-IgM and IL4 to compare the effects of TLR-mediated activation to antigen receptor engagement.

In contrast to our prediction, we found that the well characterized inhibitor of mTOR, rapamycin, reduced the levels of cell surface CLIP under all of our activating conditions, thereby ruling out the possibility that the processing of CD74 into CLIP and the resulting cell surface expression of CLIP results from autophagy. However, to further substantiate this conclusion, we treated our cell cultures in each of our stimulating conditions with HCQ, an established inhibitor of autophagy. In every case,

co-culture with HCQ increased the level of cell surface CLIP, thus supporting the interpretation that autophagy regulates the cell surface expression of CLIP in MHC-II once activated. One possible explanation for this result is that CLIP in the groove of cell surface MHC-II prevents the loading of exogenous peptide until physiological inhibition of mTOR occurs. Potential physiologic conditions that would inhibit mTOR include, but are not limited to, cellular starvation, metabolic stress, appropriate growth-controlling cytokines, such as IL-10 or TGF-β (Wu et al., 2016), or growth factor withdrawal of proliferation-promoting cytokines, such as IL-4.

The downregulation of cell surface CLIP on B cells by inhibiting mTOR suggests that macroautophagy may prime B cells to present peptidic antigens. To determine if autophagy promotes peptidic antigen presentation by B cells stimulated via TLR ligands or via antigen receptor engagement, we utilized a synthetic peptide with a higher binding constant for the peptide binding groove of MHC-II than the CLIP peptide. We expected that if rapamycin "primed" the activated B cells for presentation of exogenous peptides, then the ratio of CLIP to the synthetic peptide would decrease with increasing concentrations of biotinylated peptide. We found that under all of the stimulation conditions, treatment with rapamycin facilitated an increase in antigenic peptide loading when compared to the control, while HCQ reduced the antigenic peptide loading.

Under nutrient rich conditions, mTOR is known to be recruited to the surface of the lysosome where it forms a complex with "raptor", creating the mTOR complex 1 (mTORC1) that not only regulates lysosomal fusion with the autophagosome, but also

controls the lysosomal function by regulating the important lysosomal protein v-ATPase, which is known to regulate lysosomal acidification (Sancak et al., 2010; Zoncu et al., 2011). So, based on this function, we predicted that active mTOR would inhibit exchange of peptides in the late endosome because of reduced lysosomal activity, as well as inhibition of lysosomal fusion with the early endosome. Rapamycin treatment inhibits mTOR, resulting in activation of v-ATPase to increase lysosomal acidity, thus enhancing the exchange of antigenic peptide with the CLIP in the MHC-II peptide-binding groove of the late endosome, and thereby resulting in loss of B cell surface CLIP under all of the activation conditions (CpG, LPS, anti-IgM + IL-4). This prediction was also supported by our observations using HCQ, a well-known inhibitor of macroautophagy by neutralizing lysosomal acidity. HCQ suppressed the antigenic peptide exchange in the late endosome, and as a result, we observed more CLIP on the cell surface.

Rapamycin treatment inhibits B cell proliferation with CpG activation, and also with BCR and IL-4 treatment. Marginal zone (MZ) and follicular (FO) B cells make up the majority of B cells in the spleen. MZ B cells are thought to carry out mostly T-independent function, while FO B cells need T cell help for their function (Li et al., 2001; Martin and Kearney, 2000). Several studies report that TLR agonists, like CpG, will activate MZ B more than FO B cells, while BCR engagement activates FO B cells more than MZ B cells (Lopes-Carvalho et al., 2005). Our observations show that rapamycin treatment inhibited the proliferation of B cells under all of the stimulation conditions, indicating that rapamycin can inhibit not only PI3K-dependent, mTOR

activation-induced proliferation by BCR engagement that mostly affects FO B cells, but also PI3K-independent, mTOR activation-induced proliferation, caused by TLR9 agonist binding to MZ B cells.

Another important finding from our work is the effect of rapamycin we observed on B cell subsets in the peritoneum. The B cell subset composition of the peritoneum is different than that of secondary lymphoid organs such as the spleen. The spleen consists of predominantly B-2 B cells (FO and MZ B cells are B-2 B cells), while the B cell subpopulation of B-1 B cells are the major B cell residents of the peritoneum. B-1 B cells, just like MZ B cells, are T-cell independent and produce IgM antibody. So, we hypothesized that B-1 B cells should have similar sensitivity to rapamycin as the MZ B cells subset of B-2 cells. To our surprise, we observed that B-1 B cells are much more sensitive to rapamycin as indicated by significant decreases in the relative frequency of B-1 B cells, relative to B-2 B cells. Previous studies had established that FO B cells are less sensitive to TLR activation, when compared to MZ B cells, thus we reasoned that increased relative frequency of B-2 B cells in the peritoneum could be accounted for by MZ B cells.

