ROLE OF ANNEXIN-1 IN PATHOGEN RECOGNITION AND IMMUNE RESPONSE

SHU SHIN LA

(BSc. (Hons.), NUS

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"When life gives you a hundred reasons to cry,

Show life that you have a thousand reasons to smile."

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SUMMARY

Sepsis is a hyperimmune response that occurs during microbial infection and is characterised by severe inflammation leading to hypotension, multiple organ failure and in some cases, death. To date, there is no perfect treatment for sepsis. Toll-like receptors (TLR) were identified as a major source that triggers sepsis-stimulated pathways. Understanding regulators of TLR signaling pathway may hold the key to managing sepsis. Annexin-1 (ANXA1) is a 37 kDa Ca^{2+} dependent, glucocorticoid-inducible anti-inflammatory protein. It also serves homeostatic role for major cellular mechanisms such as cell proliferation, apoptosis, phagocytosis, cell adhesion and migration. It was previously reported that ANXA1 may play a role in TLR mediated immune response. Our lab has identified ANXA1 to be linked to the regulation of NF-κB. To investigate the role of ANXA1 in TLR signaling further, TLR agonists that stimulate several distinct TLR pathways were chosen (CpG DNA-ODN1826 to stimulate TLR 9, LPS to stimulate TLR4 and Poly I:C to stimulate TLR3). The agonists were chosen for the capacity to stimulate either or both MyD88-depedent and MyD88-independent (TRIF) pathway. Our collective results show that ANXA1 KO macrophages show impaired IL-12 and IP-10 response after LPS and poly (I:C) stimulation, but not CpG DNA stimulation. Macrophage and B cell activation were suppressed when ANXA1 was absent. Furthermore, macrophage nuclear translocation of IRF3 and NF-κB p65 after TRIF activation was regulated by ANXA1.

Macrophages are known to undergo polarization during immune response. The polarization status are type I (or M1: pro-inflammatory) and type II (or M2: tolerogenic) polarization, and constitute two extremes of a continuum of cytokine and chemokine profile. The capacity for macrophage to polarize into M1 or M2 state plays a critical role in overall immune system polarization. Therefore macrophage polarization status in ANXA1 KO mice was investigated to determine if it contributes to the impaired immune response observed in ANXA1 KO mice compared to its wild-type equivalents. ANXA1 was observed to suppress M2 polarization.

We also investigated whether an endogenous inhibitor of NF-κB was involved in the inhibition of cytokine and chemokine production after TLR activation. PPAR-γ, an endogenous suppressor of inflammatory response, was targeted using PPAR-γ specific ligand PGJ_2 and troglitazone, as well as PPAR-γ specific inhibitor GW9662. Our data demonstrates that PPAR-γ was responsible for the suppression observed in ANXA1 KO macrophages, and therefore ANXA1 regulates PPAR-γ activity. Taken together, the thesis demonstrates ANXA1 plays an important role in regulating TRIF dependent pathway, macrophage polarization and PPAR-γ activity.

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LIST OF ABBREVIATIONS

Ac2-26	Annexin-1 peptide (Ac-AMVSEFLKQAWFIENEEQEYVQTVK)
ANXA1	Annexin-1
APC	Antigen presenting cells
CLR	C-type lectin receptors
COX2	Cyclooxygenase-2
CpG 1826	CpG 1826 oligodeoxynucleotide (CpG DNA)
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cells
DEX	Dexamethasone
EGF	Epidermal growth factor
GC	Glucocorticoid
HGF	Hepatocyte growth factor
IFN-β	Interferon Beta
IκB	Inhibitor of κB
IKK	IκB kinase
IRAK	Interleukin-1 receptor-associated kinase 1
IRF-3	Interferon regulatory factor 3
ISRE	Interferon-stimulated Response Element
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
Mal/TIRAP	MyD88 adaptor-like / Toll-Interleukin-1 receptor adaptor protein
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF/MCSF	Macrophage colony stimulating factor

MFI	Mean florescence intensity
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NEMO	NF-kappa-B essential modulator
NLR	NOD-like receptors
PAMP	Pathogen-associated molecular patterns
PBML	peripheral blood mononuclear leukocytes
PC	Phosphatidylcholine
PGE2	Prostaglandin E2
PGJ_2	15-deoxy-Δ12,14-Prostaglandin J2
PI3K	Phosphatidylinositol-3-kinase
PMN	Polymorphonuclear leukocytes
Poly (I:C)	Polyinosinic:polycytidylic acid
RIP1	Receptor interacting protein 1
PPAR	Peroxisome proliferator activated receptor
PPRE	PPAR response element
PR3	Membrane Bound Proteinase 3
PRED	Prednisone
PRR	Proline-rich region
PS	Phosphatidylserine
RIG-I	Retinoic acid-inducible gene 1
RLR	RIG-like receptors
ROS	Reactive Oxygen Species
RQ	Relative Quantification
SARM	Sterile α- and heat armadillo-motif-containing protein
SMAD	Sma and Mad Related Family
STAT	Signal transducer and activator of transcription

TCR	T-cell Receptor
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptors
TNF-α	Tumor Necrosis Factor alpha
TRAF	C-terminal Tumor necrosis factor Receptor-Associated Factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	Toll-interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β
TRPM7	Transient receptor potential cation channel, subfamily M, member 7
TRR	Toll-receptor-related
Yap-1	Yes-associated protein 1

LIST OF PUBLICATIONS

Bist, P., Shu, S., Lim, J. Y., Arora, S., Nair, S., Lee, H. Y., Dayalan J., Gasser S., Biswas S. K., Fairhurst, A-M., Lim, L. H. K. A novel role for Annexin-A1 as a regulator of the MYD88-independent Toll-like receptor signaling pathway. Manuscript submitted for publication.

Bist, P., Leow, S. C., Phua, Q. H., Shu, S., Zhuang, Q., Loh, W. T., Nguyen, T. H., Zhou, J. B., Hooi, S. C., Lim, L.H.K. Annexin-1 interacts with NEMO and RIP1 to constitutively activate IKK complex and NF-κB: implication in breast cancer metastasis. *Oncogene*. (2011) Jul 14;30(28):3174-85.

CONFERENCE PAPERS

Shu Shin La, Pradeep Bist, Lim Hsiu Kim Lina. Annexin-1: A Novel Regulator of the Toll like Receptor Signaling Pathway. Korean Association of Immunologists (2010).

Shu Shin La & Lim Hsiu Kim Lina. Macrophage Polarization Status in ANXA1 KO mice. Singapore Symposium of Immunology (2010).

CHAPTER I:

INTRODUCTION

1. INTRODUCTION

"I find it astonishing that the immune system embodies a degree of complexity which suggests some more or less superficial though striking analogies with human language, and that this cognitive system has evolved and functions without assistance of the brain."

—Niels K. Jerne, Nobel Prize in Physiology or Medicine, 1984

1.1. Inflammation

During acute inflammatory response in diseases, various factors come together to present an overall phenotype, namely a pro-inflammatory or anti-inflammatory response. Inflammation occurs through dynamically varying levels of pro- and anti- inflammatory cytokines competing for an upper hand either by activation of signaling cascades or inhibition of downstream signals.

Pro-inflammatory mediators are the causative agents (thereby the main focus of concern during an acute inflammation) while anti-inflammatory mediators are the key players in bringing inflammation down to pre- inflammation levels. While most inflammation is resolved rapidly through the production or activity of anti-inflammatory mediators, some remain as chronic inflammation either when the source of inflammation persists or insufficient anti-inflammatory response is present (Figure 1.1).

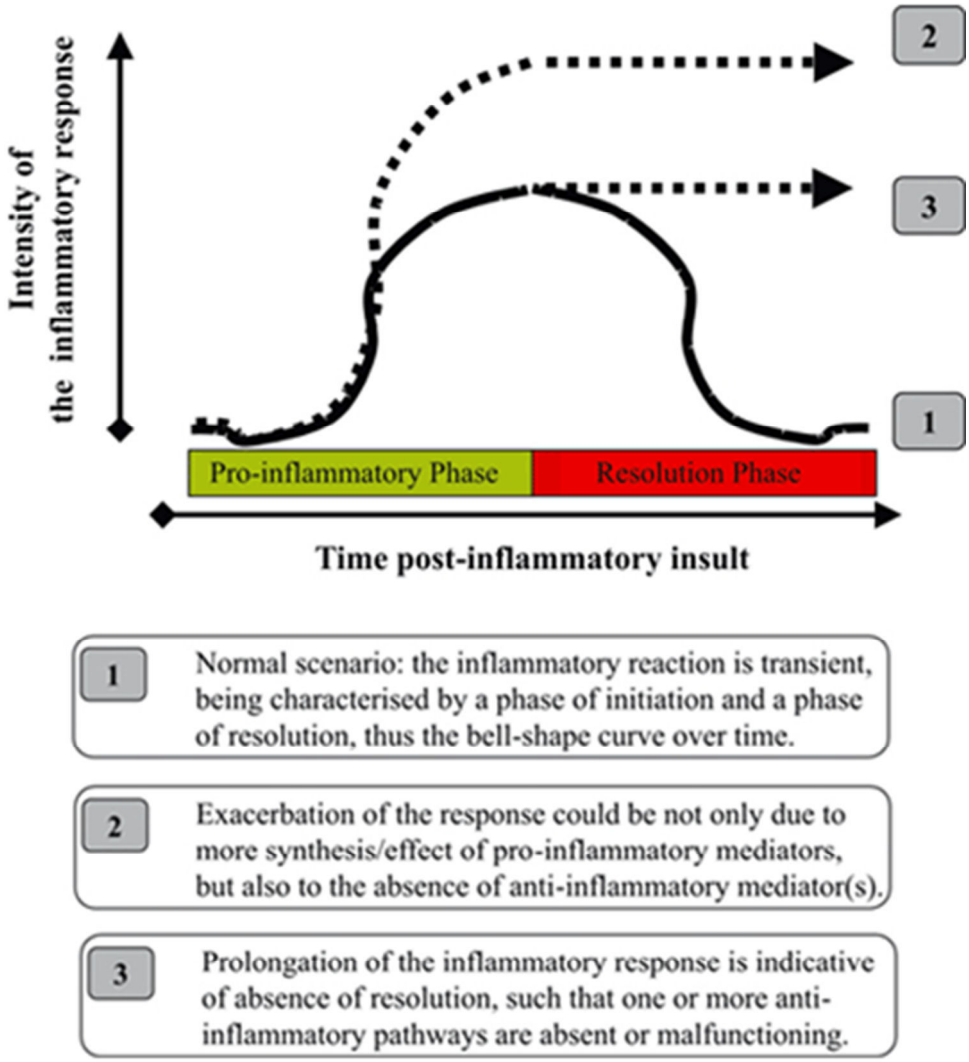


Figure 1.1. Inflammation is a dynamic process between pro- and anti-inflammatory mediators of inflammation. Prolonged inflammation is caused by the absence or limited capacity of anti- inflammatory mediators to act upon the site to resolve the inflammation. On the other hand, rapid resolution of inflammation is directly dependent on the level of activity of anti- inflammatory mediators. Therefore inflammation is caused by pro-inflammatory mediators and its resolution is dependent upon anti-inflammatory mediators (Kamal *et al*., 2005).

Induction of pro-inflammatory mediators is bound by inflammatory insults that are usually based on rapid response to external stimuli and sometimes beyond the control

of the host (e.g. systemic activation of pathogen receptor signaling cascades such as Toll like Receptors (TLRs), CLRs, Nod like Receptors(NLRs) and Rig-I like receptors (RLRs) will cause rapid build-up of pro-inflammatory response). Whether some stimuli will trigger an overwhelming pro-inflammatory response is almost impossible for the host to decipher beforehand. Anti-inflammatory mediators are molecularly existing targets that are involved in inflammation yet working to curb inflammatory response. They are therefore the present focus of research interest. In particular, anti-inflammatory mediators that exist endogenously within the host are of great interest, since their presence at physiologically relevant levels do not cause toxicity issues. Patients that may already be facing the risk of toxicity and complication through other channels of medical intervention may find such a drug invaluable for survival.

Rapid activation of an acute inflammatory response involves the concerted effort of many leukocytes. Among the many innate immune cells that mediate initial response to pathogenic stimuli found within the body, macrophages are the key players in innate immunity. Macrophages dictate the initial pro-inflammatory response during microbial infection, as it possesses a diverse array of receptors that are capable of microbial pattern recognition and immune response. (Takeuchi and Akira, 2010).

1.2. Inflammation during sepsis

Sepsis is a hyperimmune response that occurs during microbial infection (bacteria, viruses, fungi, etc.) and is characterised by severe inflammation leading to hypotension, multiple organ failure and in some cases, even death (Rice and Bernard, 2005). Sepsis continuously ranks among the top causes of illness and death worldwide. It accounts for

at least 20% of mortality in critically ill patients in U.S. and Europe (Angust *et al*., 2001; Vincent *et al*., 2006). In the United States alone, more than 210,000 deaths occurs annually and some 40% of all intensive care patients encounter sepsis during the course of their hospitalization (Skrupky *et al*., 2011). In Singapore, patients admitted to intensive care units for severe sepsis showed hospital mortality of more than 40% (Phua *et al*., 2011). However, very few therapeutic interventions exist to modulate the immune response other than clinical measures that involves maximizing oxygen delivery and prescribing broad-spectrum antibiotics to the patient. To date there is no perfect antidote to sepsis (Bernard and Bernard, 2012).

Most treatment for the management of sepsis involves down-regulation of inflammatory response during initial stages of sepsis (Pinsky, 2004). If the patient survives the initial septic shock induced by an overwhelming surge of cytokines produced by lymphocytes and macrophages, the patient goes into immune paralysis, where he completely loses the immune ability to oppose and eliminate any microbial infection he faces. The clinical outcome of such a septic patient is therefore determined both by the damage caused by the initial cytokine storm and the subsequent immune tolerance that prevents complete infection clearance (Rice and Bernard, 2005; Hotchkiss and Karl, 2003; Skrupky *et al*., 2011). As gram-negative bacteria are the major cause of sepsis, inhibiting the primary mediator of sepsis such as LPS using antibodies were initially proposed as a treatment. LPS specific antibodies were discovered and a patient cohort study based on such antibodies showed that it provided some improvement in mortality in some cases of bacteria associated sepsis, but septic patients without gram-negative bacteremia showed no treatment benefit (Fink, 1993; Ziegler *et al*., 1991). Later

on, Toll-like receptors (TLR) were identified as a major source that triggers intracellular signaling cascade which produce inflammatory cytokines upon binding microbial molecules during initial stages of sepsis (Salomao *et al*., 2008). Inhibitors for TLRs were developed to inhibit these receptors, in a hope to elucidate a treatment for bacterial sepsis. A trial for TLR4 inhibitor on septic patients showed mixed results; only severe patients showed slight improvement in mortality (Rice *et al*., 2010). Inhibiting pro-inflammatory cytokines produced during sepsis using antibodies is not a solution either. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine is persistently elevated in patients that expire after sepsis (Qiu *et al*., 2011; Reinhart and Karzai, 2001). Several clinical trials were focused on monoclonal antibodies that inhibit TNF-α over the years but were met with mediocre results (Abraham *et al*., 1995; Cohen and Carlet, 1996; Abraham *et al*., 1998). This may be partially due to the non-canonical activity of TNF-α that stimulates other immune responses Animal models with blocked TNF-α activity reduced the animal's immune system to clear microbes (Qiu *et al*., 2011). Instead, negatively regulating TLR induced signaling pathway may hold the key to managing sepsis (Ishii and Akira, 2004).

1.3. Innate immunity and TLRs

All cells of the immune system originate from the bone marrow. They include myeloid (neutrophils, basophils, eosinophils, dendritic cells and macrophages) and lymphoid (T lymphocytes, B lymphocytes and Natural Killer) cells. The immune system comprises two major arms that work synergistically to provide immunity to its host: Innate immunity (i.e. non-specific immunity), and adaptive immunity (i.e. specific immunity). While adaptive immune response require more time to respond to pathogen

invasion, innate immune response reacts almost immediately to a wide variety of organisms through the recognition of pathogen *via* pathogen associated molecular patterns (PAMPs), pattern recognition receptors (PRRs) and action of cytokines and chemokines (Cook *et al*., 2004).

PAMPs are molecular patterns derived from microorganisms that commonly invade our body. They are uniquely conserved motifs found predominantly in microorganisms but not vertebrates. Examples of PAMPs encountered by the innate immune system include lipopolysaccharide (LPS) from bacteria, bacterial DNA motif such as cysteine-phosphodiester-guanine repeat DNA stands (CpG) , double stranded RNA such as poly (I:C) and zymosan from yeast cell walls. When found inside a human body, PAMPs are distinguished by PRRs found on endothelial cells, mucosal epithelial cells, dendritic cells, macrophages and lymphocytes, and a downstream cascade of inflammatory response is immediately triggered. There are many types of PRRs found on the surface and the endosomes of cells. Among PRRs, toll-like receptors (TLRs) which comprise a family of PRRs found on both the surface and in the cytoplasm are the most diversely responsive PRRs for triggering an inflammatory response from the cells (Takeda and Akira, 2007).

Toll-like receptors gained their name from a receptor found from *Drosophila melanogaster* named Toll, which was found to play an important role in innate immunity in adult flies. TLRs possess repeated motifs high in leucine which is known as leucine-rich repeats (LRRs), and a cytoplasmic domain called the toll/interleukin 1 receptor (TIR) domain. To date, thirteen TLRs are found in mammals, and each member of the TLR family is highly specialized to bind and recognize specific PAMPs. TLR2, TLR3, TLR4,

mouse TLR7 (TLR8 for humans) and TLR9 function as signaling receptors for a diverse range of PAMPs, such as LPS, viral single-stranded RNA, CpG DNA, etc . The molecular patterns recognized by TLR family are all essential for the integrity, function and/or replication of microbial pathogens, thereby rendering TLRs difficult to evade by changing molecular sequences. For example, LPS which is recognized by TLR4, is critical to the integrity of Gram-negative bacteria such that any mutation in LPS is lethal to most species of bacteria (Rietschel *et al*., 1994). TLR3 which recognizes double stranded RNA, a central intermediate for all RNA viruses, makes it difficult for viruses to evade detection. Similar to examples afore mentioned , zymosan is recognized by TLR 2 and is also an integral component to the yeast cell wall, making it difficult for yeast infection to hide from cells from the innate immune system that possess TLRs (Cook *et al*., 2004). TLR9 is able to act as a receptor to cytosine-phosphodiester-guanine (CpG) DNA that is from bacteria (Akira, 2003).

1.3.1. TLR-specific signaling pathways

Microbial recognition of TLRs causes dimerization of TLR which triggers the activation of downstream cascade of signals through the activation of TIR domain of the TLRs. The activation causes a TIR domain containing adaptor MyD88 to induce inflammatory cytokine production such as TNF-α and IL-12, and is true for all members of the TLR family except TLR3. TLR3 is activated through a MyD88 independent, TIR domain-containing adaptor inducing IFN-β (TRIF) adaptor protein which subsequently triggers IRF-3 dependent anti-viral response.

There are distinct and specific pathways which exist to activate and/or trigger a cascade of downstream activity for the unique stimuli a cell is exposed to. Amongst many major pathways that are known for their capacity to cause transcription of inflammatory response, focus shall be placed on the following signaling molecules and transcription factors: retinoid acid-inducible gene 1 (RIG-I), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), IRF-3, STAT-1 and other pathways ANXA1 is associated with (Takeda and Akira, 2007).

1.3.2. MyD88 pathway and TRIF pathway

Engagement of TLRs by various PAMPs leads to the activation of MyD88 pathway, a controller for TLR-mediated responses for all TLRs except TLR 3 (Akira *et al.,* 2006). The MyD88 pathway is capable of activating NF-κB, a master regulator of inflammation. Upon activation of TLR by ligand-receptor binding, MyD88 adaptor protein binds to cytosolic end of MyD88 depdendent TLRs. MyD88 then recruits IL-1 receptor-associated kinases (IRAK), which leads to activation of Mitogen-activated protein kinase (MAPK) pathway. IRAK activation also causes activation of Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6), an E3 ubiquitination ligase that ubiquitinates itself. This ubiquitination of TRAF6 attracts ubiquitin binding NEMO, an important subunit of Inhibitor of kappa B kinase (IKK) complex which is required for NF-κB activation. This leads to degradation of inhibitor of κB (IκB) protein, which leads to activation of NF-κB.

TRIF dependent pathway activates both IRF-3 and NF-κB. However, TRIF is distinct and unique for its activation of IRF-3 which induces interferon beta transcription,

leading to anti-viral responses to be activated. TRIF recruits TBK1 and IKKi (or IKKε) which catalyses the phosphorylation of IRF-3 at specific serine residues in the C-terminal regulatory domain. Dimerization of IRF-3 allows for nuclear translocation and interaction with transcriptional coactivator p300 or CREB-binding protein in the nucleus, leading to transcription of IFN-β. MyD88 and TRIF dependent pathways therefore signal through different adaptor proteins leading to activation of different transcription factors (Figure 1.2).

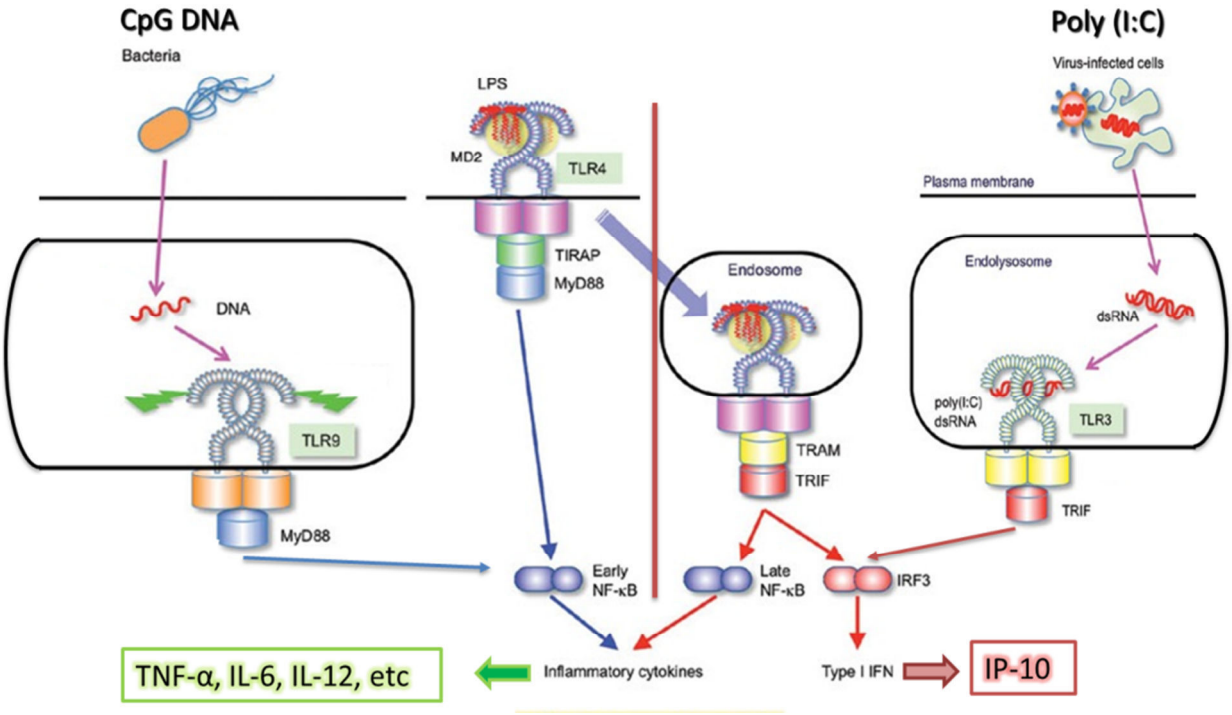


Figure 1.2: The MyD88 dependent and TRIF dependent pathways. Bacterial peptides such as CpG DNA bind to TLR9 and recruits MyD88 to trigger only MyD88 dependent pathway, causing early phase NF- κB activation. TLR 2 and TLR5 also activates specifically MyD88 pathway only. On the other hand, virus double stranded RNA or poly (I:C) binds to TLR3 to activate TRIF dependent pathway. LPS can trigger both MyD88 and TRIF pathways through the formation of TLR4-MD2-LPS complex, which can then internalize into an endosome to recruit TRAM and TRIF adaptor proteins. This leads to activation of late phase NF-κB and IRF-3 which lead to induction of type 1 interferon (TRIF-dependent pathway) (Adapted from Kawai and Akira, 2010).

1.3.3. NF-κB

NF-κB's role and association in linking immunity, inflammation and cancer is unprecedented. Known as the "master regulator of inflammation" or the "central mediator of inflammatory process", the triggering of NF-κB leads to a signalling cascade that releases countless downstream activity leading to an overall pro-inflammatory response by the activated cell (Baldwin, 1996). NF-κB is believed to target more than 250 genes in the mouse or human genome (Natoli *et al*., 2005). First discovered in 1986 by Baltimore's group as a B-cell-specific transcription factor, NF-κB has the capacity to translocate into the nucleus to bind directly to DNA, yet it is bioavailable in the cytoplasm as an inactive form. A wide range of surface receptors relay signals to activate the NF-κB pathway, notably the TNF receptor, TLR, IL-1 receptor and antigen receptor superfamilies. Growing number of intracellular receptors which are able to activate NF-κB are elucidated, and amongst them the known pathways are responses to DNA damage, reactive oxygen species as well as recognition of intracellular pathogens through NOD-like and RIG-I-like family of receptors (Hayden and Ghosh, 2012).

The NF-κB/Rel family consists of NF-κB1 (p50/p105), NF-κB2 (p52/p100), p65 (RelA), RelB and c-Rel (Chen *et al*., 1999). The most prevalent, activated form of NF-κB is a heterodimer consisting of a p50 or p52 subunit and p65, which contains all the necessary domains for transcriptional activation and gene expression. It is believed that each member of the Rel/NF-κB family play different roles *in vivo* (Ruan and Chen, 2012). Mice deficient in p65 (RelA) are embryonically lethal (Berg *et al*., 1995), and RelB-deficient mice suffer from severe autoimmune-like inflammatory diseases (Gerondakis *et al*., 1996). Mice that are NF-κB2 deficient suffer from severe developmental defects

(Caamaño *et al*., 1998). Mice deficient in c-Rel have severe impairment in T cell immune response but possess normal non-lymphoid organs (Liou *et al*., 1999), while NF-κB1 (p50) deficient mice are susceptible to bacterial infections due to compromised B cell responses (Sha *et al*., 1995). The knockout mice reflect the importance of each member of the NF-κB family in physiological systems ranging from healthy organ development to homeostatic immune function and regulation. NF-κB exists in the cytoplasm associated with inhibitory protein IκB. The IκB is made of IκBα, IκBβ and IκBε. Each of the IκB subunits inhibit specific subsets of NF-κB. The inhibition of NF-κB by IκB is released upon phosphorylation. The phosphorylating kinase was identified as IKKs. IKKs are made up of three subunits that forms the IKK complex: IKKα, IKKβ and IKKγ (NEMO). NF-κB activation always involves activation of IKK complex which phosphorylates IκB. Once IκB is phosphorylated and degraded, a point of no return is reached: NF-κB translocates to the nucleus and proceeds with transcription of NF- κB target genes (Gilmore, 2006).

NF-κB affects both innate and adaptive immune response (DiDonato *et al.,* 2012). It is activated by a diverse range of external stimuli which causes inflammatory response, and is particularly important in TLR signaling, as all the TLR except TLR3 activates the MyD88 adaptor protein that triggers a downstream cascade leading to degradation of IκB and activation of NF-κB.

NF-κB plays an important role in immune response and inflammation. T cells from transgenic mice that lack NF-κB/Rel signaling pathway exhibit a delayed Th 1 activation and response. Known as one of the most important regulators in pro-inflammatory gene expression, it induces the synthesis of pleiotropic pro-inflammatory

cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-12, pro-inflammatory mediating enzyme prostaglandin-endoperoxide synthase 2 (COX-2), adhesion molecules and inducible nitric oxide (iNOS). NF-κB is able to act in concert with other pro-inflammatory transcription factors such as AP-1, which is capable of phosphorylating MAPK/JNK pathways. It is also known that some of the corticosteroid action used in the treatment of inflammatory diseases, are mediated through the inhibition of NF-κB activation (Tak and Firestein, 2001).

1.3.4. IRF-3

Interferon regulatory transcription factor 3 (IRF-3) belongs to the IRF family of proteins, which all share significant homology in their DNA binding domains. To date, there are 9 members in the IRF family (IRF-1 to IRF-9), and all play a role as transcription mediators of virus, bacteria and other interferon-inducible activation (Zhao *et al.,* 2007). It therefore plays a critical role in antiviral defence and immune response. IRF-3 is activated by infected cells upon recognition of double stranded RNA, either by surface TLR3 receptors or internalized cytoplasmic receptors (RNA helicase RIG-I and MDA-5). Both receptors eventually activate a signaling cascade leading to the phosphorylation of IRF-3 at serine residue position 386 (Ser 386) by two non-canonical IκB kinases, TBK-1 and IKKε. The activated IRF-3 homodimerizes or heterodimerizes with IRF-7 and translocates to the nucleus, where it stimulates activation of IFN-β (Paun *et al.,* 2007). Mice with homozygous deletion of IRF-3 exhibit impairment in mounting a type 1 interferon anti-viral response against viral infection.

1.3.5. STAT-1

STAT-1, or Signal Transducer and Activator of Transcription 1, is a 91 kDa protein belonging to the JAK-STAT signaling family and plays a key role in facilitating gene transcription upon activation of type I and type II interferon receptors. Phosphorylation of STAT-1 by receptor associated kinases activates STAT-1, which forms homodimers that translocates to the nucleus to act as transcription activators. STAT-1 deficient mice are resistant to cecal ligation and puncture (CLP) induced septic shock resulting in a survival rate of 80% in STAT-1 deficient mice versus 10% for wild-type mice (Herzig *et al.,* 2012).

1.3.6. PPAR-γ

Peroxisome Proliferator- Activated Receptor gamma (PPAR-γ), is a nuclear receptor and transcription factor from the steroid family. Its natural ligand is 15-deoxy-δ(12-14)-prostaglandin J_2 (PGJ_2). Artificial ligands for PPAR-γ are thiazolidinediones widely used to treat diabetes. Upon activation of PPAR-γ by its ligand, PPAR-γ heterodimerizes with retinoid-X receptor α and binds to PPAR specific DNA response elements, or PPRE. It is widely known for its capacity to regulate fatty acid storage and modulate glucose metabolism in cells, as PPAR- γ knockout mice are unable to generate adipose tissues even when fed with a high-fat diet (Jones *et al.,* 2005). More importantly, it is found to increase sensitivity to insulin by upregulating glucose transporter 4 (Wu *et al*., 1998). It therefore plays an important role in adipogenesis and diabetes.

Its involvement in inflammation regulation was revealed only in the past decade. A variety of immune cells such as macrophages, dendritic cells, neutrophils and other

lymphocytes express PPAR-γ. In many experimental studies, PPAR-γ is capable of inducing anti-inflammatory activity, and is found to have therapeutic potential in regulating the immune system (Ohshima *et al.,* 2012). PPAR-γ has many inflammation associated ligands: unsaturated fatty acids, oxidized and nitrated fatty acids, arachidonic metabolies, 15-deoxy-$\Delta^{12,14}$-PGJ_2 $_{(15d\text{-}PGJ2)}$, thiazolidinediones (TZDs) , phospholipid cyclic phosphatidic acid, lysophosphatidic acid and oxidized low-density lipoprotein components are all ligands to PPAR-γ. PPAR-γ is expressed in various immune cells, such as primary peritoneal macrophages, dendritic cells, and T cells. The major role of PPAR-γ is to negatively regulate Th 1 specific genes and PAMPs that signal through pattern recognition receptors. (Welch *et al*., 2003). PPAR-γ is able to suppress monocyte dependent inflammatory cytokine production at doses similar to those used for adipogenesis. Both natural ligand PGJ_2 and TZDs are capable of inhibiting macrophage activation (Jiang *et al*., 1998). PPAR-γ inhibits transcriptional activity of NF-κB activity through inhibition of the p65/RelA subunit in macrophages (Chinetti *et al.,* 1998), and upon activation of PPAR-γ, IFN-β production is inhibited through prevention of IRF-3 binding to the IFN-β promoter (Zhao *et al*., 2011). In dendritic cells, 15d-PGJ_2 activates PPAR-γ, causing reduced stimulation of DCs via TLR ligands 2, 3, 4 and 7 while inhibiting MAP kinases and NF-κB pathways but not PI3 kinase/Akt signaling pathway. Interestingly, PPAR-γ -/- embryonic stem cells were found to have suppressed expression of inflammatory genes such as iNOS and COX_2 (Chawla *et al*., 2001). PPAR-γ therefore plays a role in modulating both innate and adaptive immune responses.

There are many artificial PPAR-γ activators available today due to the importance of PPAR-γ in insulin and adipocyte associated diseases, such as ciglitazone, rosiglitazone,

troglitazone. These are widely-used drugs in the treatment of diabetes (Sasaki *et al.,* 2005). TZDs are found to possess the same anti-inflammatory properties of PPAR-γ activating ligands which are known for its anti-inflammatory effects, such as PGJ_2. Ciglitazone reduces systemic inflammation in microbial sepsis by modulation of NF-κB and AP-1 pathways (Zingarelli *et al*., 2003) Rosiglitazone is another PPAR-γ activating ligand which also possesses anti-inflammatory properties. Rosiglitazone plays a role in in reducing pancreatic inflammation in obese mice (Pini *et al*., 2012). Troglitazone is found to be capable of preventing lymphocyte adhesion to endothelial cells, which is considered as a hallmark anti-inflammatory effect (Sasaki *et al.,* 2005). Based on the activity of PPAR-γ specific antagonist, it is evident that PPAR-γ is an important suppressor of pro-inflammatory response in both immune and non-immune cells.

1.4. Chemokines and cytokines involved in inflammation

There are myriad of paradigms available for displaying a pro-inflammatory phenotype by choosing cytokines and chemokines that would aid in explaining the inflammatory process. In this experiment, we have chosen the cytokine / chemokine IL-6, IL-12, IP-10 (CXCL-10), and TNF-α. We shall review through the cytokines and chemokines relevant to this study and understand their significance during inflammation.

1.4.1. IL-6

First discovered as a hepatocyte stimulating factor (Gauldie *et al*., 1987), IL-6 was also found later to function as a plasmacytoma growth factor (Suematsu *et al*., 1992). IL-6 is a multifunctional acute phase response cytokine, playing an important role in

signaling pathogen invasion in host during sepsis and endotoxemia (Meyer *et al.,* 1995). IL-6 deficient mice are highly susceptible to sepsis by common microbial pathogen such as *Streptococcus pnenumoniae* (van der Poll *et al*., 1997).

IL-6 is secreted by T cells and monocytes such as dendritic cells and macrophages, and is involved in immunoglobin secretion by mature B cells, activation of cytotoxic T-cells and other inflammatory responses (Kestler *et al.,* 1995). Its receptors are IL-6 receptor and gp130 (Ciapponi *et al.,* 1995). IL-6 is capable of being suppressed by glucocorticoid action (Fried *et al.,* 1998).

IL-6 has been implicated in the generation and propagation of both acute and chronic inflammation. IL-6 trans signaling promotes acute inflammation by increasing expression of endothelial leukocyte adhesion molecules (VCAM-1, ICAM-1) which increases leukocyte accumulation (Kaplanski *et al*., 2003). It therefore facilitates the transition from neutrophils to mononuclear-cell infiltrate, which is a hallmark of acute inflammation. IL-6 is also responsible for chronic inflammation, as it rescues T cells from apoptosis, thus postponing the clearance of mononuclear inflammatory cell infiltrate (Curnow *et al*., 2004). IL-6 is therefore important in driving local inflammation.

1.4.2. IL-12

IL-12 is a heterodimeric cytokine produced by phagocytic cells, B lymphocytes and other myelomonocytic cells (D'Ambrosio *et al.,* 1998). It was formerly known as "cytotoxic lymphocyte maturation factor" and "natural killer cell stimulatory factor". The bioactive form of IL-12 comprises a heterodimeric molecule made up of 40 kDa (p40) and 35 kDa (p35) subunits, and undergoes substantial post-translational modification

which accounts for 10 to 20% of carbohydrate by mass and a disulphide bridge that allows heterodimer formation for it to become bioactive (Podlaski *et al.,* 1992). The p35 subunit is more ubiquitously expressed even in lymphocytes not associated with IL-12, while the p40 subunit is produced only by lymphocytes that are known to produce IL-12. The NF-κB/Rel protein binding site within the IL-12 p40 promoter is responsible for p40 subunit specific production during NF-κB activation (Sanjabi *et al*., 2000).

In response to pathogens, PRRs such as TLRs are activated and IL-12 is produced by phagocytes (monocytes/macrophages and neutrophils) and dendritic cells. Specifically, IL-12 p40 subunit is produced from TLR activation by TLR ligands or agonists such as LPS. When both LPS and IFNg are present to stimulate activation, IL-12 p70 heterodimer is produced. IL-12 is a critical factor for development of Th 1 immunity and cell mediated responses against diverse pathogenic insults (Trinchieri, 1995). IL-12 stimulates increased proliferation and colony formation of haematopoietic progenitors (NK cells, NKT cells and T cells) and their production of cytokines, in particular interferons (e.g. IFN-γ). As IFN-γ can also induce transcription of IL-12, it presents a positive feedback loop for IL-12 production during inflammatory response. As a cytokine it is unique in its capacity to direct Th 1 cell development and cellular immunity, it is important to detect its levels during inflammatory and autoimmune disease research (Adorini *et al.,* 1997).

Products from microorganisms such as bacteria (LPS), bacterial DNA and CpG-containing oligonucleotides are strong inducers of IL-12 production by macrophages and other monocytes, neutrophils and dendritic cells (DCs). Intrinsic defects in macrophage IL-12 production are associated with immune dysfunction. For example, patients with

lower IL-12 production are highly susceptible to mycobacterial infection (Uzzaman and Fuleihan, 2012) leshmaniasis (Alleva *et al*., 1998) and candidiasis (van de Veerdonk *et al*., 2011). IL-12 deficiency in humans is associated with recurrent pneumonia, sepsis and other infections in the absence of fevers from a very young age (Haraguchi *et al*., 1998).

1.4.3. IP-10 (CXCL-10)

Interferon-inducible protein-10 (IP-10) or C-X-C motif chemokine 10 (CXCL10) is a 10 kD secreted protein identified from abundant RNA induced by interferon gamma and LPS. It is a member of –C-X-C- motif chemokine family of secreted proteins. Secretion of IP-10 by leukocytes, neutrophils, eosinophils, monocytes, epithelia, endothelial and stromal cells is associated with inflammation (Luster and Ravetch, 1987).

IP-10 activates the CXCR3 receptor predominantly expressed on activated T cells, B cells, NK cells, DCs and macrophage cells, and acts as "homing" beacon to attract CXCR3-positive cells. Th1 cells produce IFN-γ, which induces IP-10 production, and provides a positive feedback loop to attract and recruit more Th1 cells (Campbell *et al*., 2004). IP-10 is also produced upon induction by IFN-β produced through TLR3-TRIF-IRF3 dependent pathway activation (Petry *et al*., 2006).

Recent studies have elucidated the mechanism of IP-10 intracellular signaling pathways. Interaction of IP-10 with its receptor CXCR3 results in p38/MAPK and PI3K signaling pathway activation (Shen *et al*., 2006; Shahabuddin *et al*., 2006). IP-10 can also activate cAMP-dependent protein kinases A (PKA) singaling pathways (Jinquan *et al*., 2000), and affect cell migration and proliferation through activation of Ras/ERK, Src and PI3K/Akt pathways (Bonacchi *et al*., 2001). In murine macrophages, JAK1,

JAK2/STAT1 activation plays an important role in upregulating IP-10 production (Han *et al*., 2010). It can modulate adhesion molecule expression and stimulate monocytes, NK-cells and T-cell migration. (Kim *et al.,* 2012a). IP-10 is induced in a variety of conditions such as psoriasis, fixed drug eruptions, hypersensitivity reactions and encephalomyelitis (Luster *et al.,* 1995).

1.4.4. TNF-α

TNF-α or tumour necrosis factor-alpha, is an acute phase, pleiotropic cytokine involved in systemic inflammation produced mostly by activated macrophages, $CD4^+$ T-cells and NK-cells. Large quantities of TNF- α are produced in response to LPS and other bacterial products (Walsh *et al.,* 1991). TNF- α binds TNF receptors and trimerizes the receptors to dissociate inhibitory protein Silencer of Death Domains (SODD) from the intraceullar death domain. When the death domain is exposed, adaptor protein TNF-α Receptor Death Domain (TRADD) binds to it and initiates downstream pathways such as NF-κB pathway, MAPK pathway and caspase 8- dependent death signaling (Chen and Goeddel, 2002).

1.4.5. Interferons

Interferons (IFN) are proteins with potent anti-viral activity which plays a crucial role during early viral response to infections. Type 1 interferons (IFN-α, -β, and -ω) bind to the interferon receptors IFN-α R1 and IFN-α R2. Type 2 interferon IFN-γ interacts with IFN-γR1 and IFN-γR2. The expression of type 1 intereferon is strictly regulated by activation of transcription factors IRF-3, NF-κB and Activating transcription factor 2

homerdimerized with c-Jun (ATF-2-cJun) (Yoneyama *et al*., 2004). IRF-3 deficient cells exhibit greatly attenuated type 1 interferon production. IFNs are able to regulate cells of both innate and adaptive immunity. Of importance is IFN-γ which activates an extensive array of antimicrobial functions of phagocytes and plays an important role in the immune response against bacteria, fungi and other common intracellular pathogens. (Trinchieri, 2003).

1.5. Macrophage polarization

Macrophage polarization encompasses the diverse phenotypes of macrophage ranging from classical activation categorized at one polar end termed M1 polarization, and alternative type of macrophages that appear to be actively producing anti-inflammatory cytokines termed M2 polarization which is placed at the other end of the spectrum. All other intermediary phenotypes based on their cytokine / chemokine profile are placed in between. The two diametrically opposite properties of macrophages were well documented (Mantovani *et al*., 2005) but lacked a framework to encompass the diverse properties of macrophages depending on its polarization status.

Classical response by macrophage is termed M1 polarization. M1 macrophages produce pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-1β. There are M1 polarization specific surface markers such as CD80 and CD86 (ibid.). The importance of M1 polarized macrophages during bacterial infection is evident from mice deficient for components of either the interferon gamma or the IL-12 pathway. The mice became highly susceptible to *Mycobacteria* and S*almonella* (Jouanguy *et al.,* 1999).

While M1 macrophages are capable of recruiting Th1 response with potent microbial properties, M2 macrophages support Th2- associated responses, and are considered incompetent to eliminate pathogens. M1 polarization is characterized by high IL-12 and low IL-10 production while M2 polarization is categorized by low IL-12 and high IL-10 production. M1 and M2 macrophages are also segregated by the expression of alternative activation markers. There are M2 markers such as arginase-1 which is only expressed in M2 macrophages (Mills *et al.,* 2000).

M2 macrophages are further divided into M2a, M2b, M2c and M2d cells, based on the different stimuli it is elicited from (Figure 1.3). M2a and M2b are macrophages that exert their immune functions to drive Th2 responses. M2c macrophages play a role in immune suppression, and M2d macrophages or tumour associated macrophages (TAM) which accumulates at the tumour site due to tumour-derived signals such as macrophage colony stimulating factor (M-CSF) and monocyte chemoattractant protein (MCP-1) (Martinez *et al.,* 2008).

While the concept of macrophage polarization is new, it is capable of presenting a clear-cut explanation on certain macrophage phenotypes under complex stimuli such as live pathogens. For example, some pathogens such as *Yersinia enterocolitica* are capable of programming macrophages into M2 polarization. The pathogen-induced reprogrammed macrophages express M2 markers such as arginase-1, and produce M2 cytokines such as TGFβ and IL-4 in BALB/c mice (Benoit *et al.,* 2008). Other pathogens such as chronic Q fever inducing *Coxiella burnetii* induces macrophages to produce IL-10, TGFβ and CCL18, thus reprogramming the macrophage into M2 polarization status after successful and chronic infection. Moreover, the macrophages do not express M1-

associated cytokines such as TNF-α, IL-12 and surface expression marker CD80 and CCR7, and do not produce nitric oxide (Benoit *et al.,* 2008).

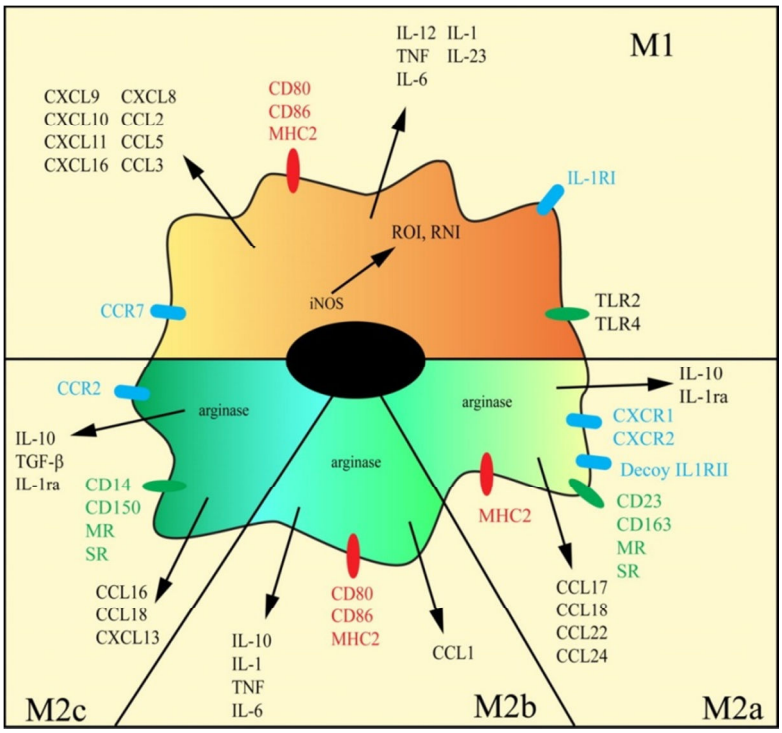


Figure 1.3: M1 and M2 polarization in macrophages. M1 macrophages, or macrophages with classical activation response are induced through LPS or other microbial products, and induces inflammation through the production of pro-inflammatory cytokines and chemokines such as IL-6, IL-12, TNF-α and CCL5. Release of reactive oxygen species and nitrogen intermediates (ROI and RNI) are also hallmarks of M1 polarization in macrophages. Alternatively activated macrophages (M2) are classified into M2a induced by IL-4/IL-13, M2b induced by immune complexes and TLR agonists, and M2c induced by IL-10 and glucocorticoid hormones, and M2d induced by tumour microenvironment (not shown in diagram) (Diagram from Benoit *et al.,* 2008)

1.6. ANXA1: A calcium and phospholipid binding molecule

ANXA1 belongs to the annexin superfamily of proteins. Under electron microscopy, ANXA1 is seen to form highly organized and symmetric scaffolds that consolidate membrane domain complexes. The annexin family is one of the three major membranous calcium binding proteins. The other two families binding to calcium are EF-hand proteins, and the C2-domain proteins (Lim and Pervaiz, 2007).

Annexins bind calcium through its signature core domain repeats, and its calcium binding face docks onto the membrane when bound to calcium (Figure 1.4). Some annexin families (e.g. Annexin-A5) even anchor directly to the hydrocarbon lipid chain of the plasma membrane. All members of the annexin family possess homologous repeats of 70-80 amino acids which form the core of the protein length that is usually repeated 4 or 8 times, and represents more than 80% of the protein (Hunter, 1988).

ANXA1 in particular exhibits the most pronounced conformational change, i.e. the N-terminal domain is usually buried deep inside the core repeats and remains inaccessible to any external interaction. Upon binding to calcium ions, the N-terminus is pushed out and exposed to the surface for presentation and binding with proteins, such as S100A, an EF-hand family of proteins. ANXA1 and Annexin-2 (ANXA2) are usually bound to bilayers of phosphatidylserine (PS)/ phosphatidylcholine (PC) mixtures as protein clusters, and also found on endosomal compartments (Gerke and Moss, 2002).

ANXA1 KO studies have demonstrated that multivesicular endosomes can form without ANXA1 but they possess fewer internal vesicles than their wildtype counterparts (Gerke *et al.,* 2005).

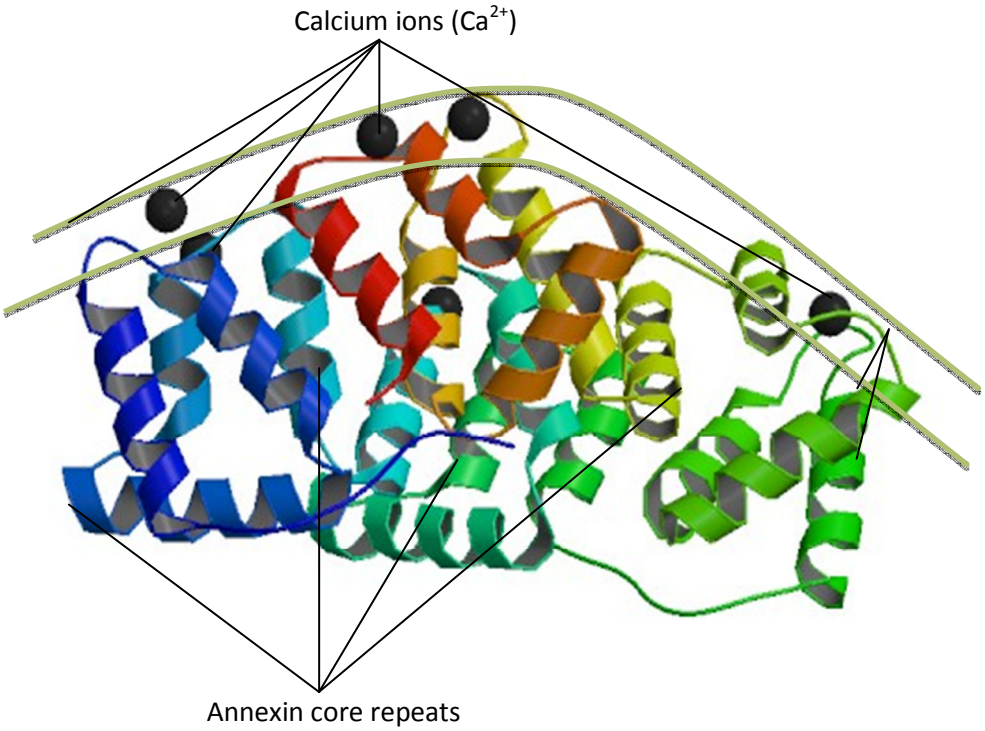


Figure 1.4. Structure of ANXA1. Black spheres denote calcium ions. Upon binding calcium, calcium binding face of ANXA1 adheres to the plasma membrane (modified from "annexin-1", from Protein Data Bank, www.pdb.org).

The N-terminus determines the uniqueness of the annexins within the annexin family. For ANXA1, the N-terminus spans exactly 33 amino acids (Flower *et al.,* 1994), and has extensive association with the membrane region of cells, in particular phospholipid vesicles (Hoekstra *et al.,* 1993; Wang and Creutz*.,* 1994). Cleavage of ANXA1 at N-terminus occurs endogenously through the help of membrane bound proteinase 3 (PR3) (Pederzoli-Ribeil *et al.,* 2010). N-terminus of ANXA1 presented

exciting avenues of anti-inflammatory research as the N-terminus was sufficient to elicit anti-inflammatory properties of its parent protein. A peptidomimetic molecule of the first 26 amino acids of ANXA1 (Ac2-26) was made to further investigate its anti-inflammatory effects, and was proven to be anti-inflammatory in an *in vivo* mouse model (Cirino *et al.,* 1993). The N-terminus of ANXA1 is a natural ligand for the activation of different receptors in the FPR family (Ernst *et al.,* 2004).

Intracellular calcium has evolved into a messenger for a diverse range of signals. Sensitivity of ANXA1 to Ca^{2+} signals is dependent on the truncation of the N-terminus of ANXA1 (Monastyrskaya *et al.,* 2007). The truncated C-terminal end with the core annexin repeats on the other hand, upon binding to Ca^{2+} undergoes conformational change to aggregate onto the plasma membrane (Rosengarth and Luecke, 2003).

1.6.1. ANXA1 is a glucocorticoid inducible protein

ANXA1 was first discovered in the 1970s as the protein responsible for anti-inflammatory activity of glucocorticoid (GC) treatment through inhibition of phospholipase A_2 (PLA_2) activity, and was named "lipocortin-1" based on its function and the source of induction. Later on, the protein was discovered to "annex" (i.e. to bind) to cellular membrane in a Ca^{2+} dependent manner, and was renamed "annexin-1" (Lim *et al.,* 2007). GCs are anti-inflammatory and immunosuppressive agents used in immune associated diseases. In U937 cells, GC drugs such as dexamethasone (DEX) cause induction of ANXA1 and a biphasic translocation of ANXA1 from cytosolic onto cell membrane regions (Solito *et al.,* 1994), and this observation is also consistently observed in periphery and central tissues *in vivo* (Philips *et al.,* 1997). The stimulation of

glucocorticoid receptor causes induction of ANXA1 and its phosphorylation at serine residue position 27, and translocates to the membrane with the help of protein kinase C (PKC), phosphatidylinositol-3-kinase (PI3K) and MAP kinase activity (Solito *et al.,* 2003a). It was not known at that time which protein was responsible for the phosphorylation of ANXA1 that caused this translocation. It is now known that serine residue position 27, 34 and 45 are recognition sites for the kinase PKC, and that phosphorylation at position 27 and 45 is essential for the translocation of ANXA1 to the membrane and its activity to modulate adrenocorticortrophic hormone (ACTH) (McArthur *et al.,* 2009). There is little doubt on how important ANXA1 is as a GC-induced corticosteroid hormone modulator, as its capacity to control ACTH activity been proven *in vitro* (Taylor *et al.,* 1993) and *in vivo* (Loxley *et al.,* 1993). The capacity of ANXA1 to mediate anti-inflammatory action from GC-based drugs became evident when monoclonal antibodies targeting ANXA1 could inhibit inflammation drivers prostaglandin E2 (PGE_2) and Leukotriene B4 (LTB4) production from peripheral blood mononuclear leukocyte (PBML). The inhibitory results were similar to GC drug induced such as dexamethasone (DEX) and prednisolone (PRED) inhibition of PBML (Almawi *et al.,* 1996).