We evaluated the contribution of CMA to displacement of CLIP and the capability of antigen presentation in B cells. Because Dr. Janice Blum's group had shown the importance of CMA in antigenic peptide presentation (Deffit and Blum, 2015), we evaluated the level of cell surface CLIP expression using 6-AN, an activator of CMA, along with TLR9 or TLR4 stimulation, or with BCR engagement. Our results show that CLIP expression on the surface of the B cells is reduced with 6-AN under all

of the activation conditions, but surprisingly 6-AN also reduced cell surface MHC-II in both LPS and anti-IgM + IL-4 stimulated B cell subsets, while 6-AN did not affect the level of MHC-II expression on the CpG-activated group. These results indicate that CpG likely upregulates the LAMP-2C isoform, which according to Dr. Blum's work inhibits CMA-mediated lysosomal degradation, while macroautophagy-mediated processes are unaffected. Thus, we believe that CpG overrides the 6-AN effect and continues to inhibit CMA, based on the observation that MHC-II expression is unchanged (Perez et al., 2016). Because BCR cross-linking increases the expression of LAMP-2A, a translocator complex on the lysosome that increases the CMA process (Perez et al., 2016; Zhou et al., 2005), we believe that both LPS activation as well as anti-IgM + IL-4 increase LAMP-2A, resulting in CMA induction, and thereby results in a decrease in CLIP in the groove of MHC-II. Based on the reduction in MHC-II levels that we have observed in these activation groups, we suggest that CMA may not contribute to antigen presentation in our system.

CD74 is an important component in the assembly of MHC-II molecules and their transport to the intracellular compartments and to the cell surface. In addition, CLIP, the proteolytic cleavage product of CD74, is essential in inhibiting binding of self-antigens in the cells. So, in our study, we evaluated how the loss of function of CD74 would affect the B cell response to activators and inhibitors of macroautophagy and CMA activators. Our initial evaluation of the immune cell sub-populations from the CD74 deficient ($CD74^{def}$) mice showed that these animals have fewer B cells in the spleen and in the peritoneum, and fewer CD4+ T cells compared to the BL6 WT mice. The cell

surface MHC-II expression on B cells from CD74^{def} mice is significantly lower than in BL6 WT B cells. This finding is consistent with previous findings by other groups that have shown that CD74 is essential for B cell maturation and that lack of CD74 results in less MHC-II and less T cell selection due to the lack of variation in MHC-II bound peptide repertoire, thus affecting the positive selection of these cells in the thymus (Bikoff et al., 1993; Matza et al., 2002b).

We activated splenocytes isolated from the CD74^{def} mice with similar strategies used with BL6 WT splenocytes, including CpG, LPS and anti-IgM + IL-4. We also treated them with rapamycin, HCQ, and 6-AN. Because these animals have no CD74, there was no detectable CLIP on the surface of the B cells, so we evaluated how the expression of MHC-II changes on the B cells and the B cell frequency in all the activated groups treated with rapamycin or HCQ. Rapamycin treatment did not induce increased levels of MHC-II expression under any of the stimulation conditions, indicating that CD74 is essential for macroautophagy-induced exogenous peptide presentation. Because 6-AN treatment significantly increased MHC-II expression in TLR4- and TLR9-activated cells from the CD74^{def} mice, the data suggest that these animals can readily utilize CMA for processing and presentation of antigenic peptides by B cells. The data also show that anti-IgM + IL-4 mediated activation results in the reduction of surface MHC-II on B cells and thus may indicate that exogenous antigens are limited to those recognized by the antigen receptor. This is consistent with reports that CD74^{def} mice are resistant to autoimmunity conditions (Sun et al., 2010).

Mechanisms required for processing of CD74 and generating CLIP expression on activated B cells

There are several very distinct functions of CD74. Collectively, CD74 is a molecule involved in multiple processes in both innate and adaptive immune responses. First, CD74 is well characterized in APCs that also express MHC-II. Within the Golgi apparatus and endoplasmic reticulum of APCs, including B cells, CD74 is involved in the assembly of MHC-II molecules, and serves as a chaperone for MHC-II transport within the cell to the lysosome, and to the cell surface**.** However, under conditions of inflammation, CD74 serves as the cell-surface receptor for MIF. As such, when MIF binds to CD74, the complex is internalized, and this event initiates downstream innate immune signaling, resulting from activation of the SPPL2a enzyme (Schneppenheim et al., 2013). The SPPL2a enzyme cleaves the CD74 N-terminal fragment (NTF) at cytoplasmic tail resulting in a 42-amino acid peptide which produces CD74 intracellular domain (ICD) and serves as a transcription factor that stimulates NF-κB activity (Matza et al., 2002a). Stimulation of NF-κB promotes inflammation associated with an innate immune response (Hayden et al., 2006). The remaining carboxy-terminal fragment resulting from SPPL2a cleavage of full-length CD74 contains the amino acid sequences that comprise MHC Class II Invariant Peptides (CLIPs), however the fate of the carboxy terminus is unknown. In addition to SPPL2a cleavage of CD74, there are 2 other distinct mechanisms by which CD74 can be cleaved into CLIPs: **(**1) When cell surface MHC-II molecules are internalized into phagolysosomes, containing cathepsins. Specifically cathepsin S in B cells, dendritic cells, and macrophages, cleaves MHC-II-associated

CD74 into CLIP (Riese et al., 1996). Once cleaved, the CLIP peptide occupies the antigen-binding groove of MHC-II until HLA-DM (H-2M in mice) catalyzes the replacement of CLIP with antigenic peptides (Denzin and Cresswell, 1995). Replacement of CLIP allows the antigenic peptide to be recognized by a CD4+ T cell, the hallmark of the first-step in an antigen-specific adaptive immune response (Garcia and Teyton, 1998). (2) Within the proteasome, proteins, including CD74, are proteolytically cleaved into peptides containing 7-9 amino acids (Schrader et al., 2009). These peptides can be presented by MHC-II via cross presentation (Houde et al., 2003), and, as above, replacement of MHC-II-bound, cleaved CD74 peptides with antigenic peptide, and subsequent T cell recognition of the antigenic peptide in the peptide binding groove of MHC-II, initiates an adaptive immune response. Based on these potential mechanisms for generating CLIP peptides, we designed experiments to determine the impact of each of these mechanisms on generating cell surface CLIP peptides in the MHC binding groove on activated B cells, activated by either TLR or antigen receptor driven signals.

We used PS-341, a well-known reversible inhibitor of the 26S proteasome. PS-341 inhibited CLIP on the surface of B cells under all of our stimulation conditions (CpG, LPS and anti-IgM + IL-4) and the reduction in cell surface CLIP was accompanied by lower MHC-II expression on the surface of LPS and anti-IgM + IL-4 activated cells, indicating that PS-341 may inhibit inflammatory signaling by stabilizing IκB-α, thus reducing the expression of NF-κB and inflammation, which in turn reduces CD74 synthesis and MHC-II expression (Sunwoo et al., 2001). An interesting

observation in CpG-activated splenocytes was that even though CLIP was reduced on the surface of the B cells, there was a striking increase in MHC-II cell surface expression, indicating that PS-341 likely does not regulate the CIITA-independent increase in expression of MHC-II that occurs in response to the MHC-II mRNA stabilization resulting from CpG activation (Kuchtey et al., 2003).

Cathepsins are cysteine proteases involved in the proteolysis of the C-terminal portion of CD74 that is bound to MHC-II, thus leaving behind the peptide CLIP bound to the antigen-binding groove of MHC-II (Riese et al., 1998). In our results with Cystatin-1, we observed that, under conditions of CpG activation, the expression of CLIP on the B cell surface remain unaltered, indicating Cystatin-1 does not affect protein transcription or translation, but rather physically interacts with available cathepsins to inactivate them. In support of this supposition, other groups have found that TLR-mediated activation of cathepsins is controlled by MyD88-dependent and Myd88-independent induction of TNF-α and IL-1β, that positively regulate the mRNA expression of cathepsins B and S (Creasy and McCoy, 2011). In BCR cross-linking with anti-IgM + IL-4, Cystatin-1 acts synergistically in reducing the cathepsin activity, along with BCR engagement, that is known to transiently downregulate cathepsin S activity in B cells (Lankar et al., 2002). Cystatin-1 did not affect the expression of CD74 in CpG-activated or in anti-IgM + IL-4 stimulated B cells, indicating that the activity of Cystatin-1 is not non-specific.