1.6.2. ANXA1 as an inhibitor of PLA_2

ANXA1 is well-studied for its capacity to induce corticosteroid dependent inhibition of PLA_2 activity, thereby preventing generation of arachidonic acid to create pro-inflammatory mediator prostaglandin from its precursors diacylglycerols and phospholipids (Flower and Blackwell, 1979; Peers and Flower; 1990). It therefore also limits substrate availability of arachidonic acid for COX-2 to generate pro-inflammatory

prostaglandins such as prostaglandin E_2. Hence it is also believed to play a role as cyclooxygenase-2 (COX-2) inhibitor. When endogenous levels of ANXA1 is depleted through RNA interference, PLA_2 activity increases substantially (Solito *et al.,* 1998). The route of inhibition which ANXA1 acts upon PLA_2 has also been found. ANXA1 forms a complex with S100A1 that bind to and inhibits cytosolic PLA_2 activity (Sakaguchi and Huh *et al.,* 2011). Recently, it was demonstrated that phosphorylation of ANXA1 by transient receptor potential cation channel, subfamily M, member 7 (TRPM7) kinase at serine residue position 5 (Ser 5) causes S100A1 to dissociate from ANXA1, demonstrating the complete regulatory process for ANXA1 in relation to its PLA_2 inhibition activity (Dorovkov *et al.,* 2004; Dorovkov *et al.,* 2011).

1.6.3. ANXA1 in the inflammatory response

As ANXA1 is a glucocorticoid inducible anti-inflammatory protein, its anti-inflammatory role has been extensively studied, but its mechanism of action in other cell functions is not well understood. ANXA1 KO mice exhibit lethality to LPS stimulation (10mg/kg) within 48 hours while wild-type mice do not (Damazo *et al.,* 2005). ANXA1 KO mice exhibited an increased expression of pro-inflammatory proteins, including COX-2 and cytosolic PLA_2 (Roviezzo *et al*., 2002). In a peritonitis model, ANXA1 KO mice exhibit an exaggerated response, i.e. increased granulocyte migration and cytokine production (Damazo *et al.,* 2006). Moreover, the absence of ANXA1 in a lung fibrosis mouse model demonstrated pathophysiological relevance for endogenous ANXA1 in lung inflammation (Damazo *et al.,* 2011), but the mechanism behind the inflammatory activation is unclear.

In a recent study, regulation of complement factor was demonstrated by ANXA1. Peritoneal lavage of ANXA1 KO mice exhibited more than fifteen-fold increase in C5a production, than its wild-type counterparts after treatment with zymosan (Dalli *et al.,* 2010).

ANXA1 is found in bronchoalveolar lavage fluid from asthmatics. ANXA1 deficient mice exhibit airway hyperresponsiveness in an asthma model. Hence it is believed to be an important regulator in the development of allergic disease (Ng *et al*., 2011).

In cystic fibrosis knockout mice (cftr -/-), ANXA1 was not detected in lungs and pancreas. Interestingly, cystic fibrosis patients also exhibit down-regulated ANXA1 levels, and is believed to contribute to worsening of clinical diagnosis during cystic fibrosis (Bensalem *et al.,* 2005). A recent study has found that reduced levels of ANXA1 is partially responsible for cystic fibrosis (Dalli *et al.,* 2010),but no mechanistic study was carried out to understand the relationship between cystic fibrosis and ANXA1 in detail.

1.6.4. ANXA1 in signaling

ANXA1 is also believed to be involved in ubiquitination, as it was found to be a substrate for E6AP-mediated ubiquitination, which is known to mediate ubiquitylation and degradation of p53. Domain III of ANXA1 can interact with E6AP and induce its ubqiuitylation in a calcium-dependent manner (Shimoji *et al.,* 2009)

In RAW macrophages, ANXA1 can specifically modulate ERK signaling, i.e. overexpression of ANXA1 causes constitutive activation of ERK 1/2 kinase (Alldridge *et al.,* 1999).

1.6.5. ANXA1 regulates cell migration

Neutrophils bind to endothelium and exhibit reduced migration when exposed to zymosan, a glucan with repeating glucose units connected by β-1,3-glycosidic linkages prepared from yeast cell wall. ANXA1 N-terminal peptide (Ac2-26) and recombinant human ANXA1 protein induced detachment and migration of neutrophils that were exposed to zymosan (Lim *et al*., 1998).

ANXA1 is also capable of directly affecting cell migration. In epithelial cells, the phosphorylation of ANXA1 is directed by LIM kinases upon induction by VEGF, causing enhanced cell migration (Côté *et al.,* 2010)

Another study proposes a more specific mechanism involving ANXA1. Overexpression of ANXA1 alone can abrogate the decrease in cell migration when microRNA miR-196a expression is increased. Moreover, the study suggests that ANXA1 regulates cell migration by establishing the formation of lamellipodia at the leading edge of the cell (Pin *et al.,* 2012).

1.6.6. Other interactions with ANXA1

Despite extensive research on the molecular functions of ANXA1, there are still several associations which are highlighted by recent research but no further investigations

were done to date. While the interactions may have been left behind as it may not be feasible to research at this stage of scientific discovery, there remains an important trace for future discoveries and understanding of ANXA1 as a regulator of homeostasis.

Another unknown property of ANXA1 is its nuclear localization in cancer cells. In oral squamous cell carcinoma, ANXA1 is found to be predominantly translocated into the nucleus, upon exposure to human growth factor (HGF). Pre-treatment with LY294002 PI3K inhibitor can inhibit nuclear translocation of ANXA1 substantially (88.3% inhibition) (Lin *et al.,* 2008).

ANXA1 is also found to interact and bind to EGF receptors that are internalized (Radke *et al.,* 2004), but no examination relating ANXA1 to EGF-dependent pathways were done.

ANXA1 is also associated with pain function. It was highly upregulated (12-fold) during tissue injury and acute pain (Wang *et al.,* 2009). But no further study detailing this observation was carried out.

Another study has identified ANXA1 to be important in cerebral ischemia, where ANXA1 levels were upregulated in polymorphonuclear cells after stroke, and is believed to be a key indicator in the severity of cerebral ischemia (Joseph *et al.,* 2012). It was observed in this study that prolonged gaseous hypothermia downregulates ANXA1.

There are other lesser-known interacting partners of ANXA1 which are available in protein databases that documents large-scale protein-protein interaction studies. The interacting partners from the interaction studies have been compiled into a table with the help of major protein databases in the world (IntAct, GRID, UniProt). Proteins with no

known function were not included into the table for a concise consolidation of information. Reliable molecular interaction techniques such as anti-bait immunoprecipitation (Ewing *et al.,* 2007), Tandem Affinity Purification (Bouwmeester *et al.,* 2004) or molecular sieving method (Bernhard *et al.,* 2004) are used to establish interactions, and is processed downstream with the latest mass-spectrometry technique (LC-ESI-MS/MS). Hence they are not putative interacting partners; they are experimentally established ANXA1 interacting partners ready for direct research efforts. Some of the interactions are already definitive interacting partners for ANXA1, such as S100A11. This information will provide ready-access to a rich source of interaction studies ready for future experiments on ANXA1 (Appendix A).

1.6.7. Implication of ANXA1 in disease

ANXA1 is involved in diseases from diverse backgrounds, cancer in particular. ANXA1 is upregulated in breast cancer (Cao *et al*., 2008), oral squamous cell carcinoma (Faria *et al*., 2010), urinary bladder urothelial carcinoma (Li *et al*., 2010) and down regulated in laryngeal squamous cell carcinoma (Silistino-Souza *et al*., 2007), cervical cancer (Wang *et al*., 2008), gastric cancer (Yu *et al*., 2008), and prostate cancer (D'Acunto *et al*., 2010). ANXA1 is also implicated in lung squamous cell carcinoma (Nan *et al*., 2009), and treatment of leukaemia (Falini *et al*., 2004).

ANXA1 is also involved in autoimmune diseases. Auto-antibodies for ANXA1 was detected in patients with systemic lupus erythematosus (Hiraata *et al*., 1981) Parkinson's disease (Knott *et al*., 2000) and Crohn's disease (Beattie *et al*., 1995). ANXA1 is highly expressed in T cells from rheumatoid arthritis patients (D'Acquisto *et*

al., 2008) and was identified in lesions of multiple sclerosis plaque and correlated with the degree of disease (Probst-Cousin *et al*., 2002).

1.6.8. ANXA1 and neutrophils

Neutrophils (or PMN) are the most abundant circulating leukocytes in the human body. They are considered the first line of response and defence during pathological insult by bacteria and fungus. Reduced count of neutrophil during bacterial and fungal infection is known as neutropenia, and is considered a good indication of whether bacterial or fungal pathogenesis has already overwhelmed the host immune response. Neutrophils are armed with a myriad of antimicrobial agents such as reactive oxygen species (ROS) producing Neutrophil. The accumulation of neutrophils is one of the main pathological markers during lung injury or disease, chelators of vitamins and minerals, and also welds enzymes capable of degrading microbial proteins and cell wall components, but is detrimental to the neutrophil producing host tissues.

Immune-mediated damage is caused by uncontrolled or over-activated neutrophil activity, especially pronounced in autoimmune diseases (Németh and Mocsai, 2012). Neutrophils are major players during inflammation in lung injuries and insults (Opal, 2010; Da Cunha *et al.,* 2012). ANXA1 and neutrophil activity is closely related in many ways, and is most extensively studied among leukocytes. This is likely because ANXA1 constitutes close to 2-4% of protein found within the gelatinase granules in the cytoplasmic portion within neutrophils, and its expression is found to be lower in extravesated neutrophils (Perretti and Flower, 2004) They are also known to release ANXA1 along with gelatinase granules as exogenous ANXA1.

Ac2-26 plays a role in potent inhibition of PMN accumulation and degranulation process, and the release of free arachidonic acid, a biomarker of PMN activation (Perretti *et al.,* 1995). Treatment of GC DEX in vivo inhibits PMN accumulation at inflammatory sites via IL-1 signaling, but this anti-inflammatory effect can be abrogated completely by immunization of mice with specific antibodies against ANXA1. While the full length protein or the anti-inflammatory Ac2-26 mimetic peptide does not activate Erk, calpain-1 dependent, self-secreted form of ANXA1 induces ERK 1/2 activation in neutrophils (Williams *et al.,* 2010). Therefore the role of ANXA1 in neutrophils during inflammation may be dependent on the site of cleavage. ANXA1 can also act as a ligand to regulate neutrophil extravasation through binding with Formyl peptide receptors (FPR), which are widely known to be involved in binding and detecting bacterial N-formyl peptides (Walther *et al.,* 2000). ANXA1 was also demonstrated to bind strongly to lipoxin A(4) receptor of the FPR family, and can halt polymorphonuclear neutrophil (PMN) diapedesis (Peretti *et al.,* 2002). Monocytes express the receptors of all three FPR families which ANXA1 is capable of binding as an N-terminal peptide, which reduces the sensitivity of monocyte response to bacterial peptides (Ernst *et al.,* 2004). ANXA1 also appears to play a critical role in PMNs to modulate responses towards hormones. Premenopausal women exhibit higher expression of ANXA1 on the surface of PMNs than males. It was found that ANXA1 dependent mechanism was responsible for the effect of estrogen on PMNs (Nadkarni *et al.,* 2011).

Neutrophils are capable of developing membrane tubulovesicular extensions (TVE) which attaches to other cells and invading bacteria. The physiological significance of TVE has been recently understood to be bactericidal secretory in function.

Interestingly, disruption of TVE releases ANXA1. It is not known whether ANXA1 is part of the bactericidal mixture or is required for the formation of extensions such as TVEs (Galkina *et al.,* 2012).

1.6.9. ANXA1 in T-cells and dendritic cells

Dendritic cells (DC) and T-cells are the other key mediators of inflammation, which transfers innate immune response to the adaptive immune response. DCs are capable of priming a T-cell response, and T-cells are involved in producing a Th-1 or Th-2 response. Generally, a Th-1 response produces pro-inflammatory interferon-gamma while a Th-2 response produces IL-4, IL-5, IL-10 and IL-13, which results in an anti-inflammatory effect. Whether the immune response skews towards a pro- or anti-inflammatory response greatly depends on the signal of the immune cells. When T cells were exposed to exogenous ANXA1 peptide, it was found that ANXA1 is a novel regulator of T-cell receptor (TCR) signaling *via* FPRL-1, as it activates both ERK/MAPK and Akt/PKB pathways, causing an increase in T-cell proliferation, differentiation (CD25 and CD69 expression) and IL-2 production (D'Acquisto *et al.,* 2007).

ANXA1 KO bone marrow derived dendritic cells exhibit an increased number of $CD11c^{+}$ cells with high levels of maturation markers such as CD40, CD54 and CD80. The dendritic cells derived from ANXA1 KO mice also lost some of its antigen uptake capacity compared to the wild-type derived dendritic cells (Huggins *et al.,* 2009). ANXA1 KO derived dendritic cells exhibit a downregulation of maturation markers, decreased migratory activity *in vivo*, and decreased pro-inflammatory cytokines such as IL-1 β, TNF-α and IL-12 production when compared to wild-type derived dendritic cells

upon treatment with LPS. Furthermore, ANXA1 KO derived dendritic cells also exhibit impaired NF-κB/DNA binding activity, and substantially decreased ERK 1/2 and Akt pathway activation after treatment with LPS compared to wild-type derived dendritic cells. These results point to the importance of ANXA1 expression in the function of dendritic cells, and may indicate the relevance of ANXA1 in the modulation of adaptive immune response during pathogen-induced T-cell-driven immune diseases.

1.6.10. ANXA1 and macrophages

ANXA1 is expressed by different types of tissue-specific macrophages such as alveolar macrophages (Ambrose *et al.,* 1992), peritoneal macrophages (Peers *et al.,* 1993), synovial macrophages (Yang *et al.,* 1998) and microglial cells (Minghetti *et al.,* 1999). Macrophages express ANXA1 regardless of the tissue-specific nature of macrophages suggesting that ANXA1 plays a general role in macrophages and its expression is not a tissue-type specific phenotype (Kamal *et al.,* 2005).

Exogenous application of ANXA1 inhibits proliferation in RAW macrophage cells (Alldridge *et al.,* 1999). Clones with increased ANXA1 levels exhibit constitutive activation of MAPK/ERK pathway (Mansour *et al.,* 1994). p38 and JNK activities remained unchanged. ANXA1 may initiate such control through association with upstream adaptor protein Grb 2. When a membrane-permeable, plasmid-based expression of ANXA1 named Tat-ANX1 is expressed in Raw 264.7 cells, inflammatory cytokine and enzyme production are inhibited, and was found to be blocking both NF-κB and MAPK pathways (Lee *et al*., 2011). When ANXA1 expression is upregulated in RAW 264.7 macrophages and treated with LPS, upregulation of iNOS protein and activity was

observed (Smyth *et al.,* 2006). This indicates that ANXA1 can modulate NO levels during LPS treatment. Nitric oxide generation by macrophages is part of the innate immune system, and is triggered by TLR agonists such as LPS. Over-expression of inducible nitric oxide gene (iNOS) occurs early in sepsis (Lange *et al*., 2010; Yin *et al*., 2007). When ANXA1 is applied exogenously to J774 murine macrophages, nitric oxide generation by macrophages was inhibited, indicating that ANXA1 may play a role in nitric oxide synthesis (Ferlazzo *et al.,* 2003). Moroever, exogenous application of ANXA1 can also increase IL-10 mRNA levels while IL-12 mRNA levels decrease.

ANXA1 and its peptide derivatives can also affect macrophage phagocytosis of apoptotic cells (Scannell *et al.,* 2007), such as that of apoptotic neutrophils. Specifically, in ANXA1 KO macrophages, phagocytosis of zymosan, *Neisseria meningitides* or sheep red blood cells is significantly impaired when compared to wild-type macrophages with a concordant increase in IL-6 and TNF-α production (Yona *et al.,* 2006).

In response to LPS administration, ANXA1 KO bone marrow derived macrophages exhibit greater IL-6 and TNF-α production (Yang *et al.,* 2009). This presents a direct association between macrophage cytokine production and ANXA1 levels. ANXA1 KO macrophages produce more pro-inflammatory cytokines. Consistent with this result, an *in vivo* study which examined peritoneal, mesenteric and alveolar macrophages using experimental endotoxemia in ANXA1 KO mice also exhibited increased IL-6 and TNF-α production in blood serum after 24 hours when compared to wild-type mice (Damazo *et al.,* 2005).

The increased production of pro-inflammatory cytokines (IL-6, TNF-α and ERK activation) in ANXA1 KO macrophages is in direct contrast to decreased pro-

inflammatory cytokines (IL-6, TNF-α and ERK activation) in ANXA1 KO dendritic cells. Moreover, several studies mentioned earlier have demonstrated that decreased ANXA1 levels cause impaired phagocytic activity. It is unclear how ANXA1 modulates pro-inflammatory activity when exposed to TLR agonists.

Therefore the aims of this PhD thesis are:

1. To determine the TLR signaling mechanism which ANXA1 modulates when exposed to TLR agonists using peritoneal macrophages derived from mice

2. To characterise immune response of ANXA1 KO mice and monocyte derived macrophages after TLR agonist activation.

3. To identify the key components in TLR signaling mechanism which ANXA1 regulates directly, and propose an overarching theme that characterises macrophages in ANXA1 KO mice.

CHAPTER II:

MATERIALS AND METHODS

2.1 Materials

2.1.1. Animals

All experimental procedures were approved by the Animal Ethics Committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research and with the guidelines of the National Advisory Committee for Laboratory Animal Research (NACLAR), Singapore.Male and female BALB/c mice (8 to 12 weeks-old) were fed with standard laboratory chow and water *ad libitum*. The protocol (Protocol number 011/08) was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore.

2.1.2. Media and buffers

2.1.2.1. PBS

PBS buffer was commercially obtained from 1st base (Singapore) as a 10X stock, ultrapure grade. The 10X stock consists of 137mM NaCl, 2.7mM KCl and 10mM phosphate buffer. The stock was diluted 9 parts with sterile water to give 1X PBS. pH of the buffer was 7.4.

2.1.2.2. FACS buffer

FACS buffer consists of 1X PBS supplemented with 1% FBS and 5mM EDTA. 0.05% sodium azide was added as a preservative to permit use

and storage under nonsterile conditions. The pH of the buffer was adjusted to 7.4.

2.1.2.3. Red Blood Cell (RBC) Lysis Buffer

The following components were added to make a 10X RBC lysis stock solution

a) Ammonium Chloride 8.3g

b) Sodium Bicarbonate 0.84g

c) EDTA 0.03g

The components were dissolved in water, the pH adjusted to 7.4, filter sterilised and made up to 100ml. For use as a 1X solution, the 10X stock solution was diluted with water.

2.1.2.4. Wash buffer for Western Blotting (TBST)

Wash buffer for Western Blotting was 1X Tris-Buffered Saline (TBS) with 0.5% v/v Tween-20, pH adjusted to 7.2 to 7.4.

2.1.2.5. Buffer for ELISA

Wash buffer for ELISA was 1X PBS with 0.5% v/v Tween-20, pH adjusted to 7.2 to 7.4. Blocking buffer was 1X PBS with 1% w/v BSA, unless or otherwise stated by the ELISA kit.

2.1.2.6. Complete DMEM for cell culture

Complete DMEM was prepared using Dulbecco's Modified Eagle Medium with L-Glutamine supplemented with 10% v/v FBS, heat inactivated for 30 mins at 55^{o}C.

2.1.2.7. Complete DMEM for Bone Marrow Macrophages

Complete DMEM was prepared using Dulbecco's Modified Eagle Medium supplemented with 10% v/v FBS, heat inactivated for 30 mins at 55^{o}C, 20% v/v L929 conditioned media, and 50μM of β-Mercaptotethanol with L-Glutamine

2.1.2.8. L929 Conditioned Media

L929 cells are maintained in T-75 filter cap flasks in DMEM + 1% penstrep + 10% FBS at 7.5% CO_2 incubator. The FBS used was the same as the one used to cultivate bone marrow derived macrophages.

Cells were split from 1 T-75 flask into 5 T-75 flasks when it was more than 90% confluent, using trypsin. The culture was incubated in CO_2 incubator overnight, until suitable confluency was reached. When the 5 T-75 flasks were more than 90% confluent, the cells trypsinised, and seeded equally into 13 T-175 flasks, each with 50 ml of DMEM media with 10% FBS. When these 13 T-175 flasks were also confluent the cells were incubated for an additional two days to condition the media. The supernatant of all 13 T-175 flaskswere filtered to remove all trace of cells from the supernatant. The filtered supernatant was then aliquoted into 50 ml Falcon tubes and stored in -20 ^{o}C freezer until use.

2.1.3. Reagents

The following reagents were used in this study:

Reagent	Company	Reagent Type
Phosphate buffer saline (PBS)	1st Base (Singapore)	Buffer
Tris-buffered saline (TBS)	1st Base (Singapore)	Buffer
Fetal bovine serum (FBS)	Hyclone (Logan, UT, USA)	Cell culture additive
L- glutamine	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Cell culture additive
Penicillin-streptomycin (Pen-strep)	Gibco®-Invitrogen (Carksbad, CA, U.S.A.)	Cell culture additive
Sodium pyruvate	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Cell culture additive
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Cell culture media
Trypsin	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Cell detachment
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Chelating agent
Bovine serum albumin (BSA)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Confocal blocking buffer
Triton-X		Detergent
Tween-20		Detergent
Thioglycollate broth, 4%	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Elicitation of macrophages for peritoneal lavage
Formalin solution, neutral buffered, 10%	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Fixative
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Florescent probe
1M Nitrile Standard (0.1M sodium nitrite in water)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Griess reagent
NED Solution (0.1% N-1-napthylethylenediamine dihydrochloride in water)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Griess reagent
Sulfanilamide Solution (1% sulfanilamide in 5% phosphoric acid)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Griess reagent
BAY-11 (BAY-11-7082)	Cayman Chemicals	Inhibitor drug

	(Ann Arbor, MI, U.S.A.)	
ProLong® Gold Antifade Reagent	Invitrogen (Carksbad, CA, U.S.A.)	Mounting media for confocal microscopy
Prolong Gold Antifade Reagent	Molecular Probes, Invitrogen (Carksbad, CA, U.S.A.)	Mounting media for microscopy
100 bp DNA ladder	Promega (Fitchburg, WI, U.S.A)	PCR product quantification reagent
25 mM MgCl2	Promega (Fitchburg, WI, U.S.A)	PCR product quantification reagent
GoTaq® Flexi DNA Polymerase	Promega (Fitchburg, WI, U.S.A)	PCR product quantification reagent
GoTaq® Flexi DNA Polymerase Buffer	Promega (Fitchburg, WI, U.S.A)	PCR product quantification reagent
15-deoxy-Δ12,14-Prostaglandin J2 (PGJ2)	Cayman Chemicals (Ann Arbor, MI, U.S.A.)	PPAR-γ agonist
Troglitazone	Selleck Chemicals (Houston, TX, U.S.A.)	PPAR-γ agonist
GW9662	Cayman Chemicals (Ann Arbor, MI, U.S.A.)	PPAR-γ antagonist
PCR Primers (Real-time and RT-PCR)	1st Base (Singapore)	Primer DNA
Bradford protein assay dye reagent	Bio-Rad (Hercules, CA, U.S.A.)	Protein sample quantification
BSA Standards	Thermo-Scientific (Hudson, NH, U.S.A.)	Protein sample quantification
SYBR Green Master Mix	Applied Biosystems (Foster City, CA, U.S.A.)	Real-time PCR reagent
β-Mercaptotethanol	Merck (Whitehouse Station, NJ, U.S.A.)	Reducing agent
10mM dNTP mix	Promega (Fitchburg, WI, U.S.A)	Reverse transcription reagent
5X M-MLV reverse transcriptase buffer	Promega (Fitchburg, WI, U.S.A)	Reverse transcription reagent
M-MLV reverse transcriptase enzyme	Promega (Fitchburg, WI, U.S.A)	Reverse transcription reagent
Oligo dT	Promega (Fitchburg, WI, U.S.A)	Reverse transcription reagent
RNasin Plus RNase inhibitor	Promega (Fitchburg, WI, U.S.A)	Reverse transcription reagent
Chloroform	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	RNA extraction
Ethanol	Merck (Whitehouse Station, NJ)	RNA extraction

Isopropanol	QRëc (New Zealand)	RNA extraction
TRIZOL®	Invitrogen (Carksbad, CA, U.S.A.)	RNA extraction
Ammonium Chloride	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Salt
Sodium Bicarbonate	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	Salt
Methanol	Merck (Whitehouse Station, NJ)	Solvent
CpG 1826 oligodeoxynucleotide	InvivoGen (San Diego, CA, U.S.A.)	TLR agonist
Lipopolysaccharide (LPS)	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	TLR agonist
Poly (I:C)	InvivoGen (San Diego, CA, U.S.A.)	TLR agonist
Zymosan	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	TLR agonist
Zymosan A *Saccharomyces cerevisiae*	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)	TLR agonist
Nitrocellulose membranes	Bio-Rad (Hercules, CA, U.S.A.)	Western blotting
SuperSignal West Pico Chemiluminescent Substrate	Thermo-Scientific (Hudson, NH, U.S.A.)	Western blotting
CL-XPosure Film	Thermo-Scientific (Hudson, NH, U.S.A.)	X-ray film for Western Blotting

Table 1: List of reagents used in this study.

Note: 15-deoxy-Δ12,14-Prostaglandin J2 (PGJ_2) and Troglitazone were kindly provided by Dr Alan Premkumar's lab.

2.1.4. Antibodies

The following antibodies were used in this study:

Name of antibody	Used for	Company
Rabbit anti- IRF-3	Immunofluorescence staining for confocal studies	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.)
Rabbit anti- NF-κB	Immunofluorescence	Cell Signaling

	staining for confocal studies	Techonlogy Inc (Danvers, MA, U.S.A)
Rabbit anti- STAT-1	Western Blotting	Cell Signaling Techonlogy Inc (Danvers, MA, U.S.A)
Rabbit anti- phospho STAT-1 (Tyr701)	Western Blotting	Cell Signaling Techonlogy Inc (Danvers, MA, U.S.A)
Rabbit anti-RIG-I	Immunofluorescence staining for Duolink®	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.)
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	Western Blotting	Sigma-Aldrich Co (St. Louis, MO, U.S.A.)
Alexaflor 488 anti- rabbit IgG	Immunofluorescence staining for confocal studies	Invitrogen (Carksbad, CA, U.S.A.)
Alexaflor 633 anti- rabbit IgG	Immunofluorescence staining for confocal studies	Invitrogen (Carksbad, CA, U.S.A.)

Table 2: List of antibodies used in this study.