The ICD produced by SPPL2a processing of NTF is known to induce inflammation by its translocation to the nucleus and induction of the NF-κB pathway.

Because there are no strong inhibitors of SPPL2a available, we used the fungal antibiotic Brefeldin A (BFA) to inhibit the translocation of the ICD to the nucleus, as reported in the literature (Matza et al., 2002a). BFA treatment resulted in the reduction of CLIP on the cell surface, without affecting the level of cell surface CD74. It is important to note that BFA has many non-specific effects, such as blocking protein transport from the Golgi and inhibition of cytokine production (Arioka et al., 1991; Schuerwegh et al., 2001). Therefore, CLIP reduction due to BFA treatment could be the result of multiple effects of this inhibitor.

Inhibition of MIF binding to CD74 reduces cell surface CLIP and facilitates antigenic peptides loading into MHC-II on B cells

Intact CD74 also plays a key role in innate immunity via its actions as a cell surface receptor for MIF. In this context, MIF binding to CD74 leads to an association with CD44, as a signaling element, and to the internalization of the MIF/CD74 complex. Once MIF is bound and internalized, the CD74 molecule is cleaved by SPPL2a enzyme (Bucala and Shachar, 2015).

To address the possibility that MIF-dependent processing of CD74 contributes to cell surface CLIP on B cells, we employed two inhibitors of MIF known as ISO-1 and MIF-098. For these experiments, we utilized our system of TLR activation with LPS or CpG and the use of antigen receptor engagement and IL-4, to determine the impact of MIF binding on the induction of cell surface CLIP on B cells. We found that B cells from the MIF-KO animals have constitutive levels of cell surface CLIP prior to the

addition of any of our stimuli. When we injected the MIF-KO mice *in vivo* with CpG there was no increase in the level of CLIP. We performed *in vitro* experiments using the MIF inhibitor ISO-1. CpG or anti-IgM + IL-4 activated splenocytes, when treated with ISO-1, displayed a reduction in cell surface CLIP on B cells, without an affect on the level of cell surface CD74. When we repeated the same *in vitro* experiment using MIF-098, we observed a consistent and significant decrease in the cell surface CLIP expression and an increase in MHC-II expression on B cells under all three stimulation conditions, CpG, LPS, and anti-IgM + IL-4, indicating that MIF inhibition could lead to macroautophagy, and potentially antigenic peptide presentation. This interpretation was confirmed by using our competitive peptide-binding assay. MIF-098 treatment, under all the three stimulation conditions, increased the ratio of APi1702 to CLIP on the surface of B cells. This is an important finding because the contribution of MIF binding to antigenic peptide presentation has never been identified in B cells, although there has been studies indicating the loss of autophagy resulting in MIF secretion in innate immune cells (Lee et al., 2016).

From these experiments, we learned that both ISO-1 and MIF-098 treatments decreased the amount of cell surface CLIP on B cells. These results have several important implications. Our data are the first to demonstrate the link between CD74's contribution to an innate, inflammatory response and the expansion of B cells that express cell surface CLIP in MHC class II. Our results also demonstrate the importance of MIF inhibition in macroautophagy and antigenic peptide presentation in adaptive the immune system.

Regulation of cell surface CLIP expression: application to autoimmune disease and cancers

Our work has demonstrated that the processing of CD74 into CLIP can be regulated specifically by mTOR inhibition-dependent autophagy, thereby resulting in reduced levels of CLIP and increased antigen presentation. Furthermore, by using a synthetic peptide with a high binding constant for cell surface MHC-II, we have shown that this mTOR inhibition-dependent reduction of CLIP selectively favors antigenic peptide presentation of exogenous peptides. In parallel with these studies, we have found that MIF inhibition reduces cell surface CLIP, thereby connecting the pro-inflammatory innate immune response with the antigen-specific adaptive immune response by creating and facilitating increased exogenous antigenic peptide loading into MHC-II.

In contrast, we have found that TLR activation that signals via the adaptor complex MyD88, and its selective activation of LAMP2C and CMA, controls endogenous, ie "self" antigen presentation. This result has important implications for the development of new therapeutic targets for treating autoimmunity by limiting endogenous self-antigen presentation, or for cancer therapy, by increasing exogenous antigens on the tumor to promote anti-tumoral immune responses. Thus, there are at least two clinical implications of our findings as they relate to "self" versus "non-self" discrimination: the involvement of CLIP in self-antigen presentation and the potential involvement of CD74, and its processing into CLIP, in preventing anti-tumoral immune responses.