2.1.5. ELISA kits

The following ELISA kits were used in this study:

Name of ELISA kit	Cross-reactivity	Company
Murine IL-12 Standard ELISA Development Kit	Mouse IL-12 p40, IL-23 p40, IL-12 p70	Peprotech Inc. (Rocky Hill, NJ, U.S.A)
Mouse TNF-α ELISA Ready-Set-Go!®	No significant cross-reactivity, including species cross-reactivity	Ebioscience Inc. (San Diego, CA, U.S.A)
Mouse IFN-α ELISA Ready-Set-Go!®	No significant cross-reactivity, including species cross-reactivity	Ebioscience Inc. (San Diego, CA, U.S.A)
Mouse IFN-β ELISA Ready-Set-Go!®	No significant cross-reactivity, including species cross-reactivity	Ebioscience Inc. (San Diego, CA, U.S.A)
Mouse IFN-γ ELISA Ready-Set-Go!®	No significant cross-reactivity, including species cross-	Ebioscience Inc. (San Diego, CA, U.S.A)

	reactivity	
Mouse IL-6 ELISA MAX™ Standard	Rat IL-6	BioLegend, Inc. (San Diego, CA, U.S.A)
Quantikine ® ELISA Mouse CXCL10/IP-10 Immunoassay	No significant cross-reactivity, including species cross-reactivity	R&D Systems (Minneapolis, MN, U.S.A)

Table 3: List of ELISA kits used for this study

2.1.6. Primers

The following primers were used in this study:

Primer Name	Sequence	Company
Mouse IL-6 (Forward)	5'-GGG ACT GAT GCT GGT GAC AA-3'	1st Base (Singapore)
Mouse IL-6 (Reverse)	5'-TCC ACG ATT TCC CAG AGA ACA-3'	1st Base (Singapore)
Mouse IL-12 p40 (Forward)	5'-GGA AGC ACG GCA GCA GAA TA-3'	1st Base (Singapore)
Mouse IL-12 p40 (Reverse)	5'-AAC TTG AGG GAG AAG TAG GAA TGG-3'	1st Base (Singapore)
Mouse β-actin (Forward)	5'-CTT AGG AA TGC CTC TGG GAG GTC C-3'	1st Base (Singapore)
Mouse β-actin (Reverse)	5'-GCA GAC GCG AGG AAG GAG-3'	1st Base (Singapore)
Mouse FIZZ1 (Forward)	5'-GGC CCA TCT GTT CAT AGT CT-3'	1st Base (Singapore)
Mouse FIZZ1 (Reverse)	5'-CCC AGG ATG CCA ACT TTG AA-3'	1st Base (Singapore)
Mouse Arginase-1 (Forward)	5'-GCT GTC TTC CCA AGA GTT GGG-3'	1st Base (Singapore)
Mouse Arginase-1 (Reverse)	5'-ATG GAA GAG ACC TTC AGC TAC-3'	1st Base (Singapore)
Mouse TNF-α (Forward)	5'-GGC AAG GAT GAG CCT TTT AGG-3'	1st Base (Singapore)
Mouse TNF-	5'-TTG GTT TGG GAG GAA AGG G-	1st Base

α (Reverse)	3’	(Singapore)
Mouse IP-10 (Forward)	5’-GGA CGG TCC GCT GCA A-3’	1st Base (Singapore)
Mouse IP-10 (Reverse)	5’-GCT TCC CTA TGG CCC TCA TT-3’	1st Base (Singapore)

Table 4: List of primers used in this study

2.2. Methods

2.2.1. Animal Derived Cell Techniques

2.2.1.1 Macrophage Recruitment using Thioglycollate

Mice were injected with 3 ml of 4% thioglycollate broth directly into the intraperitoneal cavity using a 27 gauge needle. The mice were harvested after three days for macrophages in peritoneal cavity by peritoneal lavage.

2.2.1.2. Peritoneal lavage

Peritoneal lavage was done on mice with the intent of flushing out peritoneal fluid from the mice. Euthanized mice were first sterilized in a beaker with 70% ethanol, then placed in the tissue culture hood immediately. Small incision was made to expose intraperitoneal cavity. Ice cold 1X PBS (5 ml) was injected into the mouse peritoneal cavity in the lower abdominal area to wash (lavage) the intraperitoneal cavity with PBS. Scissor was used to create a small opening in the peritoneal cavity for the extraction of peritoneal fluid using sterile pasteur pipettes. The cell suspension collected was then pooled with three other mice with the same genotype and experimental conditions before cell count was done. For peritoneal macrophages cell count, cells were first plated

for at least 4 h and only adherent cells were considered peritoneal macrophages.

2.2.1.3. Splenic B Cell isolation

Splenic cells were obtained by mashing the spleens over 70uM mesh filters . The single cell suspension obtained spun at 3,000 rpm for 5 min and the cell pellet was resuspended at a concentration of 1 x 10^8 cells/ml. EasySep Mouse B Cell Enrichment Kit (Stemcell Technologies, Vancouver, B.C.) was used thereafter, according to manufacturer's protocol.

2.2.1.4 Bone Marrow Derived Macrophages

8-12 week old mice are euthanized and hind limbs (femur and tibia) were removed and placed into a small petri dish with sterile tweezers. Muscles attached to bones were removed using a guaze and the bones were transferred to a second petri dish containing 70% ethanol for one minute and then transferred to a third petri dish containing DMEM media. The two ends of each bones were cut just off at the joint to expose the bone marrow while minimizing loss. The bone marrow was flushed out into a 50 ml Falcon tube by inserting a 26-gauge needle attached to a 5 ml syringe filled with DMEM media at the ends of the cut bone. Flushing was done until the bone turns from red to white, indicating that all the marrow has been expelled. The collected bone marrow suspended in DMEM media was then passed through a 70 um cell strainer (BD, Franklin Lakes, NJ, U.S.A.), and then 300g for 10mins at 4degrees Cell pellet collected was then resuspended in 1 ml of red blood cell lysis buffer and incubated on ice for 5 min, and then diluted with

approximately 6 ml of DMEM media before centrifuging at 2,000 rpm for 10 min. The collected cell pellet was white as there werr no red blood cells present in the cell pellet. The cells are then counted and seeded onto either 24-well plate or 10 cm plate with L929 conditioned media (L cell media), and incubated for seven days. In between the seven days, on the third day, an additional and equivolume of L929 conditioned media was added to the tissue culture plate. On the seventh day of incubation, the cells are ready for experiment after 1X wash with plain DMEM media. 70% of the Cells derived from this technique have been shown to be $F4/80^{+}$,which is a macrophage specific surface marker as evaluated by flow cytometry, on the seventh day.

2.2.3. Cell culture techniques

2.2.3.1. Cell culture

All cell cultures were done in 37 ^{o}C incubator with CO_2 injection maintained at 5% in a CO_2 cell culture incubator (Thermo Scientific Steri-Cycle® CO2 incubator Model 381). Cells were observed for changes in morphology, adhesion to cell culture surface and confluency every day.

2.2.3.2. Trypsinization

Flask or 10 cm plates with strongly adherent cells are ideal for trypsinization. First, supernatant was aspirated and the surface (flask or 10 cm plate) was washed once with 1X PBS. Thereafter, 1~3ml of 1X trypsin was added to the surface of adherent cells. The surface (flask or 10 cm plate) was then placed in 37 ^{o}C incubator for one min, and then tapped lightly on the side of the surface to dislodge the cells from surface. Full media (e.g. DMEM with

10% FBS added) was added at 1:1 volume of trypsin to neutralize trypsinization. Cells are then spun down at 3000 rpm for 5 mins and supernatant was removed before suspending the pellet with the appropriate media for the next batch of cell culture.

2.2.4. Bacterial co-culture studies

Cells were co-cultured with log-phase culture of *E. coli* at the required bacteria-to-cell ratio in antibiotics-free medium using optical densitometry. Mid-log phase bacteria were prepared by inoculating 250 µl overnight culture into 5 ml Luria-Bertani (LB) broth and allowed to grow for 2 h with constant agitation. Bacteria and cells were both incubated for 24 hours and supernatant was recovered by filtering through 0.25 um sterile filters.

2.2.5. Cell stimulation

TLR agonists used in the experiments and their concentrations, unless stated otherwise are: LPS (1 µg/ml), CpG 1826 (1 uM), poly I:C (50 ug/ml). Treatment condition for macrophage polarization to M1 (20 ng/ml of IL-4 with 100 ng/ml of LPS), were based on macrophage polarization studies done by Mantovani *et al*., 2004. Macrophages were polarized for at least 18 hours before treatment with LPS or IFN-γ.

2.2.6. Microscopy

2.2.6.1. Confocal microscopy

Cells for confocal study were seeded on round coverslips placed within wells of a 24-well plate. After completion of experimental treatment, wells containing cells adherent on round coverslips were washed twice with 1X PBS and suspended in 200 ul of 10% neutral buffered formalin at 4^{o}C for 30 min. Staining of samples for confocal were done in the following manner:

On a new 24 well tissue culture plate, 500 ul of 1X PBS was added onto the same number of wells as the number of coverslips to stain for confocal, and one coverslip was placed into one well with 500 ul 1X PBS. The 1X PBS was aspirated using a glass micropipette attached to a vacuum pump and 50 mM of ammonium chloride solution made with 1X PBS was added onto each well, and shaken in belly shaker for 2 min at 90 rpm. Thereafter the well was washed with 1X PBS twice for 2 min to remove any trace of ammonium chloride. Next, the sample was placed in 1X PBS solution with 0.5% Triton-X, and shaken in belly shaker for 30 min at 90 rpm.

The solution was then aspirated and replaced with 500 ul of blocking buffer (2% BSA, 2% FBS in 1X PBS, passed through 20μm sterile filter) and placed on belly shaker for 30 min at 90 rpm. The blocking buffer was aspirated and primary antibody (1:200 dilution in blocking buffer) was added on the coverslip by taking out the coverslip and invert it over the cover of 24 well plate dropped with 40 ul of primary antibody. The 24 well plate cover was wrapped in aluminium foil and placed into a 37^{o}C incubator for 45 min. Next, the coverslips are inverted and placed back into the 24-well plate well as

before, into 500 ul of 1X PBS with 0.2% Trion-X, and placed on a belly shaker for 2 min at 90 rpm to wash. The wash was repeated twice. The coverslips are again taken out, inverted onto a 40 ul drop of secondary antibody (1:1000 dilution in blocking buffer) on a clean spot of 24 well plate cover. It was then wrapped in aluminium foil and incubated at room temperature on a belly shaker for 45 min at 90 rpm.

The coverslips are again inverted and placed back into the 24-well plate well as before, into 500ul of 1X PBS with 0.2% Trion-X, and placed on a belly shaker for 2 min at 90 rpm to wash. Lastly, 500ul of DAPI dissolved in 100% methanol to a final concentration of 0.5ug/ml was added for 1 min, then washed with 1X PBS twice for 2 min on belly shaker at 90 rpm to remove any trace of excess DAPI and methanol. The coverslips are then removed, mounted onto glass slides using Prolong Gold Antifade Reagent (Molecular Probes, Invitrogen, USA) and viewed using using Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Germany). Images were processed using Leica confocal LAS AF software (Leica Microsystems GmbH, Germany).

2.2.6.2. Fluorescence microscopy

All slides were mounted in Prolong Gold Antifade Reagent (Molecular Probes, Invitrogen, USA) and viewed using Carl Zeiss Axio imager.Z1 fluorescent microscope (Axiocam HRM camera; Carl Zeiss Micro Imaging, Germany). Images were processed using Axiovision Rel 4.8 software (Carl Zeiss Micro Imaging, Germany).

2.2.7. Protein and Molecular Biology Techniques

2.2.7.1. Bradford assay

5 µl of protein albumin standards with known protein concentrations or 1 µl of protein sample was added to 150 µl 1X Bradford Assay Solution. Absorbance was measured at a wavelength of 595 nm and the absorbance values of the protein standards were plotted to obtain a standard curve. Absorbance of protein samples were then read from the standard curve to determine the amount of protein within the sample.

2.2.7.2. Western Blotting

Treated cells were harvested at indicated times, washed once in ice-cold PBS and scraped off the culture plates in a lysis buffer containing 75mM NaCl, 10mM Tris (HCl), 1mM EDTA, 0.5% NP40 and protease inhibitor cocktail (Pierce Biotechnology). Cells were then incubated on ice for 15 minutes before being centrifuged at 14000 rpm for 15 minutes at 4°C. The supernatant was collected and protein concentration was determined *by* Bradford Assay. 50 µg of protein per sample was loaded onto 15% SDS-PAGE gel placed at a phased voltage of 75V for 15 min and 125 V for 60 min using a gel electrophoresis cell (Mini-PROTEAN® Tetra Cell, Bio-Rad, Hercules, CA, U.S.A.). The proteins were then transferred onto nitrocellulose membranes using a wet transfer apparatus (Criterion Blotter, Bio-Rad, Hercules, CA, U.S.A.).

Following transfer, the membranes were washed with distilled water to remove traces of transfer buffer and then air-dried for several hours to allow

for firm binding of proteins to membranes. The membranes were rewetted with 1X TBS and blocked with 5% w/v milk proteins in 1X wash buffer for western blot (TBST) for 1 hr. Membranes were then washed for 15 min with 1X TBST before incubation with the appropriate primary antibody (see table below) at either 37^{o}C for 1 h or 4^{o}C overnight. Membranes were next washed thrice with 1X TBST for a total of 30 min before incubation with secondary antibodies (see table below) conjugated to horseradish peroxidase for 1 hr (1:5000 dilution). Three washes lasting 10 minutes each were carried out prior to the addition of the SuperSignal West Pico Chemiluminescent Substrate. Blots were then exposed to CL-XPosure X-ray films until bands were clearly seen after film development.

To detect phosphorylated proteins, the same blots were probed with phospho-specific antibodies, stripped and reprobed with antibodies against total proteins. All blots were incubated with mouse antibodies against β-actin to confirm equal protein loading.

2.2.7.3. RNA extraction

For RT-PCR analysis of tissue chemokine mRNA expression, total RNA was extracted from the pancreas and lungs using the TRIzol reagent following the manufacturer's instructions with some modifications. Briefly, pancreas and lung tissues were quickly harvested and homogenized in TRIzol reagent. Chloroform (200ul/ml of TRIzol) was then added to the homogenates, and samples were shaken, incubated for 5 min at 4 °C and centrifuged for 15 min at 12,000 g at 4°C. The aqueous phase was separated and RNA was precipitated by adding isopropyl alcohol. After RNA was pelleted by

centrifugation (12,000 g for 10 min at 4°C), the pellet was washed twice in 70% ethanol, air-dried and dissolved in RNase-free water. RNA was quantitated spectrophotometrically by measuring absorbance at 260 nm (A260). The purity of RNA was assessed by an A260/A280 ratio between 1.6 and 2.0.

2.2.7.4. RT-PCR

Total RNA (1 µg) was used for cDNA synthesis through reverse transcription. Primer-cDNA mix (see table below) was incubated at 70 ^{o}C for 5 min and then on ice for 1 min. Master mix made with the following, for each reaction: 2 µl of 5x M-MLV reverse transcriptase buffer, 4 µl of 25mM $MgCl_2$, 0.125 µl of RNasin Plus RNase inhibitor and 1 µl of M-MLV reverse transcriptase enzyme (200 reaction units). Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min. The tubes are then incubated at 42 ^{o}C for 50 min and then heat inactivate the reverse transcriptase at 70 ^{o}C for 10 min, and then chill on ice. The final product (1st strand cDNA) was used immediately or stored at -20 ^{o}C for 15 min. For the subsequent PCR amplifcation, the PCR amplification mix containing the following reagents are added:

Component	**Amount (µl)**
First strand cDNA reaction	1
10 mM dNTP mix	1
25 mM $MgCl_2$	2
5X GoTaq® Flexi DNA Polymerase Buffer	5
GoTaq® Flexi DNA Polymerase	11

Forward Primer	1
Reverse Primer	1
DEPC Water	28
Total	50

2.2.7.5. Realtime PCR

Real time PCR was performed on an ABI7500 Real-time PCR system using SYBR Green (Applied Biosystems, USA). The relative quantities of target gene expression were quantified by comparative CT method and normalised to GAPDH.

2.2.8. Analysis techniques

2.2.8.1. Flow cytometry

Cells for FACS staining were transferred into FACS tube and pelleted by centrifugation at 400 g for 5 mins. Tubes were inverted to discard the supernatant and were further blotted on tissue paper to remove the excess supernatant that collected near the edge of the tube after inversion. The cell pellet was then disrupted by raking on tube racks. Fc Block (2.4G2) was added at 0.2 μg per million cells and incubated for 5 mins at 4^{o}C to prevent non-specific binding of antibodies to Fc receptors. Antibodies directly conjugated to fluorophores were added to the cells at 0.02 μg per million cells and incubated in the dark at 4^{o}C for 30 mins. After incubation, cells were washed twice with at least 1 ml of FACS buffer to remove unbound antibodies. Cells

were resuspended in 350 µl of FACS buffer if analysis was performed within several hours or fixed with 1% PFA if analysed on a separate occasion. To fix with 1% PFA, the cells were pelleted and the FACS buffer supernatant discarded. To prevent the formation of cell clumps during fixation, the cell pellet was well disrupted by raking several times on tube racks and vortexed at 3000rpm while simultaneously adding 1% PFA.

2.2.8.2. Nitric oxide (NO) assay (Griess assay)

Nitric oxide formation can be measured using the diazotization reaction originally described by Griess in 1879 (Griess, 1879).

1 ml of 100 uM nitrile solution was made from 1 M Nitrile Standard stock solution by 1:10,000 dilution in DMEM. Three columns in a 96-well plate were reserved for nitrile standard reference curve. These wells were filled with 50 ul of DMEM. On the topmost rows of these three columns, 100 µl of 100 µM nitrile solution was added. Immediately thereafter, 6 serial, twofold dilutions (50 ul/well) in triplicate down the plate was made to generate the nitrile standard reference curve (100, 50, 25, 12.5, 6.25, 3.13, 1.56 uM). The last set of wells are left as blanks (0 uM). Next, the Sulfanilamide solution and NED solution was left to equilibrate to room temperature for about fifteen minutes. Thereafter 50 ul of each experimental sample was added to wells in triplicates. Using a multichannel pipettor, 50 ul of sulphanilamide solution was dispensed to all experimental samples and wells containing the nitrile standard reference curve. The mixture was incubated at room temperature away from light for 5-10 minutes. Using a multichannel pipettor, 50 ul of NED solution was added to all wells, and

incubated for another 5-10 mins away from light source. A purple/ magenta colour is formed immediately, and the resultant coloured azo compound has an absorbance which is measured at 550 nm using a microplate reader (Perkin Elmer Victor 3 Model 1420-012).

2.3. Statistical analysis

Each experiment was performed at least 3 times. Individual groups were compared using the Student's t test with a two-tailed *p*-value. A value of $P < 0.05$ was taken as significant. If a parameter is known to decrease or increase under certain stimuli, one tailed student's t test was used.

CHAPTER III:

ROLE OF ANXA1 IN INNATE IMMUNITY

The pathogen associated molecular pattern (PAMP) response is based primarily on toll-like receptor (TLR) stimulation by pathogen associated molecules. ANXA1 deficient (KO) mice are physiologically indistinguishable from its wild-type (WT) counterparts, yet its immune system exhibits an inclination towards Th2 differentiation even without exposure to stimuli (D'Aquisto *et al*., 2007) and is also particularly sensitive to immune stimuli such as LPS, whose exposure can cause death in mice within 48 hours (Damazo *et al*., 2005). In recent studies from our group and others, NF-κB was found to be regulated by ANXA1- either directly by binding to the p65 subunit of NF-κB (Zhang *et al*., 2010) or indirectly through the regulation of NEMO (Bist *et al*., 2011). Furthermore, ANXA1 KO thymocytes and T cells exhibit impaired activation of NF-κB (Paschalidis *et al*., 2010, D'Acquisto *et al*., 2008).

This led us to believe that ANXA1 may control inflammatory cytokine production through NF-κB. Huggins and colleagues (2009) reported that bone-marrow derived dendritic cells from ANXA1 KO mice exhibit a matured phenotype but lower production of pro-inflammatory cytokines such as IL-1, TNF-α and IL-12 after LPS treatment. However, Yang and colleagues (2009) reported that ANXA1 KO macrophages produce higher levels of pro-inflammatory cytokines (IL-6, TNF-α and ERK activation) compared to WT mice after LPS treatment. Taken together, it became unclear whether ANXA1 affects TLR-dependent cytokine production, and no underlying mechanism has been proposed for the discrepancy in cytokine production with respect to WT. Moreover, there are no studies that specifically characterize the role of ANXA1 in MyD88 dependent or independent activation of the TLR pathway.

There are two distinct and mechanistically segregated signaling pathways in TLR dependent activation (Yamamoto *et al*., 2003). This study investigated TLR agonists that either stimulate both NF-κB dependent and TRIF dependent TLR pathways (i.e. LPS to stimulate TLR4 and CpG DNA to stimulate TLR9) or specifically stimulate the TRIF dependent pathway only (poly (I:C) to stimulate TLR3). In order to dissect the signaling mechanism responsible for the differential immune response in ANXA1 KO macrophages, we investigated whether ANXA1 is involved in modulating MyD88/NFκB dependent or MyD88-independent/TRIF activation.

3.1 Role of ANXA1 in inflammatory cytokine production in response to TLR9 and TLR4 agonists

Our study focused on cytokine production in macrophages, as macrophages are major cytokine producing lymphocytes in the initial stages of pathogenic insult. In order to determine the cytokine response of peritoneal macrophages (PM) after exposure to the most common pathogens such as viruses, bacteria and fungi, we used specific agonists to stimulate TLRs.

To validate our experimental set-up, WT PM which were elicited by thioglycollate were first harvested, pooled (n=4 mice per experiment), quantified, and then seeded in equal quantities (1 million cells / ml) in a 24-well plate. Thereafter, various TLR agonists were added to the PM, and cytokine/chemokine production was quantified by ELISA after 24h.

Significant IL-6 production was observed after treatment with CpG 1826 or LPS. Poly (I:C) did not cause noticeable IL-6 production. A significant production of IL-12 was observed after exposure to LPS, poly (I:C) or CpG DNA (CpG 1826) (Figure 3.1a). Based on these results, we demonstrate that the TLR agonists CpG 1826, LPS and poly (I:C) induce significant IL-12 production in our model.

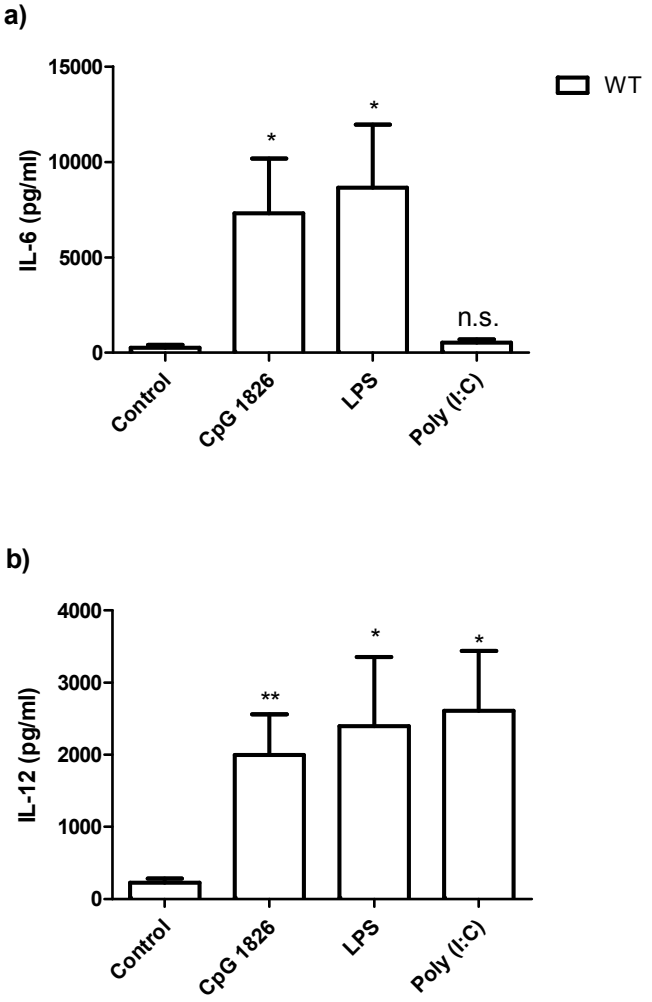


Figure 3.1. a) IL-12, b) IL-6 production in WT PM after treatment with TLR agonists (1 million cells/ml). * denotes $p<0.05$, ** denotes $p<0.01$ and n.s. denotes "no significant difference", i.e. $p>0.05$, compared to WT, thioglycollate elicited PM. Results are based on five biological repeats (mean ± S.E.M. of n=5).

3.1.1 IL-12 and IL-6 production in response to TLR9 agonist (CpG 1826)

In this experiment, the role of ANXA1 in PM activation by the TLR9 agonist CpG 1826 was investigated as CpG DNA stimulates the MyD88 dependent pathway. IL-12 and IL-6 production was compared between WT and ANXA1 KO PM before and after CPG 1826 stimulation (Figure 3.2).

After treatment with CpG 1826, IL-12 was produced in both WT and ANXA1 KO PM but no significance difference in their levels was observed. Similarly, when treated with CpG 1826, IL-6 was produced in both WT and ANXA1 KO PM with no significant difference between them (p=0.4604).

Based on this data, ANXA1 does not regulate IL-6 or IL-12 production after the activation of the MyD88-specific, TLR9 pathway.

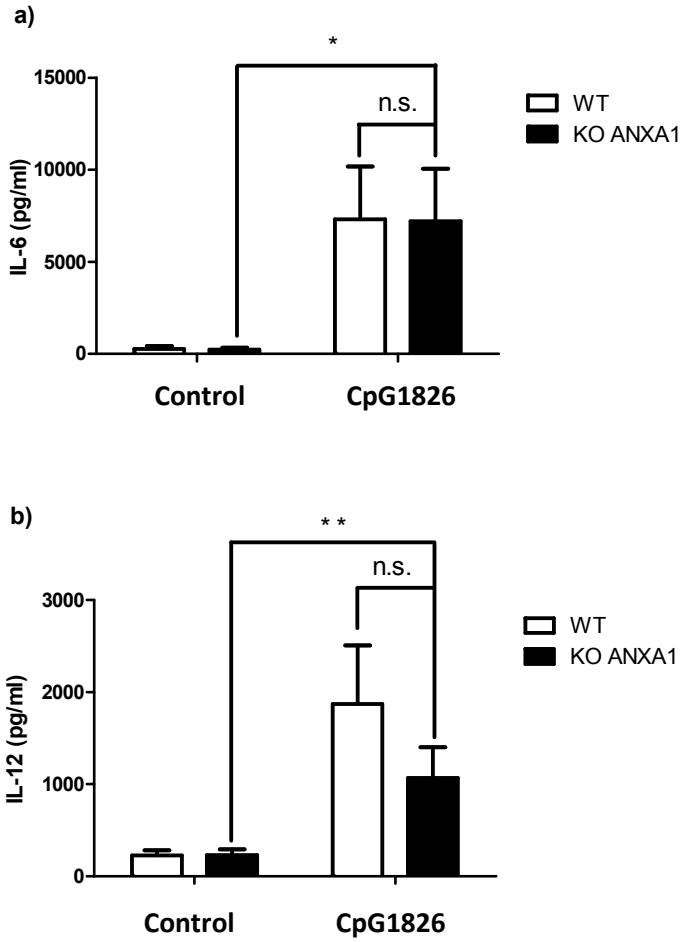


Figure 3.2. a) IL-6 and b) IL-12 production in WT and ANXA1 KO PM after treatment with TLR agonist CpG1826 (1 million cells/ml). ** denotes $p<0.01$ and n.s. denotes "no significant difference", i.e. $p>0.05$, compared to WT, thioglycollate elicited PM. Results are based on five biological repeats (mean ± S.E.M. of n=5).

3.1.2 IL-12 and IL-6 production in response to TLR4 agonist (LPS)

LPS—a TLR2 and TLR4 agonist that triggers both MyD88-dependent and independent signaling—was next used to investigate the role of ANXA1 in TLR signaling (Figure 3.3).

Consistent with WT PM, ANXA1 KO PM displayed significantly increased IL-12 production after LPS treatment when compared to untreated ANXA1 KO PM. However, ANXA1 KO PM produced less IL-12 than WT PM after LPS stimulation (p=0.0014). Following LPS exposure, a significant increase in IL-6 production was observed in both WT and ANXA1 KO PM treated with LPS; however, the difference in IL-6 production between WT and ANXA1 KO PM after treatment with LPS is not significant (p=0.4729). This suggests that ANXA1 does not directly affect the production of IL-6 through the activation of the TLR-3 and TLR-4 pathways.

Based on this result, the production of IL-12 but not IL-6 is impaired in the absence of ANXA1 in response to LPS.

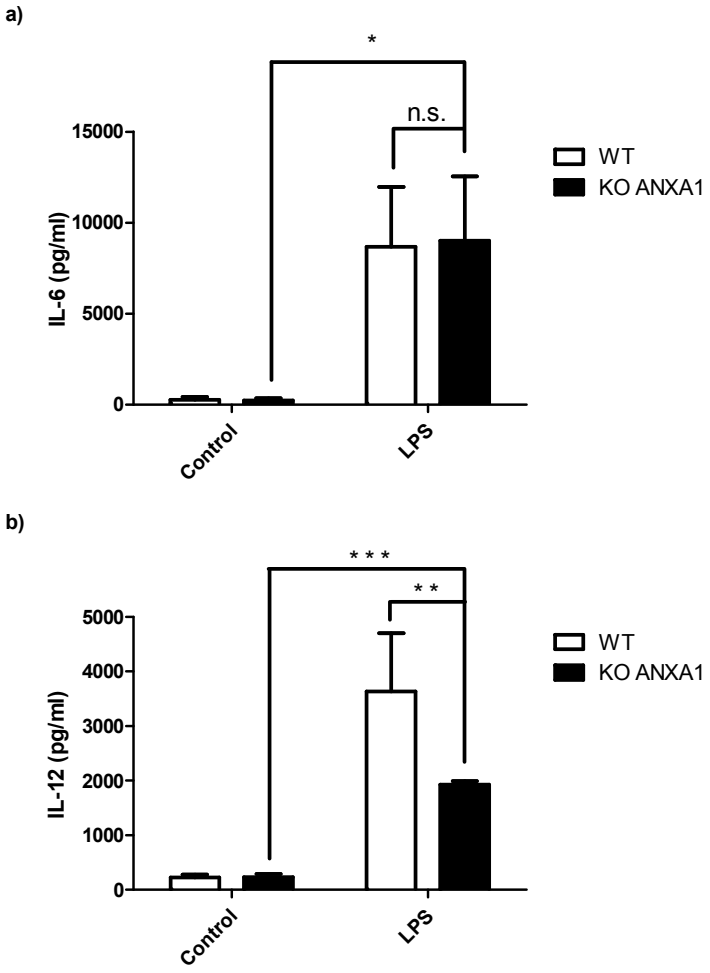


Figure 3.3. a) IL-6 and b) IL-12 production in WT and ANXA1 KO PM after treatment with TLR agonist LPS (1 million cells/ml). * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$ and n.s. denotes "no significant difference", i.e. $p>0.05$, compared to WT, thioglycollate elicited PM. Results are based on five biological repeats (mean ± S.E.M. of n=5).

3.1.3 IL-12 and IL-6 production in response to TLR3 agonist (poly (I:C)

ANXA1 did not regulate IL-6 and IL-12 production after CpG1826 activation, yet IL-12 production was affected after LPS stimulation in ANXA1 KO macrophages. CpG1826 induces TLR9 stimulation which is MyD88-dependent, while LPS stimulates both TLR3 and TLR4, which are specifically segregated into MyD88-independent and dependent pathways, respectively.

Therefore, we wished to investigate if ANXA1 could regulate the MyD88-independent cytokine production using a TLR3 specific agonist, poly (I:C). Exposure to poly (I:C) induced significant IL-6 and IL-12 production (Figure 3.4). IL-6 was produced after poly (I:C) treatment in both WT and ANXA1 KO PM, but no significant difference was observed between the treated samples. Similar to the results with LPS stimulation, poly (I:C) treated ANXA1 KO PM exhibit significantly lower IL-12 production than WT PM.

Based on these observations, ANXA1 may play a role in TLR3 associated pro-inflammatory cytokine production, which is linked to an MyD88-independent pathway.

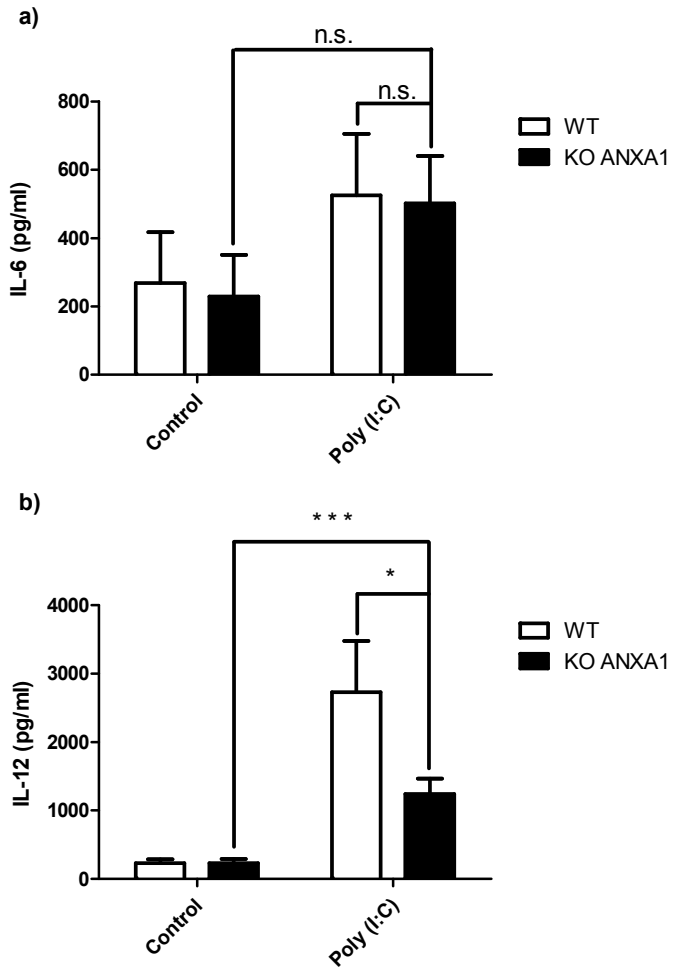


Figure 3.4. a) IL-6 and b) IL-12 production in WT and ANXA1 KO PM after treatment with TLR agonist poly (I:C) (1 million cells/ml). * denotes $p<0.05$, *** denotes $p<0.001$ and n.s. denotes "no significant difference", i.e. $p>0.05$, compared to WT, thioglycollate elicited PM. Results are based on five biological repeats (mean ± S.E.M. of n=5).

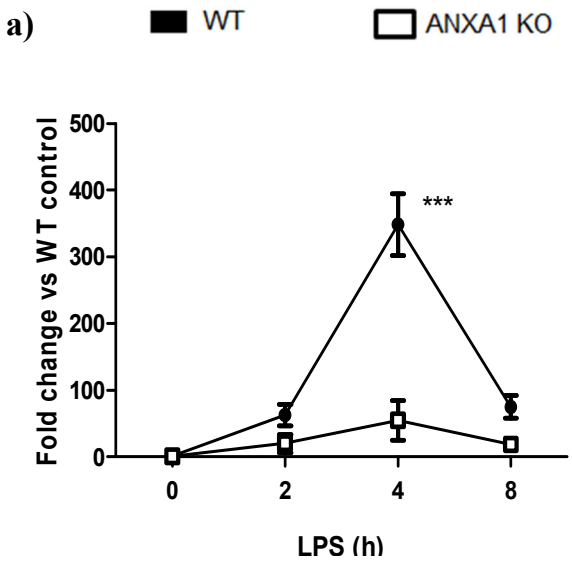
3.2 ANXA1 regulates TRIF dependent cytokine production

Stimulation of TLR3 results in the activation of the endosomal adaptor protein TRIF, resulting in the phosphorylation of IRF-3 by TBK1 and the translocation of the activated IRF-3 in the form of an IRF-3 dimer into the nucleus. There, the IRF-3 dimer acts as a transcription initiation factor for the type I interferon response, inducing the transcription of cytokines such as IFN-β. IFN-β acts on its receptor IFNR1 to induce the production of IFN-β inducible chemokines such as IP-10. We next examined whether ANXA1 directly regulates production of MyD88-independent or TRIF dependent pathway by investigating the levels of IP-10 production.

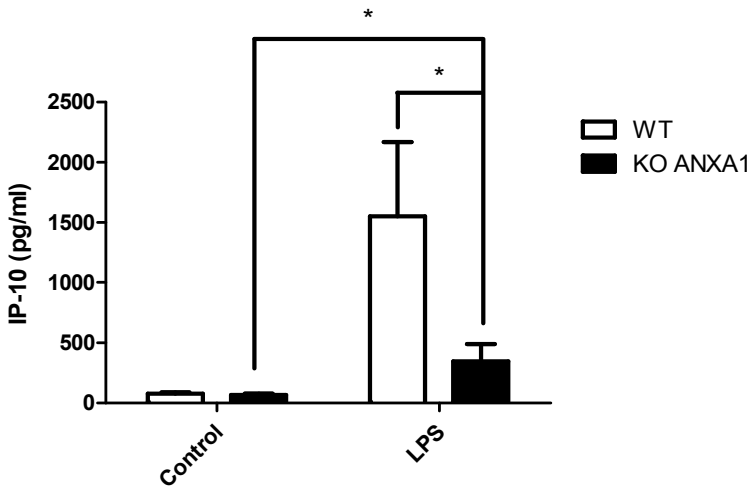
The mRNA level of IP-10 after a 4-hour treatment with LPS was significantly attenuated in ANXA1 KO when compared to WT (Figure 3.5a). In addition, LPS treatment induced significant IP-10 production in WT but not in ANXA1 KO PM (p=0.0419, Figure 3.5b). Similarly, IP-10 was produced in WT but not in ANXA1 KO PM after poly (I:C) treatment (p<0.001, Figure 3.5c). To verify that a selective MyD88-dependent activator does not induce IP-10 production, IP-10 levels were investigated after stimulation with CpG1826. As expected, CpG1826 stimulation did not produce IP-10 production (Figure 3.5d).

Therefore, we show that the absence of ANXA1 directly impairs IP-10 production in ANXA1 KO PM after treatment with TRIF- pathway activators.

a)


WT
ANXA1 KO
Fold change vs WT control
500
400
300
200
100
0
***
0
2
4
8
LPS (h)

b)


*
*
IP-10 (pg/ml)
2500
2000
1500
1000
500
0
Control
LPS
WT
KO ANXA1

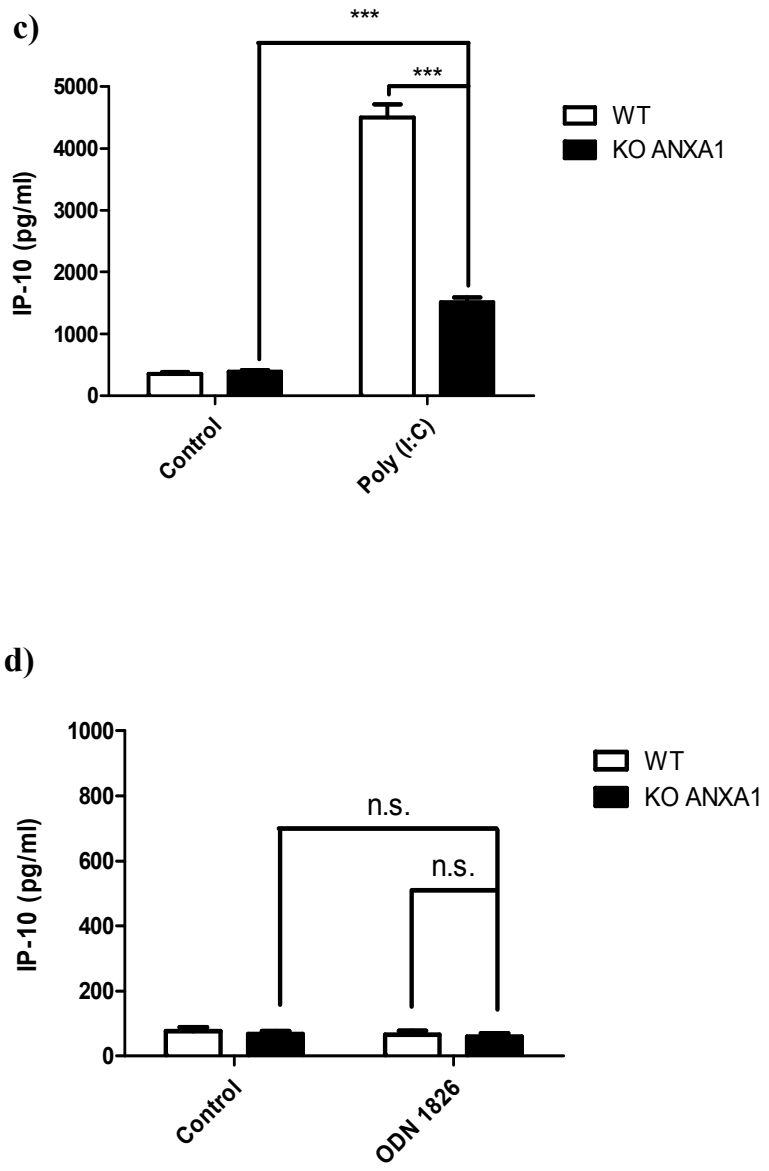


Figure 3.5. IP-10 mRNA expression and production from WT and ANXA1 KO PM. a) mRNA profile of IP-10 over time. IP-10 production after treatment with b) LPS, c) Poly (I:C) or d) CpG 1826 (ODN 1826). * denotes $p<0.05$ ** denotes $p<0.01$ and *** denotes $p<0.001$. *n.s.* denotes "no significant difference". Results are based on 3-5 biological repeats (mean ± S.E.M. of n=3-5).

3.3 Cytokine response against live *E coli.* co-culture

LPS, a bacterial endotoxin, and CpG DNA are capable of inducing cytokine production. However our data thus far does not provide concrete proof that ANXA1 is clinically relevant in modulating cytokine response during exposure to gram negative bacteria. To establish ANXA1 as a novel regulator during TLR associated pathogenesis, a co-culture system of pathogen and macrophages was used. To mimic bacterial pathogenesis, live bacteria (*E. coli* DH5α strain) was co-cultured with macrophages.

Once again, IL-6 and IL-12, as well as TRIF-specific chemokines IP-10 were assessed using ELISA. Co-culture with bacteria DH5α caused significant IL-6 production for WT and ANXA1 KO PM, with both exhibiting similar levels of IL-6 production (Figure 3.6a). Statistical difference is observed between IL-12 production in WT and in ANXA1 KO PM co-cultured with DH5α. ANXA1 KO PM displayed significantly less production of IL-12 compared to its WT counterparts (Figure 3.6b). In both WT and ANXA1 KO PM, IP-10 is produced substantially upon co-culture with DH5α. However higher IP-10 production is observed in WT than in ANXA1 KO PM, similar to that of LPS stimulation (Figure 3.6c). Taken together, these results highlight the importance of ANXA1 in regulating IL-12 and IP-10 production in a clinically relevant setting.

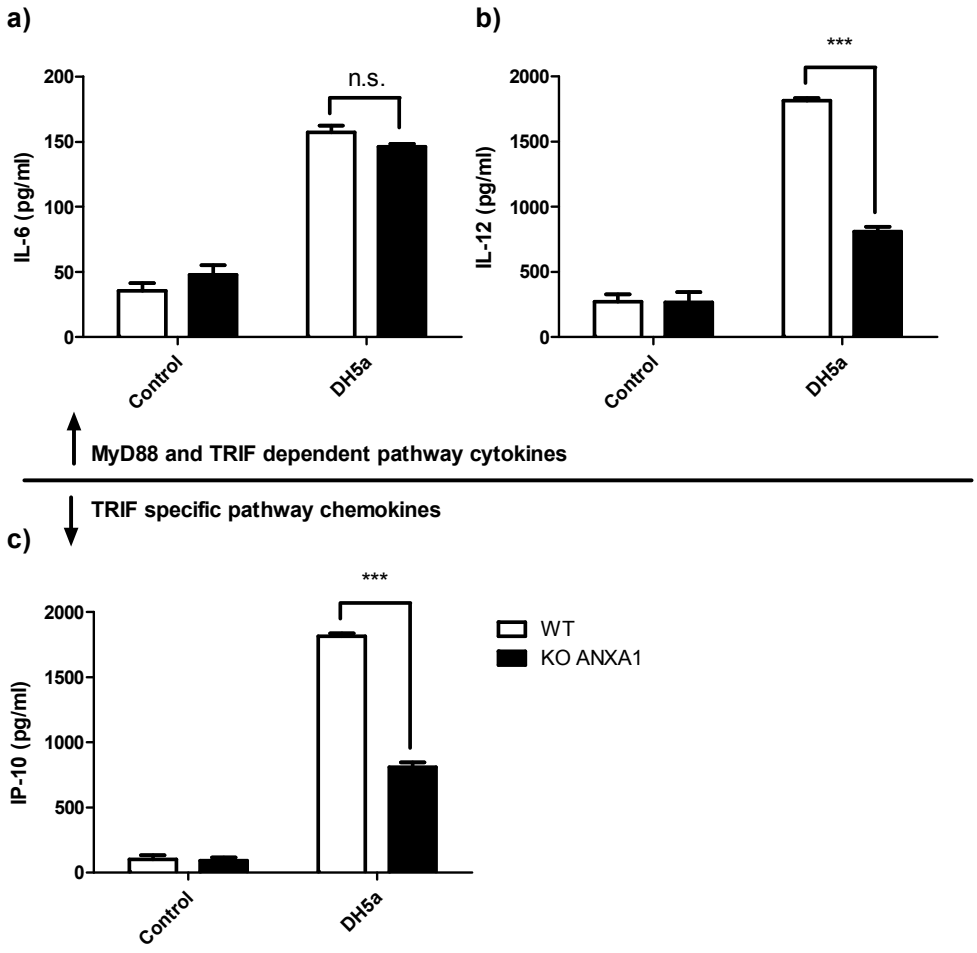


Figure 3.6. Co-culture of PM with DH5α. Results are observed for a) IL-6 and b) IL-12 c) IP-10 production after the co-culture experiment. 10 million bacteria were added to 1 million PM for co-culture duration of 24 hours. "n.s." denotes "no significant difference", i.e. $p>0.05$, Results are based on at least three biological repeats (n=3).

3.4 Cellular activation of PM and B cells after TLR agonist treatment

Next, we investigated if antigen presenting cells harvested from ANXA1 KO mice undergo similar dynamics of innate-to-adaptive immune responses as WT equivalents through activation and up-regulation of cell surface markers that can trigger adaptive immune response such as T cell activation. Macrophages are major players in this aspect and indicators of activation through initial stimulation by TLR agonist can be observed through co-stimulatory molecules and MHC on the cell surface. While there are studies which focused predominantly on dendritic cell activation for ANXA1 KO mice (Huggins *et al*., 2009), we intend to place emphasis on macrophages and splenic B cells by investigating their activation level and antigen presentation capacity, in order to dissect what happens upstream (macrophages as first line of defence) and downstream (B cell activation and response). Hence, increase in activation markers such as $CD86^{+}$ in PM and $CD\ 69^{+}$ in splenic B cells, and changes in MHC II surface expression levels in both macrophages and B cells were chosen for analysis by comparing surface expression level between immune cells of wild type and ANXA1 KO mice.

3.4.1 MHC II surface expression after TLR agonist treatment

In this experiment, we studied MHC class II surface expression in PM and splenic B cells of ANXA1 KO mice. In WT PM (Figure 3.7 a) the expression of MHC II increased after treatment with Poly (I:C) (79.7%) or LPS (82.9%) when compared with untreated control (47.7%). Likewise, the basal levels of MFI values (28.1) increases after treatment with Poly (I:C)

treatment (51.4) or LPS treatment (72.6). In ANXA1 KO PM, an increase in MHC II surface expression upon treatment with poly (I:C) or LPS was observed, but it was not as pronounced as the response observed for WT PM. Both poly (I:C) and LPS elicited only a marginal increase (less than 10% of the population) in MHC II level versus ANXA1 KO control and MFI values are similar between untreated and poly (I:C) or LPS treated PM. Based on these results, ANXA1 KO PM are incapable of up regulating MHC II surface expression even after treatment with TLR agonists poly (I:C) or LPS, compared to WT PM.

Such correlation is also noted in splenic B cells (Figure 3.7 b). In WT control B cells, MHC II positive population constitutes slightly more than half of the population (54.6%) and this population increased to 70.5% after poly (I:C) exposure and 87.9% after LPS exposure. Likewise for MFI values, from a baseline value of 740, treatment with poly (I:C) and LPS increased the MFI values nearly two fold, to 1204 in poly (I:C) and 1493 in LPS, indicating an increase in MHCII expression level in cell populations after treatment with TLR agonists. ANXA1 KO B cells exhibited a reduced MHCII upregulation after stimulation with poly (I:C) when compared with WT B cells. ANXA1 KO B cells after stimulation with poly (I:C) exhibited a reduced population of B cells with MHCII positivity (61.5%) compared to WT B cells (70.5%).

Based on these data, PM of ANXA1 KO mice have reduced phagocytic activity after stimulation with poly (I:C) and LPS, while B cells of ANXA1 KO mice have reduced phagocytic activity after stimulation with poly (I:C).

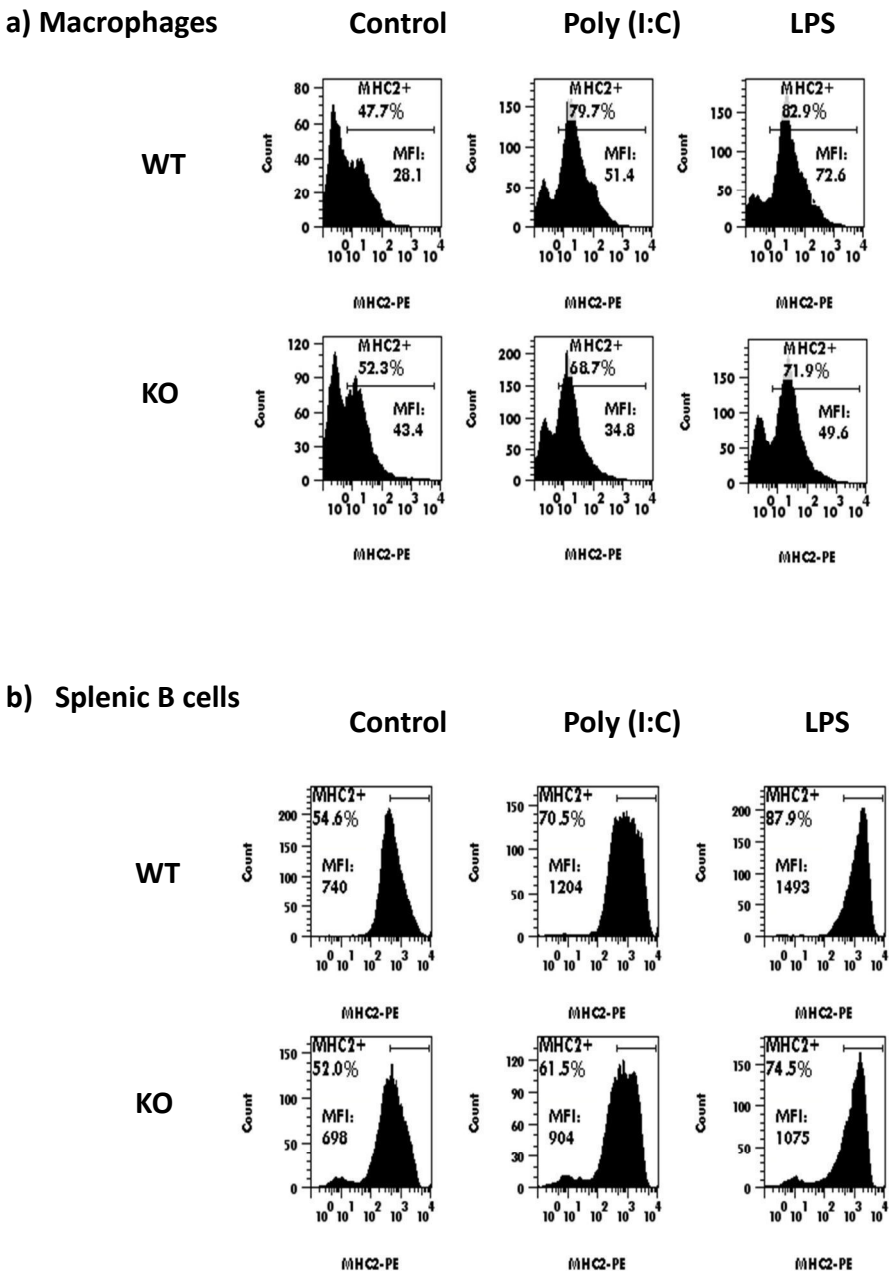


Figure 3.7. Flow cytometry analysis of MHC II expression levels on PM and B cells. a) PM, identified through prior gating for F4/80^{+} cells, after FSC/SSC gating for macrophage sized cells. b) Splenic B cells, identified through prior gating for CD19^{+} cells, after FSC/SSC gating for splenic B cell sized cells. Cell count: 1 million cells / sample. Result is a representative data based on pooled samples from four mice (n=4) with two independent biological replicates done on a different day.

3.4.2 CD86 and CD69 expression after TLR agonist treatment

Next, the level of activation markers on macrophages and B cells was investigated. We examined $CD86^+$ as an activation marker for PM and $CD69^+$ as an activation marker for splenic B cells.