To confirm the clinical relevance of these findings, and based on the discovery that MyD88 signals result in increased endogenous peptide presentation of cell surface CLIP peptide, we examined the levels of cell surface CLIP on a well-established mouse model of autoimmune disease, the B6.*lpr* model. Strikingly similar to the MIF-KO splenocytes, the splenocytes from the B6.*lpr* animals constitutively expressed higher than wild type levels of CLIP on the B cell surface. However, when either the B6.*lpr* cells or MIF-KO cells were stimulated with TLR agonists, the stimulation caused very minimal increases in the level of cell surface CLIP. However, stimulation with antigen receptor caused a more significant increase in cell surface CLIP in the B6.*lpr* mice. Of importance, when the cells were treated with rapamycin, only stimulation with antigen receptor engagement and IL-4 were strongly capable of presenting exogenous antigen. The TLR9-activated cells from B6.*lpr* mice were non-responsive to the mTOR inhibitor rapamycin with respect to displaying exogenous antigenic peptides. Thus, these data suggest that in this strain of autoimmune prone-animals, the B cells are limited to the presentation of endogenous, self-antigens.

In the tumor cells, we observed that treatment with 6-AN reduces CLIP expression on the tumor cell surface, while the level of cell surface MHC-II expression was unaltered (Figure 28), suggesting that induction of CMA may increase the immunogenicity of these cancer cells, potentially making them more susceptible to the host immune anti-tumor response via the loss of CLIP on the tumor cell surface. Another significant finding from our studies is that in tumor cells that become resistant to the proteasomal inhibitors PS-341 or MG-132 proteasomal degradation drugs (Dowell et al.,

2007; Hideshima et al., 2005) can still be targeted for an immune response because PS-341 reduces CLIP, making the cancer cells good targets for MHC-II mediated extrinsic cell death, a potential promising immune therapy.

Figure 29 illustrates our interpretation, and the potential importance, of our findings. Under normal/healthy conditions, when the B cell surface antigen receptor recognizes antigen, antigens presented are restricted to the presentation of antigens that have been recognized by the BCR, with a high number of the remaining peptides bound in the groove likely comprised of CLIP molecules. Once mTOR inhibition occurs, the antigen specific B cell presents predominantly exogenous antigens, Panel A. Panel B illustrates that under autoimmune conditions, the lysosomal degradation of exogenous antigens and their loading into MHC-II is suppressed, with most of the antigen loaded into MHC-II coming from endogenous/cytosolic sources. Under these conditions the surface expression is predominantly endogenous antigens, including some CLIP peptides, suggesting that these B cells more prone to present cytosolic autoantigens. MIF binding to the surface CD74 can also potentiate self/cytosolic antigen presentation, while reducing exogenous antigen processing and presentation. Our data shows that blocking mTOR with rapamycin, and inducing mTOR-dependent autophagy, increases the exogenous antigen processing and presentation of exogenous peptides on the B cell surface, thus reducing the probability of presenting auto-antigens to T cells. In addition, by blocking MIF binding to CD74, our data suggest that we can induce autophagy and, thus promote better exogenous antigen presentation.

Figure 29: Autophagy induces exogenous antigen presentation. This figure illustrates activation of autophagy can improve exogenous antigen presentation in autoimmune conditions. Panel A shows BCR and antigen presentation on the B cells and effect of rapamycin. Panel B shows antigen presentation by a B cell in autoimmune conditions and effect of rapamycin.