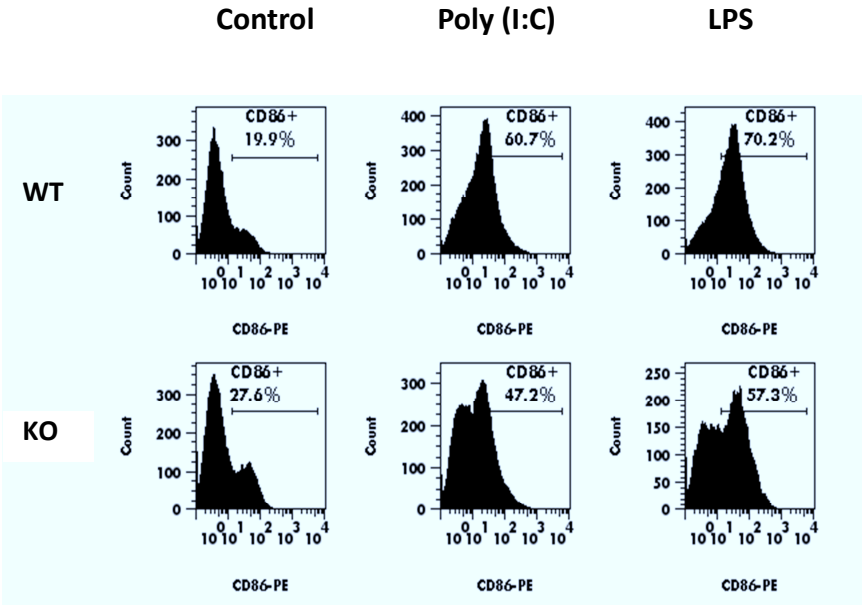
For PM, as seen in Figure 3.8 a), a small population of PM was activated (19.9%) in WT controls and this marks a basal level of activation. In contrast, more than half (60.7%) of the population was activated upon treatment with poly (I:C) and likewise a slighter larger proportion of activation for LPS treatment (70.2%). This also places a fundamental doubt raised earlier to rest: that thioglycollate based-elicitation of PM may activate the PM. Based on this result, it is clear that the macrophages are not activated as a whole population.

In ANXA1 KO PM, close to a third of the untreated control population already possesses activation markers (27.6%). While an increase in activation upon treatment with poly (I:C) (47.2%) or LPS (57.3%) is observed, it is much lower than what is observed in WT PM for both poly (I:C) and LPS. Specifically, the presence of a double peak only in the ANXA1 KO macrophage population appears to hint on a substantial population that is not capable of responding at all. ANXA1 KO PM also exhibited an increase in activated macrophage population upon treatment with TLR agonists, but this increase is not as substantial as its WT counterpart PM. Hence, based on these results, ANXA1 KO PM seem to have a specific population that is not activated after TLR agonist treatment.

Splenic B cells derived from WT and ANXA1 KO mice were also investigated. In WT B cells, only a small fraction (10.2%) is $CD69^+$ and this increased marginally upon treatment with poly (I:C) (22.3%). However, a drastic increase in $CD69^+$ population was found upon exposure to LPS (55.9%), indicating that the activation of B cell is more pronounced in LPS than poly (I:C). In ANXA1 KO splenic B cells, control cells are not activated (12.5% positive) while exposure to poly (I:C) increases $CD69^+$ expression only slightly. While LPS treatment of ANXA1 KO B cells markedly increased the positive population, it is visibly lower than its WT counterparts. Consequently, this outlines the role ANXA1 plays in B cell activation, that ANXA1 is also important in the B cell dependent inflammatory response towards TLR agonist stimulation.

In conclusion, these results indicate a potential role ANXA1 may play in the activation of PM and B cells, thereby regulating the interface between innate and adaptive immunity.

a) Macrophages



b) Splenic B cells

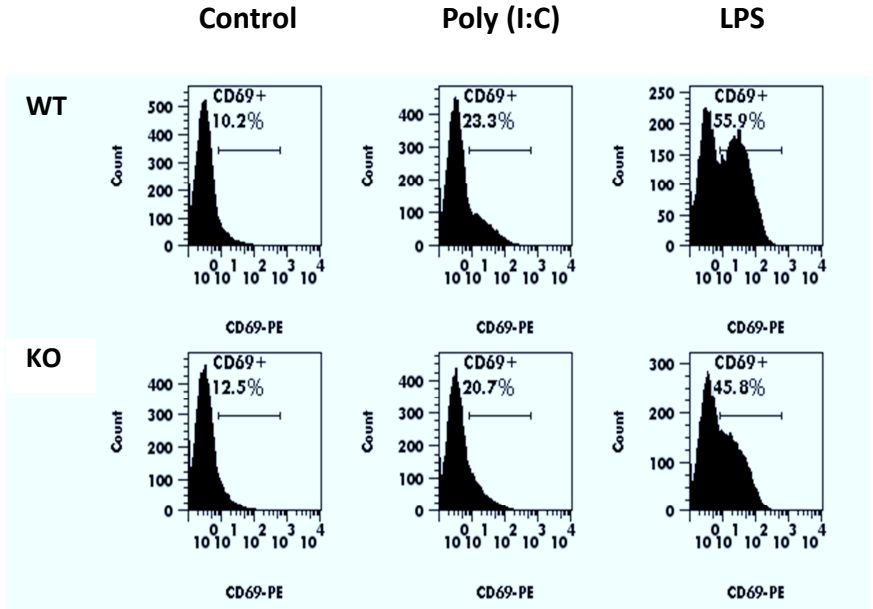


Figure 3.8. Flow cytometry analysis of activation markers for PM and B cells. a) Macrophages, identified through prior gating for F4/80$^+$ cells, after FSC/SSC gating for macrophage sized cells. b) Splenic B cells, identified through prior gating for CD19$^+$ cells, after FSC/SSC gating for splenic B cell sized cells. Cell count: 1 million cells / sample. Result is a representative data based on pooled samples from four mice (n=4) with two independent biological replicates done separately.

3.5 Role of ANXA1 in cellular activation and cytokine production after poly(I:C) administration in vivo

We next investigated whether the results observed *in vitro* can also be observed *in vivo* as well. In our earlier experiments, we demonstrated that immune cells from ANXA1 KO mice show impaired TRIF dependent activation during inflammatory response induced by TLR agonists LPS and poly (I:C). To confirm that ANXA1 plays an important role in triggering TRIF dependent activation and inflammation response *in vivo*, TLR agonist poly (I:C) (1mg/ml) was injected into mice peritoneal cavity of WT and ANXA1 KO mice. As an indicator of inflammation response *in vivo*, serum levels were measured for TNF-α, IL-6, IFN-γ, IFN-α, IFN-β and IP-10 (CXCL 10) using ELISA.

Upon treatment with poly (I:C) for 6 h and 24 h, WT and ANXA1 KO mice showed comparable levels of pro-inflammatory cytokine production in TNF-α and IL-6 (Figure 3.9 a and b). However, the production of TRIF dependent chemokines IFN-β and IP-10 at 6 h was significantly inhibited in ANXA1 KO mice when compared to WT mice (Figure 3.9 d and f). To determine whether the inhibition affects other interferons induced during antiviral and inflammation process, IFN-α and IFN-γ production level was also investigated. Serum levels of IFN-α and IFN-γ were similar between WT and ANXA1 KO mice for both 6 h and 24h (Figure 3.9 c and e). IFN-β is unique among the three interferons as its activation is IRF-3 dependent. This highlights the possibility of a TRIF/IRF-3 dependent pathway inhibition existing in ANXA1 KO mice. ANXA1 is important for activation of TRIF

pathway for the production of IFN-β and IP-10 chemokine production *in vivo* in a TLR 3 dependent manner.

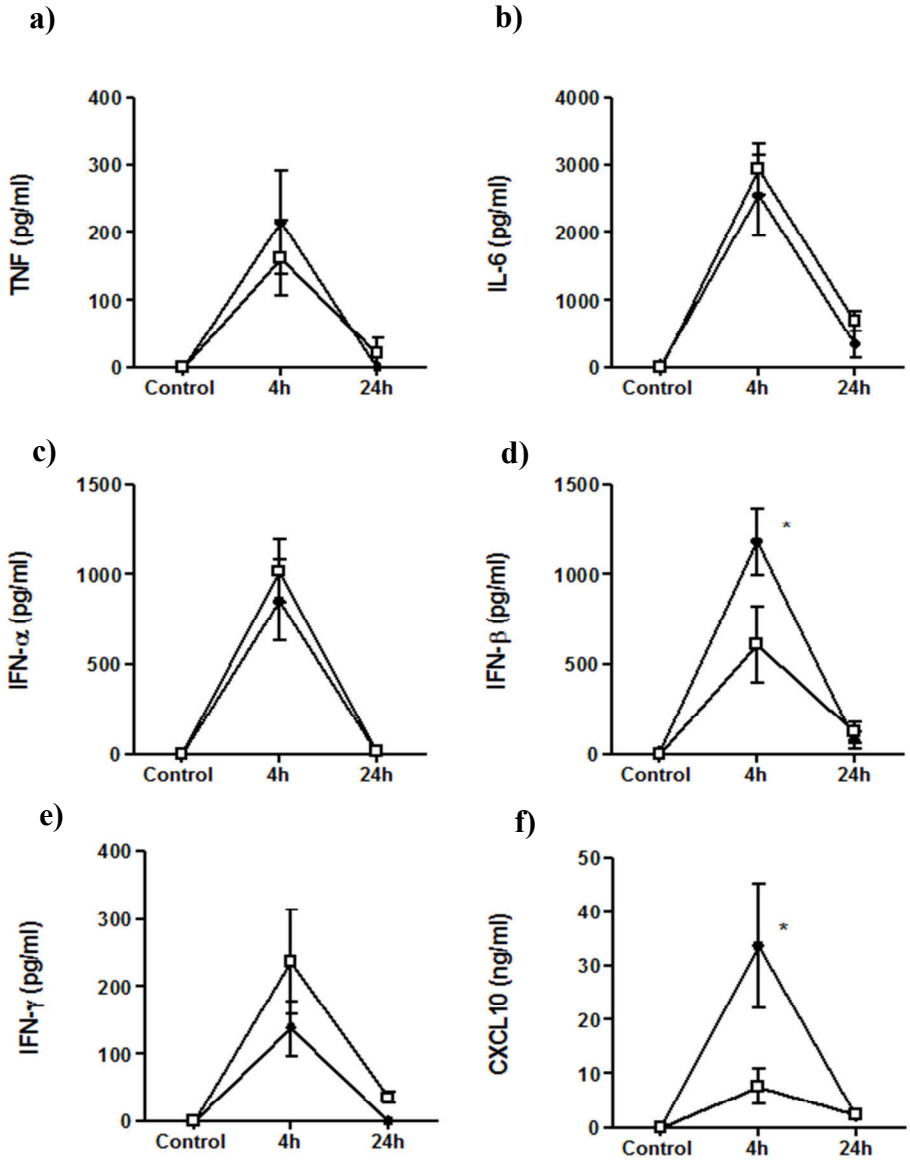


Figure 3.9 Poly (I:C) stimulation induces lower serum levels of TRIF dependent chemokines IFN-β and IP-10 (CXCL 10) in ANXA1 KO PM. Black diamonds indicate WT and white square indicate ANXA1 KO mice serum cytokine levels. * denotes $p<0.05$. Results are based on two separate experiments on eight mice (n=8).

3.6 Mechanism of action of ANXA1-dependent regulation of cytokine production

Toll-like receptors have many pathways of action. TLR receptor adaptor proteins (with TIR domains) regulate downstream response of many cytokine producing immune cells, particularly macrophages (Kawai and Akira, 2006). There are five known TLR adaptor proteins, namely MyD88, TRIF, Mal, TRAM and SARM (Kenney and O'Neil, 2008). MyD88 and TRIF are essential for LPS induced TLR3 and TLR 4 activation while TRIF is essential for poly (I:C) induced TLR3 activation.

This study has demonstrated that ANXA1 KO exhibited impaired IL-12 and IP-10 production in ANXA1 KO PM after stimulation with LPS and poly (I:C), and also impaired PM and B cell activation after stimulation with LPS. As cytokine production and upregulation of activation markers are both dependent on activation of transcription factors, we next examined whether ANXA1 is important for the activation of transcription factors.

The first imperative was to identify whether the signaling cascade upstream of cytokine production was intact in ANXA1 KO PM. There are "master switches" to examine signaling cascade vitality, such as IRF-3 and NF-κB p65 nuclear translocation. IRF-3 and NF-κB p65 activity were studied using confocal microscopy to determine if nuclear translocation of these proteins was affected in the ANXA1 KO PM, after exposure to Poly I:C and LPS.

3.6.1 Nuclear localization IRF-3 after LPS treatment

To investigate whether nuclear localization of transcription factors in ANXA1 KO PM is similar to the WT, we used LPS, the MyD88-dependent and independent stimulator, to test IRF-3 nuclear translocation. In this experiment, PM were exposed to LPS for 1 h prior to fixation. The samples were then incubated with anti-IRF-3 primary antibodies followed by that with Alexafluor 488-labeled secondary antibodies. DAPI, a common stain used to bind A-T rich regions of DNA during flourescent microscopy, was used to visualize the nucleus.

Under unstimulated conditions, both WT PM and ANXA1 KO PM did not show any localization of IRF-3 in the nucleus, which is delineated by DAPI (Figure 3.10). However, upon treatment with LPS, IRF-3 is seen to translocate substantially to the nucleus. However, such nuclear translocation of IRF-3 was not seen in ANXA1 KO PM after 1 h of exposure to LPS. This observation is similar to the result for poly (I:C) treatment, indicating ANXA1 plays an extensive role in TRIF dependent IRF-3 activation.

Therefore, ANXA1 is important for the translocation of IRF-3 to the nucleus after LPS exposure.

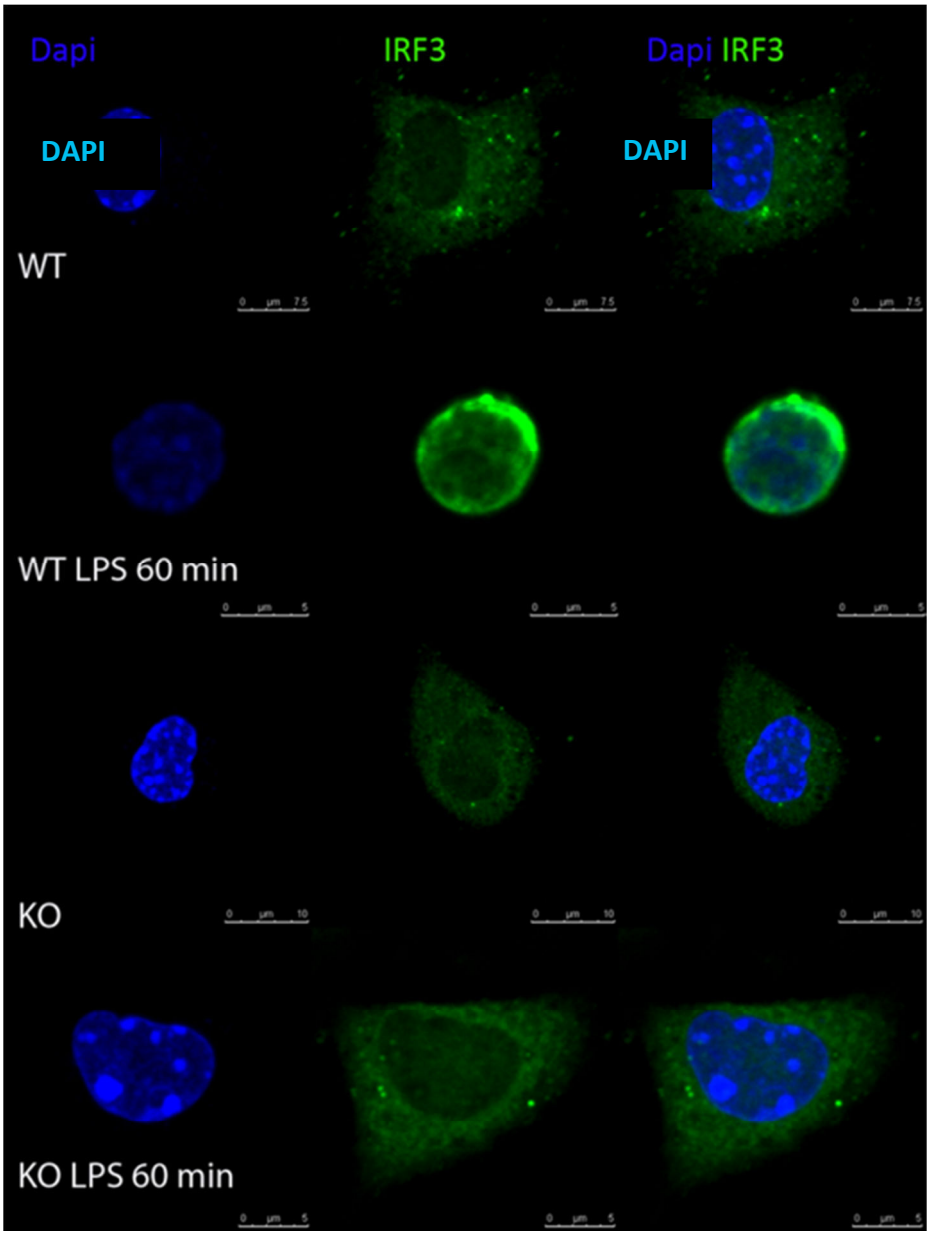


Figure 3.10. Impaired nuclear translocation of IRF-3 in ANXA1 KO PM under LPS treatment. PM were used in this confocal imaging. Stains used are DAPI for nuclear staining and rabbit anti- IRF-3 antibody for detecting localization of IRF-3 in the nucleus. WT: wild-type PM. WT LPS 60 min: wild-type PM exposed to LPS (1ug/ml) for 60 minutes. KO: PM derived from ANXA1 KO mice. KO LPS 60 min: PM derived from ANXA1 KO mice exposed to LPS (1 ug/ml) for 60 minutes.

3.6.2 Nuclear localization of IRF-3 after poly (I:C) treatment

In this experiment, PM were exposed to poly (I:C) for 1 h. In untreated, non-stimulated control WT and ANXA1 KO PM, IRF-3 was found mainly in the cytosolic region with visibly less localization within the nucleus, a region highlighted distinctly by DAPI stain (Figure 3.11). When these PM were exposed to the TLR3 agonist poly (I:C), IRF-3 translocated to the nucleus in WT but not in ANXA1 KO PM. Indeed, PM derived from WT mice exhibited prominent florescence in the nuclear region, which was brighter than that of the cytosolic regions. All the WT PM cells observed exhibited such nuclear localization. However, this was not observed in PM derived from ANXA1 KO PM after sixty minutes of poly (I:C) exposure. Instead, very little IRF-3 translocation to the nucleus was seen in these PM.

Based on these results, IRF-3 nuclear translocation is impaired in ANXA1 KO PM.

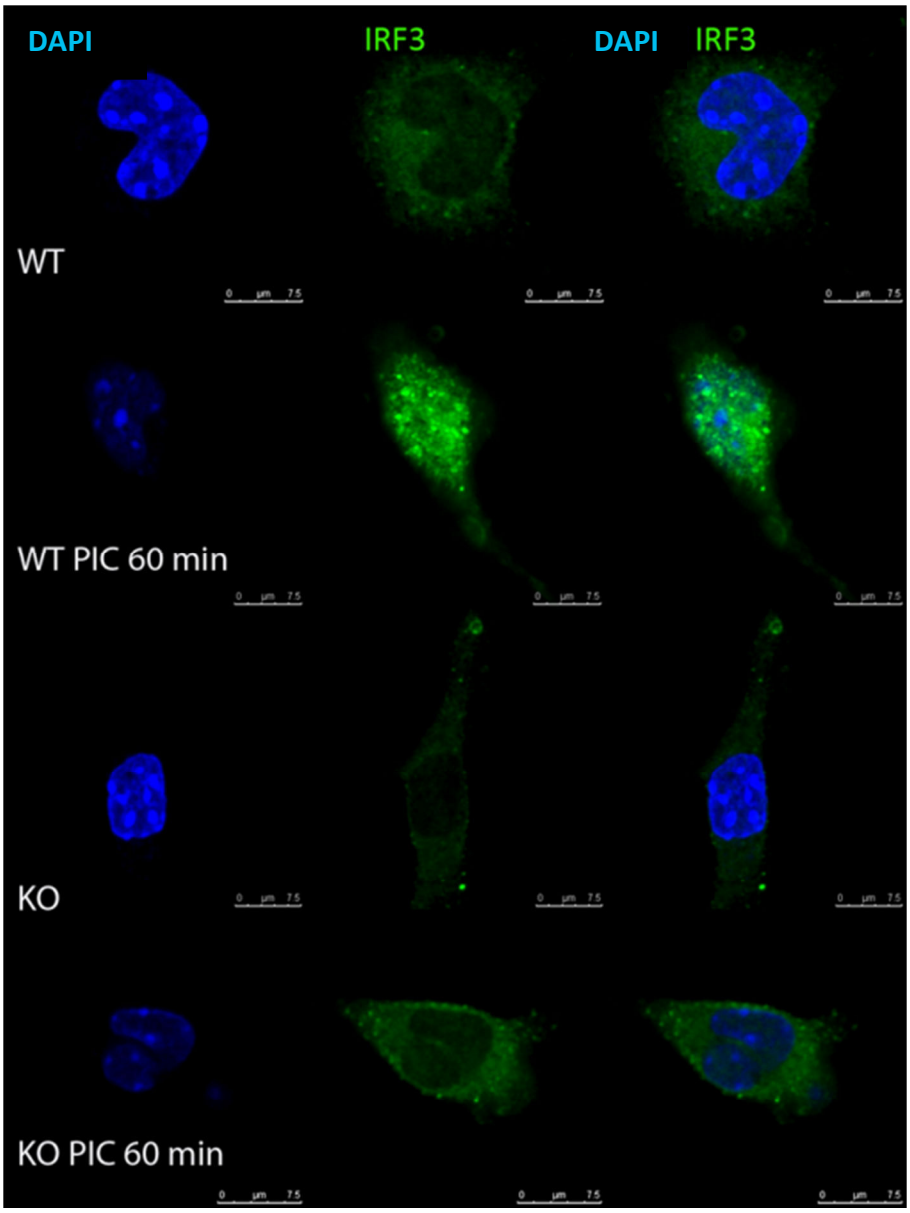


Figure 3.11. Impaired nuclear translocation of IRF-3 in ANXA1 KO PM under poly (I:C) treatment. PM were used in this confocal imaging. Stains used are DAPI for nuclear staining and rabbit anti- IRF-3 antibody for detecting localization of IRF-3 in the nucleus. WT: WT PM. WT PIC 60 min: WT PM exposed to poly (I:C) for 60 minutes. KO: PM derived from ANXA1 KO mice. KO PIC 60 min: PM derived from ANXA1 KO mice exposed to poly (I:C) for 60 minutes.

3.6.3 Nuclear localization of NF-κB p65 after LPS treatment

We next examined an active unit of NF-κB—the p65 subunit. Upon activation and release from IκB lockdown, the p65 subunit translocates to the nucleus to transcribe various pro-inflammatory genes; e.g. IL-12 in macrophages. As our laboratory has discovered that NF-κB promoter activity in PM from ANXA1 KO mice is impaired (unpublished data), it is important to determine independently whether translocation of NF-κB p65 subunit is also impaired. To clarify the exact involvement of ANXA1 in NF-κB signaling, we examined whether nuclear translocation of NF-κB after exposure to LPS is regulated by ANXA1.

Results for confocal on WT and ANXA1 KO PM which have undergone LPS-induced activation are shown in Figure 3.12. Both WT and ANXA1 KO PM showed distinctly low concentration of NF-κB in nucleus compared to cytosol at basal conditions. Upon treatment with LPS for one hour, WT PM showed clear and distinctive nuclear translocation of NF-κB, as the nuclear region stained by DAPI fluoresced brightly with the signal from NF-κB binding antibodies. ANXA1 KO PM also exhibits nuclear localization of NF-κB.

Hence ANXA1 does not appear to affect NF-κB nuclear translocation in PM significantly when stimulated with LPS.

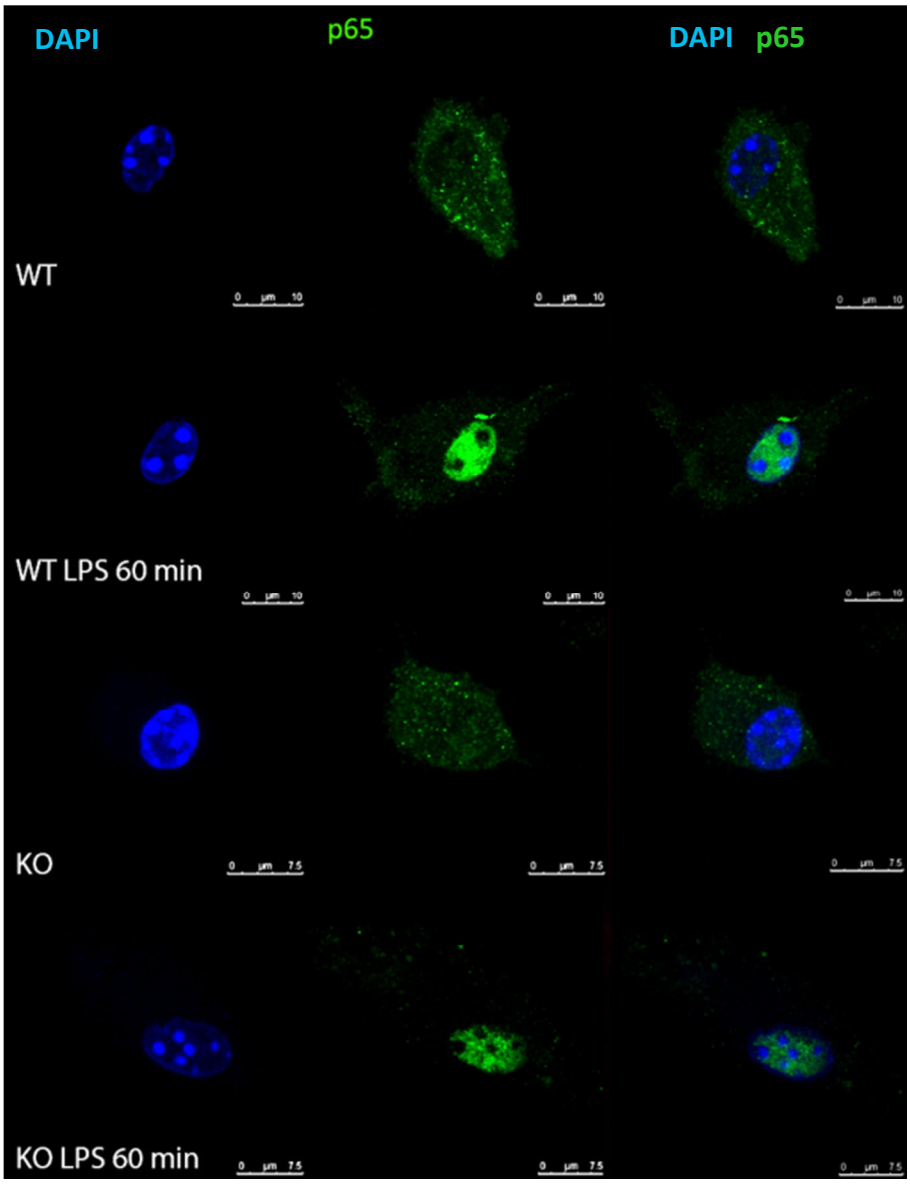


Figure 3.12. Impaired nuclear translocation of NF-κB in ANXA1 KO PM under LPS treatment. PM were used in this confocal imaging. Stains used are DAPI for nuclear staining and rabbit anti- NF-κB antibody for detecting localization of NF-κB in the nucleus. WT: wild-type PM. WT LPS 60 min: wild-type PM exposed to LPS (1 ug/ml) for 60 minutes. KO: PM derived from ANXA1 KO mice. KO LPS 60 min: PM derived from ANXA1 KO mice exposed to LPS (1 ug/ml) for 60 minutes.

3.6.4 Nuclear localization of NF-κB p65 after poly (I:C) treatment

Figure 3.13 shows the result of confocal imaging of PM derived from both WT and ANXA1 KO mice. In both types of PM, no translocation of the NF-κB p65 subunit into the nucleus was observed in untreated control PM. However, upon treatment of poly (I:C) for 60 min, only WT PM exhibited prominent translocation of NF-κB p65 subunit to the nucleus. However, this was not observed in ANXA1 KO PM after stimulation with poly (I:C). It therefore clearly implicates ANXA1 in relaying the late phase NF-κB signal through the activation of TRIF dependent pathway.

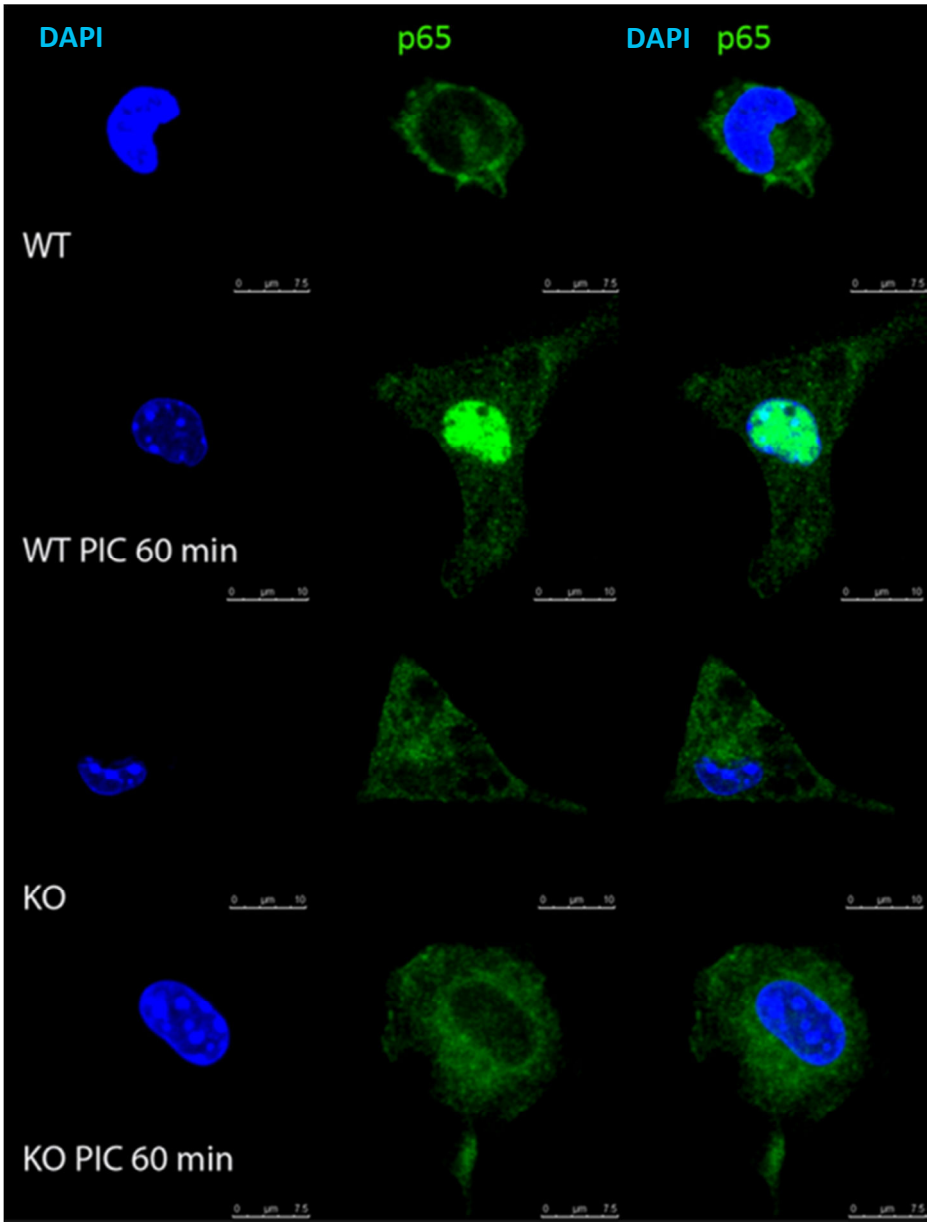


Figure 3.13. Impaired nuclear translocation of NF-κB in ANXA1 KO PM under poly (I:C) treatment. PM were used in this confocal imaging. Stains used are DAPI for nuclear staining and rabbit anti- NF-κB antibody for detecting localization of NF-κB in the nucleus. WT: wild-type PM. WT PIC 60 min: wild-type PM exposed to poly (I:C) for 60 minutes. KO: PM derived from ANXA1 KO mice. KO PIC 60 min: PM derived from ANXA1 KO mice exposed to poly (I:C) for 60 minutes.

CHAPTER IV:

MACROPHAGE POLARIZATION AND ANXA1

4. MACROPHAGE POLARIZATION AND ANXA1

In the previous chapter, we studied the role ANXA1 plays in cytokine / chemokine production and investigated possible mechanisms examined for mechanisms which may explain its functional properties in macrophages. However, there is no clear framework which can provide an explanation why ANXA1 KO macrophages are spectrally distant from wild-type macrophages in terms of both immune response and activation. As ANXA1 is also a candidate marker for aggressive cancer phenotypes (Lim and Pervaiz, 2007) and macrophages are closely associated with cancer phenotype modulation, we sought the literature for an overarching theme which could amalgamate all these observations into a tangible structure for ANXA1 to align itself in a concordant manner.

Macrophage polarization is a term coined to consolidate the gamut of macrophage phenotypes into an operationally comprehensible concept that assents both the plasticity of macrophages in microenvironments and the existence of an alternative morphologic macrophage that participates in non-immune response (Alberto *et al.,* 2005). When the term "macrophage polarization" was popularized actively in literature, there were key features which distinguished two diametric opposites of macrophages, termed M1 and M2 macrophages. M1 macrophages are macrophages that respond and behave in a classical manner, i.e. M1 macrophages are immune response cells that highly express IL-12, IL-23 and less IL-10, while M2 macrophages express low IL-12, IL-23 and high IL-10.

The lack of mechanistic elaboration and macrophage polarization specific markers, especially for M2 polarization, has plagued its well-conceptualized idea until M2 macrophage polarization specific markers such as Arginase-1 and YM-1 were established (ibid). As more research converge to include macrophage polarization as a compendious exposition for data analysis involving macrophages, pathways and mechanisms behind macrophage polarization are better understood now. M1 polarizing pathways within M1 macrophages have been found to be dependent on the activation of three major inflammation-associated transcription factors: NF-κB, IRF-3 and STAT-1 (Sica and Bronte, 2007). These transcription factors are activated to induce M1 polarization, and also provide reciprocal modulation which also provide reciprocal modulation, preventing M2 polarization markers such as Arginase-1 from being expressed. This allows pathway-driven investigation to establish whether ANXA1 directly affects macrophage polarization.

In this chapter, we examined M2 polarization specific markers to ascertain whether ANXA1 directly affects macrophage polarization. We also focused on three major transcription factors involved in M1 polarization, namely NF-κB, IRF-3 and STAT-1, and a transcription factor involved in M2 polarization, PPAR-γ. Figure 4.1 summarizes these investigations.

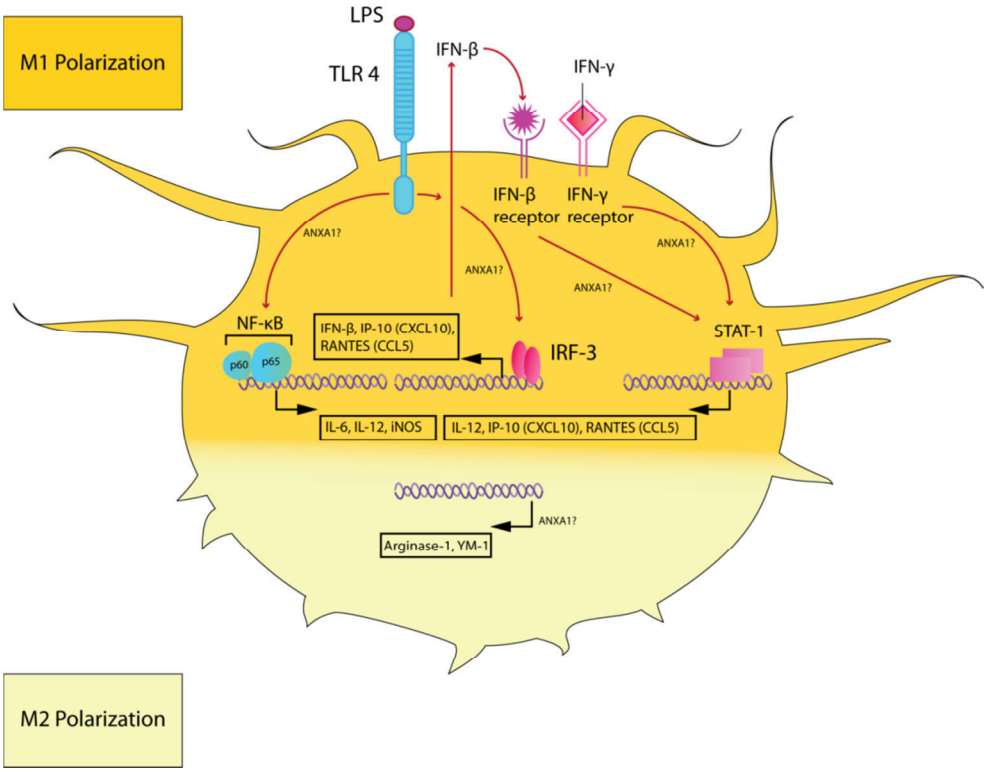


Figure 4.1. The regulation of M1 and M2 macrophage polarization by ANXA1 may be from several pathways. This diagram gives the general picture of critical components of in M1 and M2 polarization which are relevant to this study. The involvement of ANXA1 in macrophage polarization is not known. ANXA1 may be important for activating transcription factors NF-κB and IRF-3 or STAT-1. ANXA1 may also regulate M2 polarization, which can be identified by changes in expression of M2 markers such as Arginase-1 and YM-1 (Diagram by Shu Shin La).

4.1. Using Bone Marrow Derived Macrophages (BMDM) as a Model for Further Investigation

The use of PM is invaluable as an archetypal example of *in vivo* response which signals a response through TLR pathways in activated macrophages. Compared to other common methods of deriving macrophages such as bone marrow derived macrophages (BMDM), which mimics the development of macrophages and its cytokine environment but does not factor in the subtlety of other cellular interactions during its maturation, the use of PM have an added advantage of providing a global picture of macrophage response in a homeostatic state of differentiation and antigenic differences. However, to study macrophage polarization, the use of naïve macrophages BMDM was required, as mature macrophages such as PM may skew the polarization of macrophages.

In order to use BMDM for detailed mechanism studies, ANXA1 KO BMDM cells were examined and compared to ANXA1 KO PM. This allows BMDM cells to be used as a model for *in vivo* response of PM. As mentioned previously, ANXA1 KO PM exhibited impairment of both MyD88 and TRIF dependent pathways, particularly after LPS treatment, and it is important to determine if BMDM also share such impaired phenotype.

To investigate whether BMDM of ANXA1 KO also possess the same impaired phenotype for inflammatory cytokine production, MyD88 dependent cytokines (IL-6 and IL-12) and TRIF dependent chemokine (IP-10) production levels were examined.

IL-6, IL-12 and IP-10 production of WT and ANXA1 KO BMDM in untreated controls was observed to be at basal level and similar to each other (Figure 4.2). After LPS treatment, we observed a lower production of IL-6 (although insignificant), IL-12 ($p<0.05$) and IP-10 ($p<0.001$). IP-10 levels of WT and ANXA1 KO BMDM in untreated controls were was at basal level and not significantly different (Figure 4.2 c), although compared to WT PM, WT BMDM produced more than twice the levels of IP-10.

Based on these results, we postulated that ANXA1 is required for cytokine production in response to LPS in BMDM, and is required for TRIF-dependent activation.

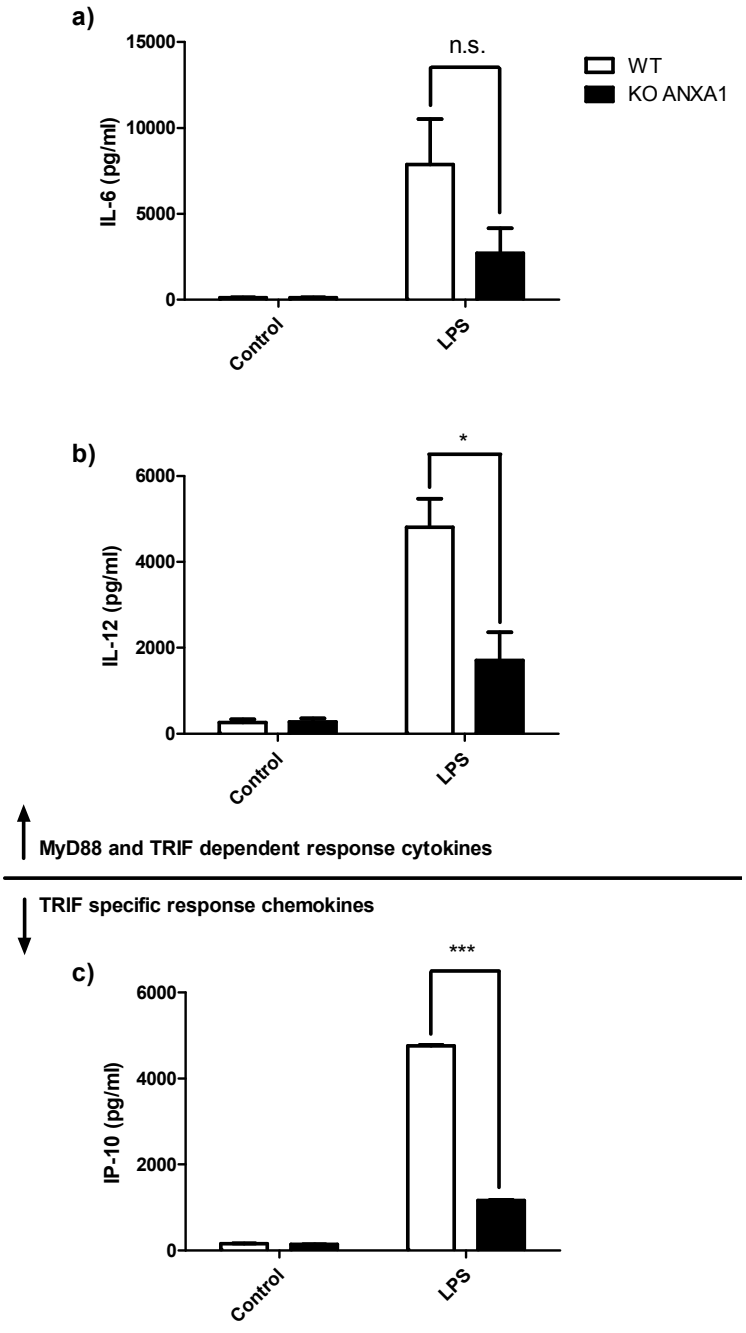


Figure 4.2. MyD88 and MyD88-independent, TRIF dependent cytokine / chemokine production in WT and ANXA1 KO BMDM after treatment with TLR agonists (1 million cells/ml). * denotes $p<0.05$, *** denotes $p<0.001$ and n.s. denotes "no significant difference", i.e. $p>0.05$. Results are based on three biological repeats (n=3).

4.2. ANXA1 is involved in supressing M2 polarization

Despite comprehensive evidence suggesting that ANXA1 affects diverse pathways involved in macrophage polarization, an association of ANXA1 to M2 polarization has not been described thus far. Macrophage polarization is a term born out of macrophages that do not respond in classical manner (M1 polarization), but there are very few methods to determine whether certain macrophages are M2 polarized, as macrophages with impairment of pro-inflammatory cytokines is the key evidence that an alternative macrophage state exists (Alberto *et al.,* 2005). However, there exist markers that can conclusively identify M2 polarization.

Arginase-1, an enzyme that converts arginine into ornithine or urea, is expressed in macrophages which actively drive an anti-inflammatory, type-2 innate response (Pesce *et al.,* 2009). Another M2 specific, anti-inflammatory macrophage associated marker is YM-1 (Raes *et al.,* 2002). The next experiment focuses on these M2 markers.

4.2.1. ANXA1 suppresses Arginase-1 and YM1 expression

To examine the mRNA expression level of M2 markers in WT and ANXA1 KO BMDM, RT-PCR was used to quantify mRNA levels in BMDM of WT and ANXA1 KO mice at basal levels without stimulation. YM-1 mRNA level is higher in ANXA1 KO macrophages compared to WT BMDM (Figure 4.3). Likewise for arginase-1, ANXA1 KO macrophages have higher mRNA expression level compared to WT BMDM. We also examined a M1 macrophage polarization marker, IL-6, which is expressed at basal level in macrophages (Mantovani *et al.,* 2005). Interestingly, ANXA1 KO BMDM has

diminished IL-6 mRNA expression compared to WT BMDM. This is in contrast to the M2 polarization markers that were up regulated in ANXA1 KO BMDM.

Therefore, ANXA1 KO BMDM exhibits a M2 polarization phenotype. These results demonstrate that ANXA1 may inhibit M2 and promote M1 polarization. In addition, IP-10 and IL-12 are M1 cytokines, and is observed in the previous chapter to be decreased in ANXA1 KO PM, thus further confirming that ANXA1 promotes M1 polarization.

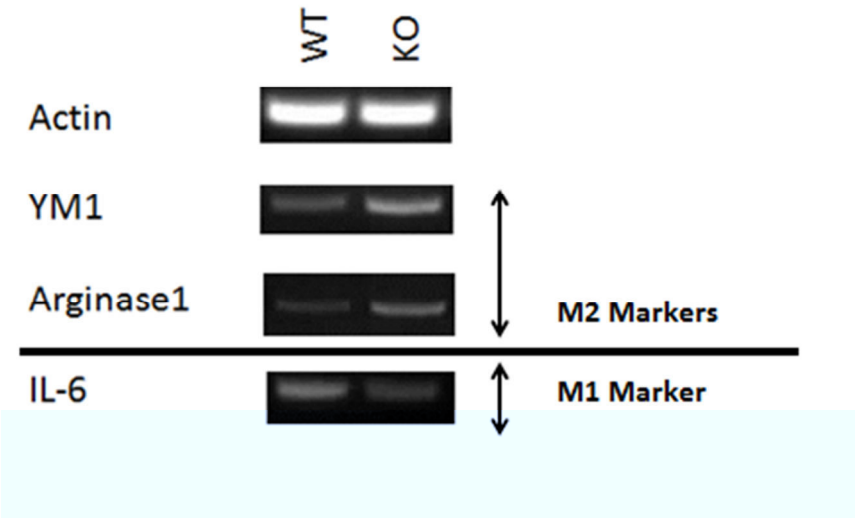


Figure 4.3. ANXA1 suppresses M2 polarization markers. a) mRNA quantification using RT-PCR to examine mRNA levels in WT and ANXA1 KO BMDM, without any prior treatment. Result shown is a representative among two biological repeats (n=2).

4.2.2. ANXA1 KO BMDM is unresponsive to NF-κB inhibitor

NF-κB is a transcription factor important in M1 polarization. To investigate whether NF-κB is directly responsible for IL-12 production during LPS exposure, and if ANXA1 KO cells are M2 polarized due to an inhibition in NF-κB, we inhibited NF-kB using a chemical inhibitor BAY-11-7082 (hereafter called BAY-11). BAY-11 is capable of blocking NF-κB activation by irreversibly inhibiting phosphorylation of IκB-α which clamps NF-κB into an inactive state unless phosphorylated. We hypothesized that should NF-κB be responsible for the difference in IL-12 production observed in ANXA1 KO macrophages, inhibiting NF-κB would not affect ANXA1 KO macrophages.

Figure 4.4 shows the result for wild-type and ANXA1 KO macrophages pre-treated with BAY-11 for 1 hour before exposure to LPS. Both wild-type and ANXA1 KO macrophages produce IL-12 upon exposure to BAY-11 alone. After exposure to LPS, when cells are pre-treated with BAY11, a significant decrease in IL-12 production is observed in WT BMDM. However, in ANXA1 KO BMDM, BAY11 does not inhibit IL-12. Therefore, BAY-11 only affects NF-κB-dependent IL-12 production in WT BMDM, but not ANXA1 KO BMDM after stimulation with LPS.

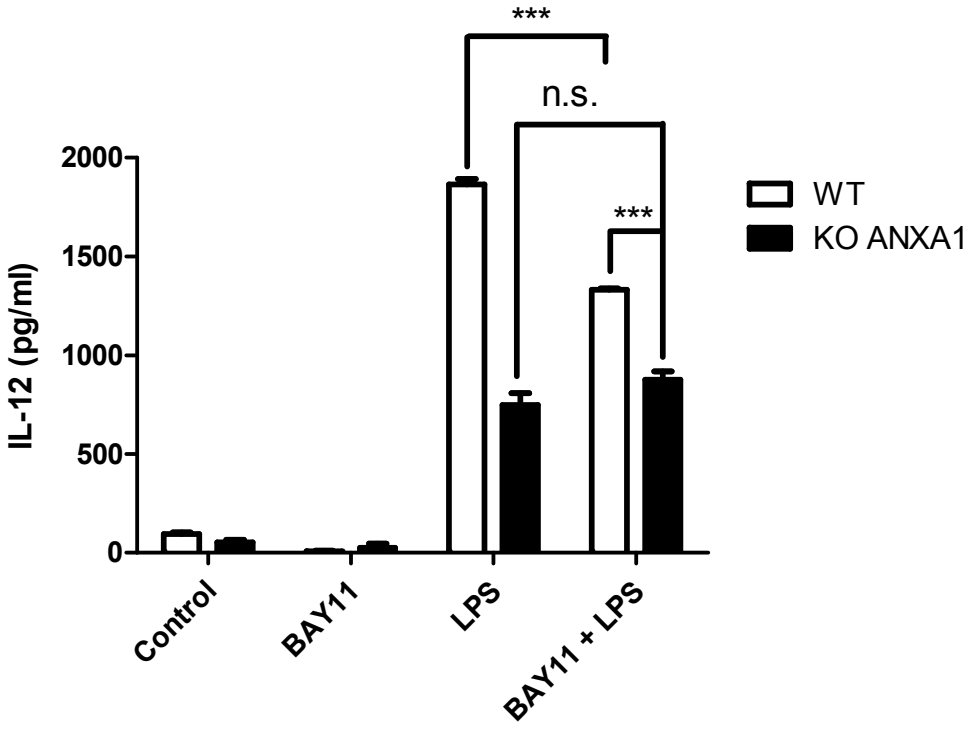


Figure 4.4. BAY-11 inhibits only wild-type BMDM for IL-12 production treated with BAY-11 prior to LPS treatment. BAY-11 (100ng/ml) was given as a pre-treatment one hour prior to addition of LPS (1 ug/ml) to samples. *** denotes $p<0.001$ and "n.s." denotes "no significant difference", i.e. $p>0.05$, Results shown is a representative among three biological repeats (n=3).

4.2.3. ANXA1 directly affects NO production in macrophages

We next examined the capacity of ANXA1 to regulate other factors that characterize M1 polarization. Macrophages are active producers of nitric oxide (NO) during inflammation, and NO production is a distinguishing characteristic of M1 polarization from M2 polarization, as NO production is greatly reduced during M2 polarization (Ho and Sly, 2009). Although NO production is independent of cytokine production trigger, it is nevertheless partly affected by NF-κB activation, as the transcription factor affects iNOS

expression level, which is one of the major inflammation-inducible genes responsible for increases in NO production (DeFronzo, 2009).

In untreated controls, both wild-type and ANXA1 KO macrophages produced small but detectable amounts of NO (Figure 4.5). In contrast, wild-type macrophages when exposed to LPS produced approximately13 μM of NO, and a smaller amount of approximately 6 μM of NO upon exposure to poly (I:C). This is significantly higher than the amount produced in ANXA1 KO macrophages when exposed to LPS or poly (I:C). Based on this result, we demonstrated that NO production is significantly inhibited in ANXA1 KO macrophages after stimulation with LPS, and poly (I:C), indicating a phenotype closer to M2 polarization.

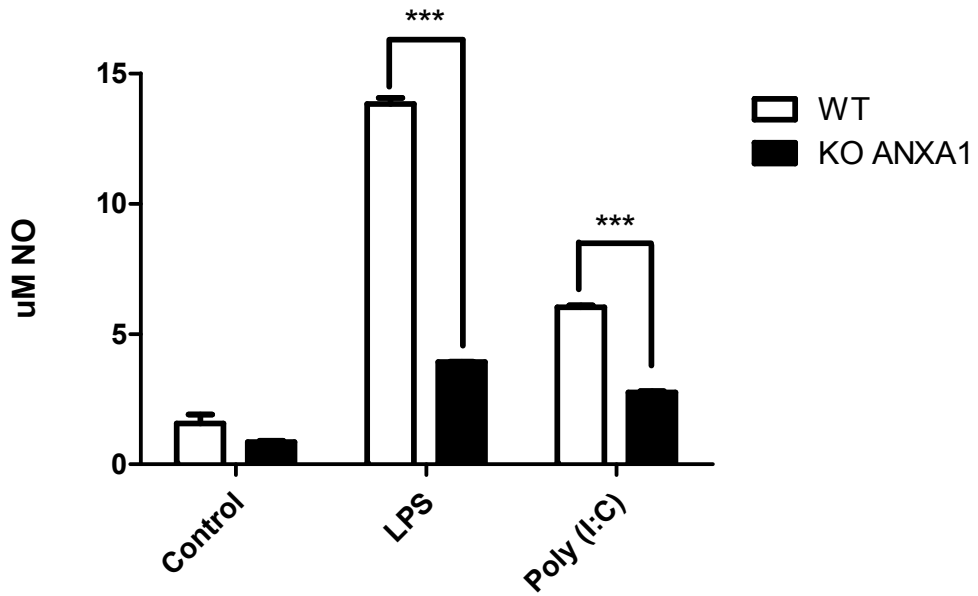


Figure 4.5. ANXA1 KO macrophages exhibited diminished NO production levels. NO levels compared to wild-type BMDM, after treatment with TLR agonists LPS (1ug/ml) and poly (I:C) (10ug/ml), based on Greiss assay. *** denotes $p<0.001$. Results shown is an average from three biological repeats (n=3).

4.2.4. ANXA1 and STAT- 1 signaling

Another major transcription activator which is directly involved in M1 polarization is STAT-1. Activation of STAT-1 through phosphorylation of tyrosine residue 701 (Y701) is important for both bacterial and viral immunity (Burbin *et al.,* 1996; Kristensen *et al.,* 2011). STAT-1 is mainly involved in triggering type I interferon response which is induced by external signal transduction *via* interferon-gamma (IFN-γ) stimulation. Thus, we examined if STAT-1 could also be responsible for ANXA1-dependent functions or *vice versa*.