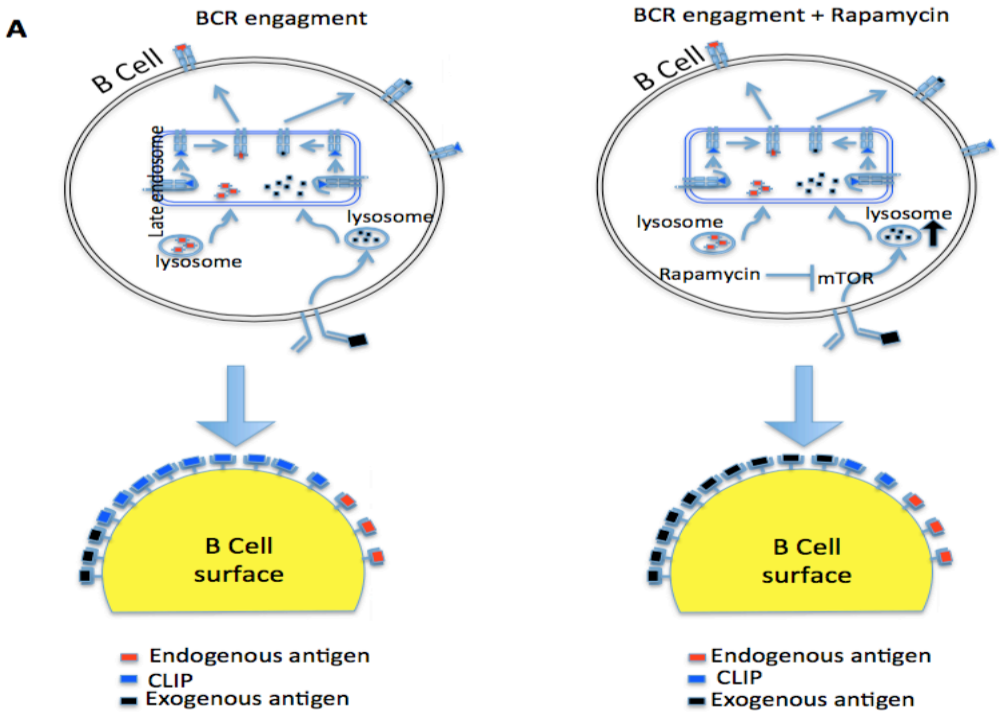
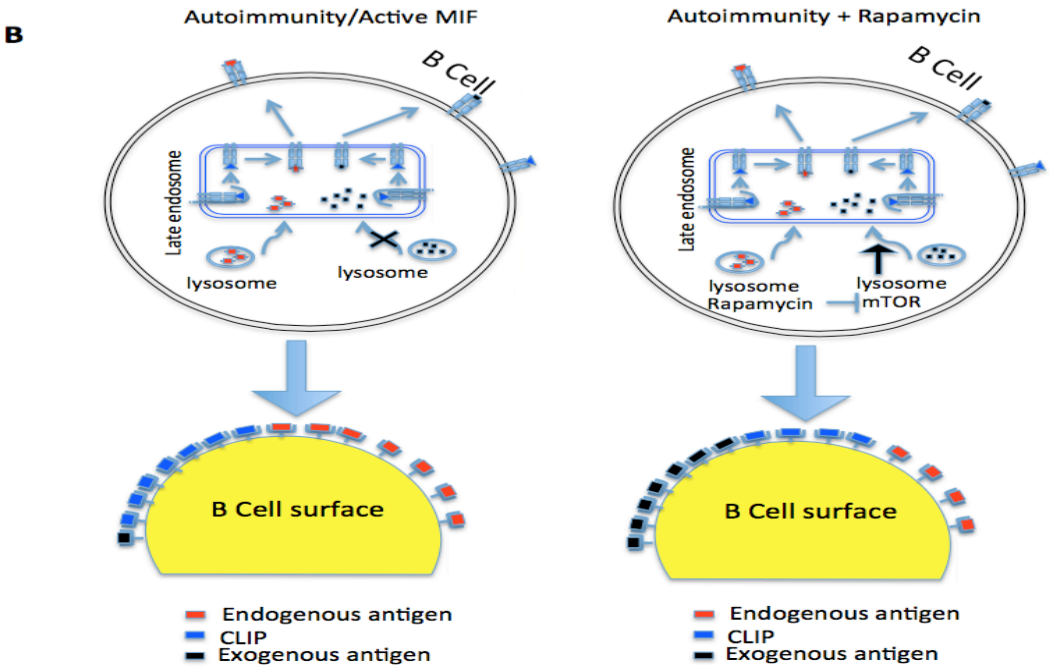


Figure 29 Continued



Future studies

For our future studies we will perform *in vivo* experiments with rapamycin and biotinylated APi1702 peptide in autoimmune disease models, including experiments in mouse models of experimental autoimmune encephalitis or in inflammatory bowel diseases. We will also utilize *in vitro* experiments to address the important question of whether rapamycin enables peptide exchange with CLIP in MHC-II on the B cell surface without the need for going though the endocytosis process. We will also plan to study the impact of rapamycin treatment on B cells as antigen presentation to CD4+ T cells and their activation. Finally, we will study the effect of PS-341 on immune cell regulation in *in vivo* mouse models of glioblastoma multiforme.

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