4.2.5. ANXA1 does not regulate IFN-γ stimulated cytokine / chemokine production.

Throughout our experimental results, a decrease in both IL-12 and IP-10 production in ANXA1 KO macrophages was observed compared to wild-type macrophages when treated with poly (I:C) or LPS. To activate STAT-1 specific activity, and to examine whether STAT-1 activation is affected by ANXA1, we stimulated the macrophages with STAT-1 activating IFN-γ (100 U/ml). We also used a known M1 polarizing treatment, i.e. IFN-γ (100 U/ml) with LPS (100 ng/ml), to examine whether the inhibition in cytokine production could be STAT-1 dependent.

Upon treatment with IFN-γ alone, an increase in IP-10 production was observed in both wild-type and ANXA1 KO BMDM. Interestingly, ANXA1 KO BMDM produce similar amounts of IP-10 compared to WT. This gave us

a good indication that IP-10 production impairment observed in ANXA1 KO macrophages during TLR agonist treatment is TLR pathway specific, and based on this data, suggests the impairment is TRIF specific, since IP-10 is produced through TRIF- dependent pathway.

Next, M1 polarizing stimulation given by exposure to both IFN-γ and LPS was examined. Similar to results for IFN-γ treatment alone, both wild-type and ANXA1 KO macrophages produce statistically identical amount of IP-10, indicating that STAT-1 activation and downstream transcription pathway is intact in ANXA1 KO macrophages. This also demonstrated that when ANXA1 is not involved in regulating STAT-1 dependent activation. To ascertain whether the production of IP-10 after M1 polarization affects STAT-1 activation compared to activating STAT-1 only through IFN-γ, we added IFN-γ 18 hours after M1 polarization to ascertain if secondary exposure to IFN-γ affects IP-10 production. WT and ANXA1 KO macrophages produce similar levels of IP-10, indicating that ANXA1 does not affect STAT-1 signaling (Figure 4.6 a).

IL-12 production was also examined. STAT-1 activation through IFN-γ alone does not trigger the production of IL-12 in both wild-type and ANXA1 KO macrophages. Interestingly, when M1 polarizing stimulation was administered (IFN-γ and LPS), both wild-type and ANXA1 KO macrophages produced substantial and statistically similar levels of IL-12, indicating the capacity of STAT-1 driven pathway is not affected in ANXA1 KO macrophages (Figure 4.6 b). Again, this gives a good indication that IL-12 impairment observed in ANXA1 KO macrophages is a highly specific mechanism which likely involves a complex that does not associate with

STAT-1 pathway. When a secondary dose was administered 18 hours after initial stimuli, IL-12 production in both wild-type and ANXA1 KO macrophages remained unchanged. This indicates a sustained M1 polarizing capability and its capacity to retain IL-12 production. As a whole, this data therefore shows that ANXA1 is not involved in STAT-1 activation.

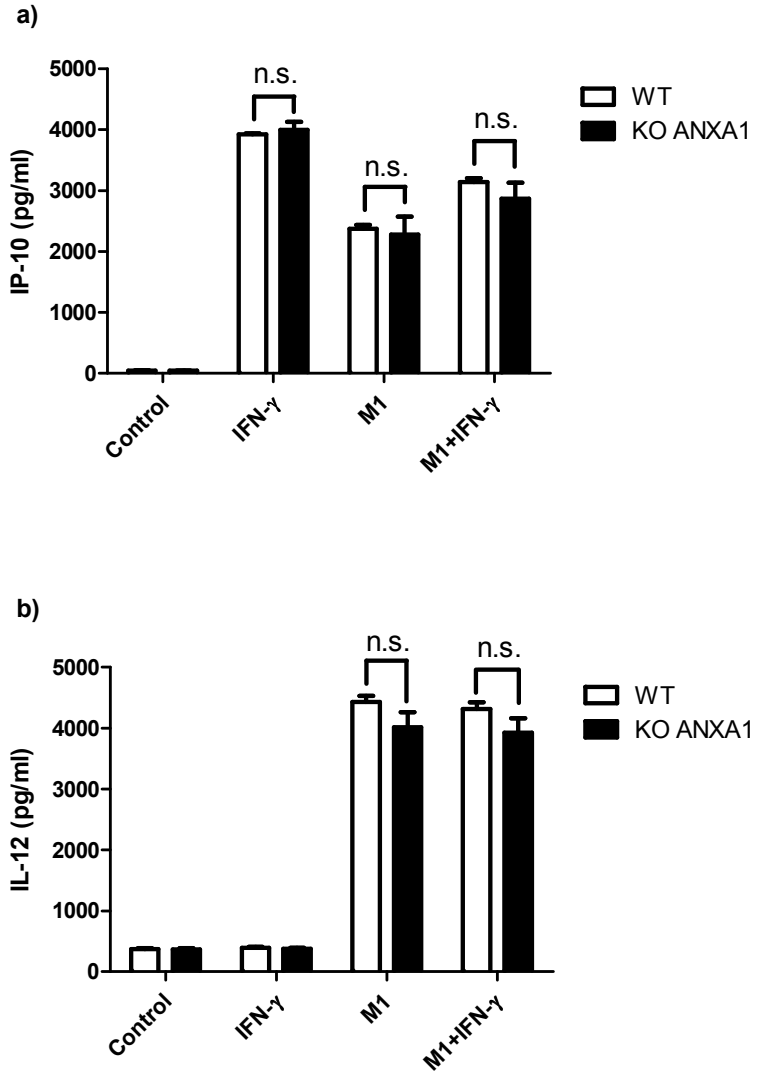


Figure 4.6. STAT-1 dependent cytokine / chemokine production is not affected by absence of ANXA1. a) IP-10 production and b) IL-12 production of wild-type and ANXA1 KO BMDM treated with IFN-γ (100U/ml) and or M1 polarizing stimulant (100 ng/ml of LPS with 100U/ml of IFN-γ). "n.s." denotes "no significant difference", i.e. $p>0.05$, Results shown is an average between three biological repeats (n=3).

4.2.6. STAT-1 phosphorylation is not affected by absence of ANXA1

We determined whether activation of STAT-1 is affected in ANXA1 KO PM. STAT-1 activation levels were next examined through the use of western blotting.

Figure 4.7 shows the results for western blot on wild-type and ANXA1 KO PM, after treatment with LPS. Without any treatment, similar levels of STAT-1 protein expression were observed in both wild-type and ANXA1 KO PM. No change in STAT-1 levels was observed in both WT and ANXA1 KO PM after LPS treatment. No phosphorylation of STAT-1 for both wild-type and ANXA1 KO macrophages was observed at 2 h of LPS. At 4 h and 24 h post LPS treatment, STAT-1 phosphorylation is observed to be at equal levels for both wild-type and ANXA1 KO macrophages.

These results demonstrated that ANXA1 is not involved in influencing STAT-1 phosphorylation and activation after LPS.

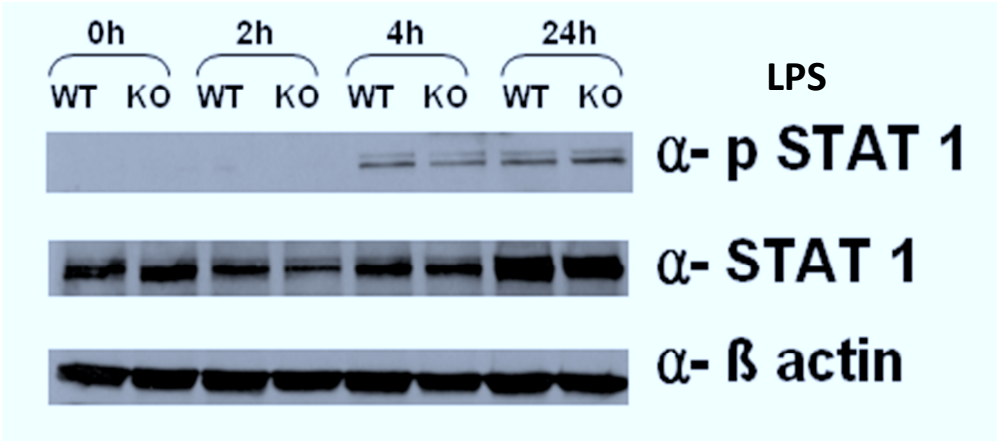


Figure 4.7. STAT-1 phosphorylation and basal expression level is unchanged by absence of ANXA1. Wild-type (WT) and ANXA1 KO (KO) PM macrophages were given LPS treatment for 2h , 4h and 24h respectively. 1 ug/ml of LPS was given as experimental treatment. Western blotting was done with a protein lysate concentration of 100 ug / ml. Results shown is a representative among two biological repeats (n=2). "α-p STAT-1" denotes antibody probe for phosphorylated STAT-1 at tyrosine residue 701. "α- STAT-1" denotes antibody probe for STAT-1. "α-Actin" denotes antibody probe for actin, as loading control.

4.3. ANXA1 and PPAR-γ signaling

There are known natural NF-κB inhibitors that would inhibit production of IL-12 and IP-10 upon LPS exposure in phagocytes. Although ANXA1 was shown to bind and inhibit NF-κB signaling in cancer cells (Zhang *et al.,* 2010), it remains unclear whether ANXA1 regulates NF-κB in healthy phagocytes but findings were limited to *in vitro* experiments using exogenous application of ANXA1 peptide on murine macrophage cell lines (Alldridge *et al*, 1999; Xu *et al.,* 2009). This led to a question whether a natural inhibitor of NF-κB signaling exists which ANXA1 may regulate directly in healthy phagocytes.

One such natural inhibitor of NF-κB is PPAR-γ, a natural NF-κB inhibitor. In addition, PPAR-γ has been shown to be a M2 polarizing stimuli. We hypothesized that PPAR- γ signaling may be dysregulated in ANXA1 KO macrophages, thus causing the impaired cytokine response, and skewing the polarization to the M2 phenotype. PPAR-γ function is involved with ANXA1 based on previous studies in our lab that has linked ANXA1 as a potential PPAR-γ regulator in cancer cells (unpublished data). As PPAR-γ is a known natural NF-κB inhibitor acting through NFkB p65/RelA subunit (Giulia *et al.,* 1998; Vanden Berghe *et al.,* 2003), and since ANXA1 was shown to interact tightly and regulate upstream elements of NF-κB signaling pathway (Bist *et al.,* 2011), the study focused on whether downregulation of both My-D88 dependent and TRIF-dependent cytokines continues to be observed when ANXA1 KO macrophages were exposed to PPAR-γ agonists. This would

determine if PPAR-γ is responsible for the attenuation in cytokine production found in ANXA1 KO macrophages.

4.3.1. Investigating PGJ_2 as a PPAR-γ specific agonist in wild-type macrophages

We first investigated a well-known, endogenously and readily bio-available PPAR-γ specific agonist 15-deoxy-Δ12,14-prostaglandin J2, or PGJ_2 in wild-type BMDM, in a dose-dependent manner. PGJ_2 is capable of inducing small amounts of IL-12 even at low doses (Figure 4.8 a). When macrophages are treated with LPS, IL-12 production is substantially increased, and upon pre-treatment for 1 hour with PGJ_2 , IL-12 production decreased in a dose-dependent manner (Figure 4.8 b). This result is consistent with literature which studied IL-12 released by macrophages induced with LPS and PGJ_2 showing a dose-dependent inhibition of IL-12 production (Alleva *et al.,* 2002), thereby providing a robust model for downstream observation and analysis of results.

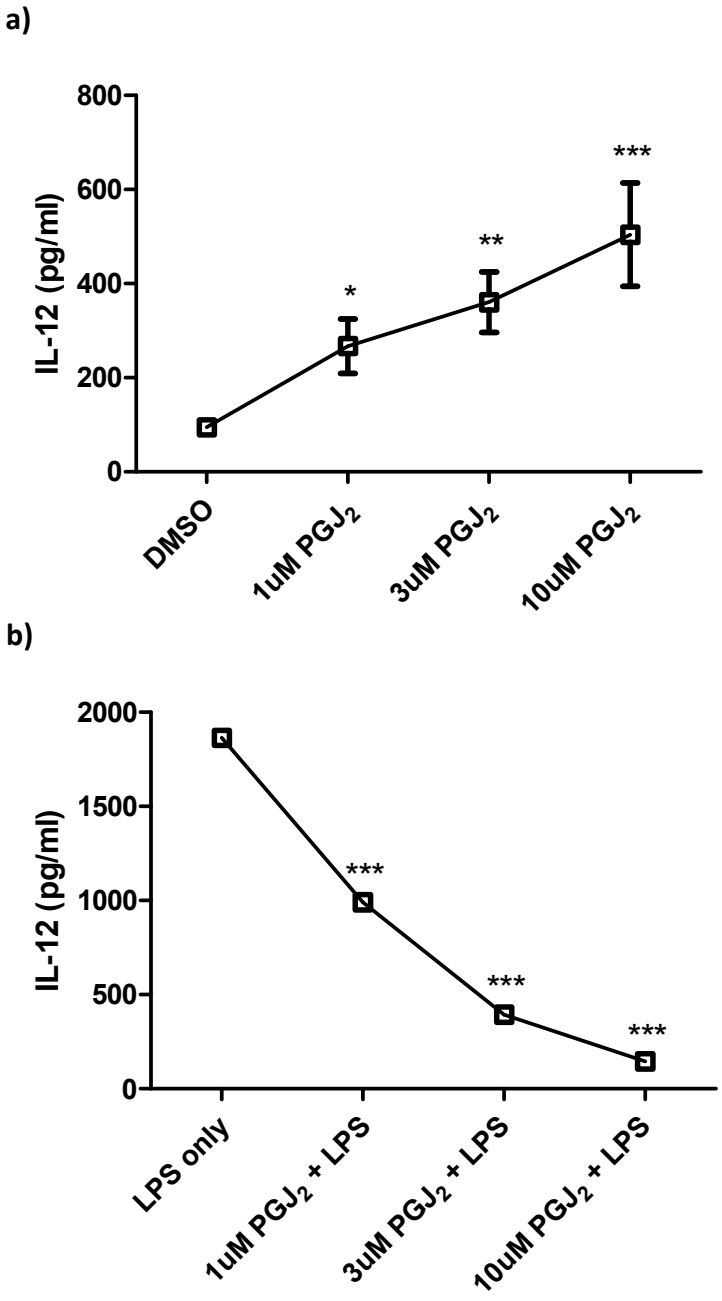


Figure 4.8. Wild-type macrophage response to PGJ_2 treatment. PGJ_2 was given as a pre-treatment 1 hour prior to addition of LPS to samples. *** denotes $p<0.001$, ** denotes $p<0.01$, * denotes $p<0.05$, "n.s." denotes "no significant difference", i.e. $p>0.05$ when compared to DMSO only (for PGJ_2 treatment alone) or LPS only (for PGJ_2 + LPS treatment). Results shown is a representative among three biological repeats (n=3).

4.3.2. Stimulating PPAR-γ with PGJ_2 inhibited IL-12 production

We next investigated the effect of PGJ_2 on ANXA1 KO BMDM. These graphs are depicted in Figure 4.9 and 4.10, showing control and PGJ_2 treated cells side by side. When cells were treated with PGJ_2 alone, IL-12 was produced significantly lesser in ANXA1 KO macrophages. After treatment with LPS, 1μM PGJ_2 inhibits IL-12 production in WT, but not in ANXA1 KO macrophages. Since PGJ_2 is a PPAR-γ agonist, this result suggests that ANXA1 may be required for the inhibitory effect induced by PGJ_2 (Figure 4.9 b).

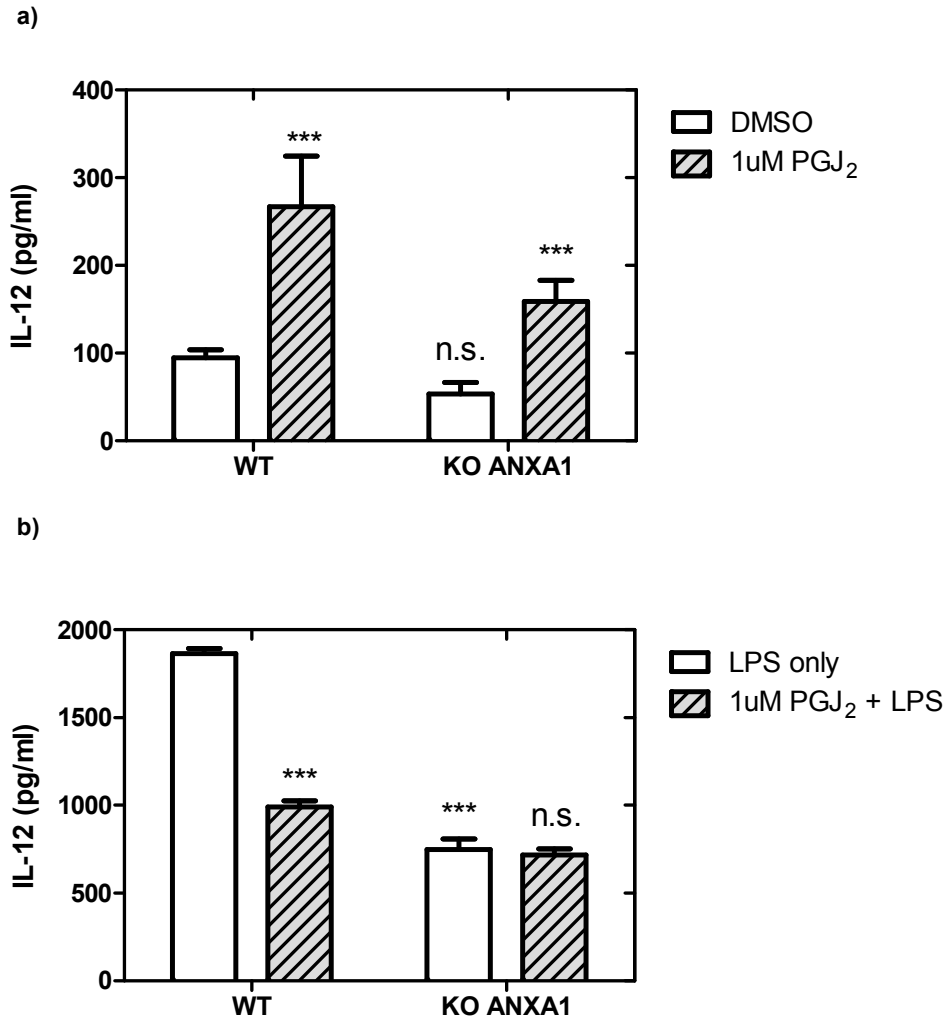


Figure 4.9. ANXA1 KO BMDM response to low dose of PGJ_2 treatment (1 μM). PGJ_2 was given as a pre-treatment 1 h prior to addition of LPS to samples. *** denotes $p<0.001$, ** denotes $p<0.01$, and "n.s." denotes "no significant difference", i.e. $p>0.05$. Results shown is a representative among three biological repeats (n=3).

As PGJ_2 is an inhibitor of IL-12 production and ANXA1 KO also exhibited inhibited IL-12 production, further analysis of our results was required to determine if the inhibition by PPAR-γ is abrogated in ANXA1 KO PM. To do so, analysis for percentage of inhibition of IL-12 for PGJ_2 treatment was performed by normalizing the production of IL-12 produced by BMDM pre-treated with PGJ_2 prior to LPS treatment by dividing it with the production of IL-12 with LPS treatment alone. This was done for both WT and ANXA1 KO BMDM for pre-treatment with 1 μM PGJ_2. IL-12 inhibition is not observed at 1 μM PGJ_2 upon treatment with LPS (Figure 4.10). Therefore ANXA1 might regulated PPAR-γ –dependent production of IL-12 in BMDMs.

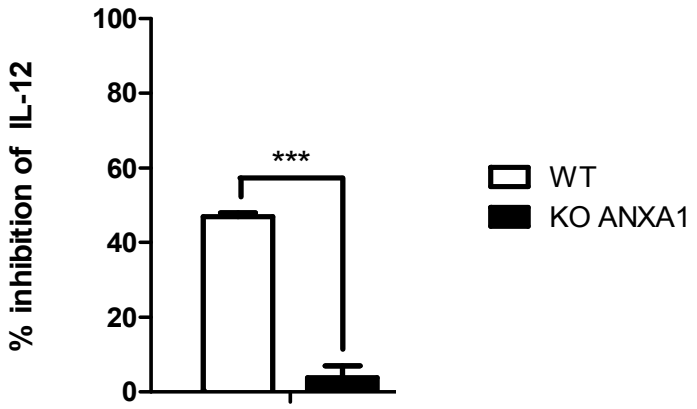


Figure 4.10. Percentage inhibition of IL-12 production after LPS stimulation with 1 μM PGJ_2 pre-treatment. *** denotes $p<0.05$ compared to WT. Results shown is a representative among three biological repeats (n=3).

4.3.3. Investigating the role of endogenous PPAR-γ in ANXA1-regulated cytokine production –use of GW9662

Next, in order to confirm PPAR-γ as an endogenous regulator involved in the impairment of MyD88 dependent IL-12 production, we inhibited PPAR-

γ through the use of PPAR-γ specific inhibitor. We hypothesized that treatment with GW9662 would abrogate the endogenous link between PPAR-γ to ANXA1, i.e. GW9662 would reverse the IL-12 inhibition in ANXA1 KO BMDM upon exposure to LPS if endogenous PPAR-γ was involved. Prior to our investigation, we first examined WT BMDM responses to PPAR-γ inhibitor GW9662 at 1μM, 3μM and 10μM levels as a pre-treatment prior to LPS exposure.

GW9662 alone inhibited the production of IL-12 in WT BMDM (Figure 4.11 a), indicating that PPAR-γ may be involved in basal IL-12 production. When used as a pre-treatment to LPS, all doses of GW9662 reduces IL-12 production levels significantly (Figure 4.11 b). Although GW9662 is an inhibitor of PPAR-γ, and despite being a PPAR-γ specific antagonist, some action of its chemistry as molecular inhibitor may yet be unknown. Moreover, this decrease in IL-12 level upon pre-treatment with GW9662 prior to exposure to LPS is consistent with findings in other laboratories (Zhang *et al.,* 2004).

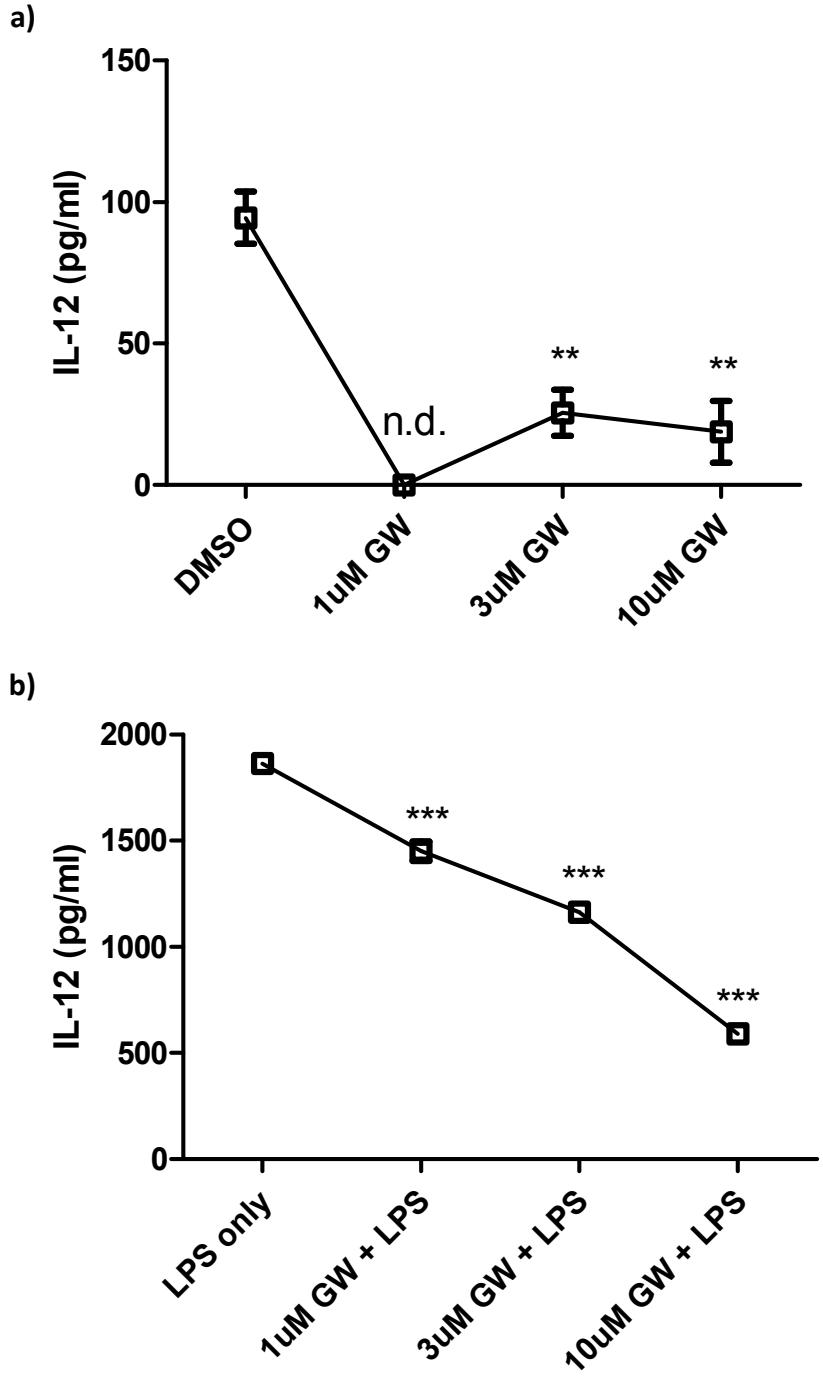


Figure 4.11. WT BMDM response to GW9662 treatment. GW9662 (GW) was given as a pre-treatment 1 h prior to addition of LPS to samples. ** denotes $p<0.01$, *** denotes $p<0.001$ when compared to DMSO only (for PGJ_2 treatment alone) or LPS only (for PGJ_2 + LPS treatment). "n.d." denotes "not detected", i.e. below detection limit of IL-12 ELISA kit. Results shown is a representative among three biological repeats (n=3).

4.3.4. Inhibiting PPAR-γ reverses ANXA1-KO IL-12 inhibition

We next examined ANXA1 KO BMDM and its response after pre-treatment with GW9662 prior to LPS exposure. Similar to wild-type macrophages, ANXA1 KO BMDM did not produce any IL-12 upon treatment with GW9662 alone, at all concentrations investigated.

Upon treatment with 1 μM of GW9662 prior to LPS exposure, ANXA1 KO BMDM exhibited a significant increase in IL-12 production compared to ANXA1 KO BMDM exposed only to LPS, albeit being lower in production compared to its wild-type counterparts in both LPS treatment alone samples and 1 μM GW9662 + LPS. This was also observed for 3 μM GW9662 treatment before LPS exposure, but the difference of IL-12 production between LPS treated and 1 μM GW9662 with LPS treated ANXA1 KO BMDM was not as marked, but remained significantly different ($p<0.05$). Pre-treatment with 1μM GW9662 before LPS increased IL-12 production level in ANXA1 KO BMDM compared to LPS treatment alone (Figure 4.12).

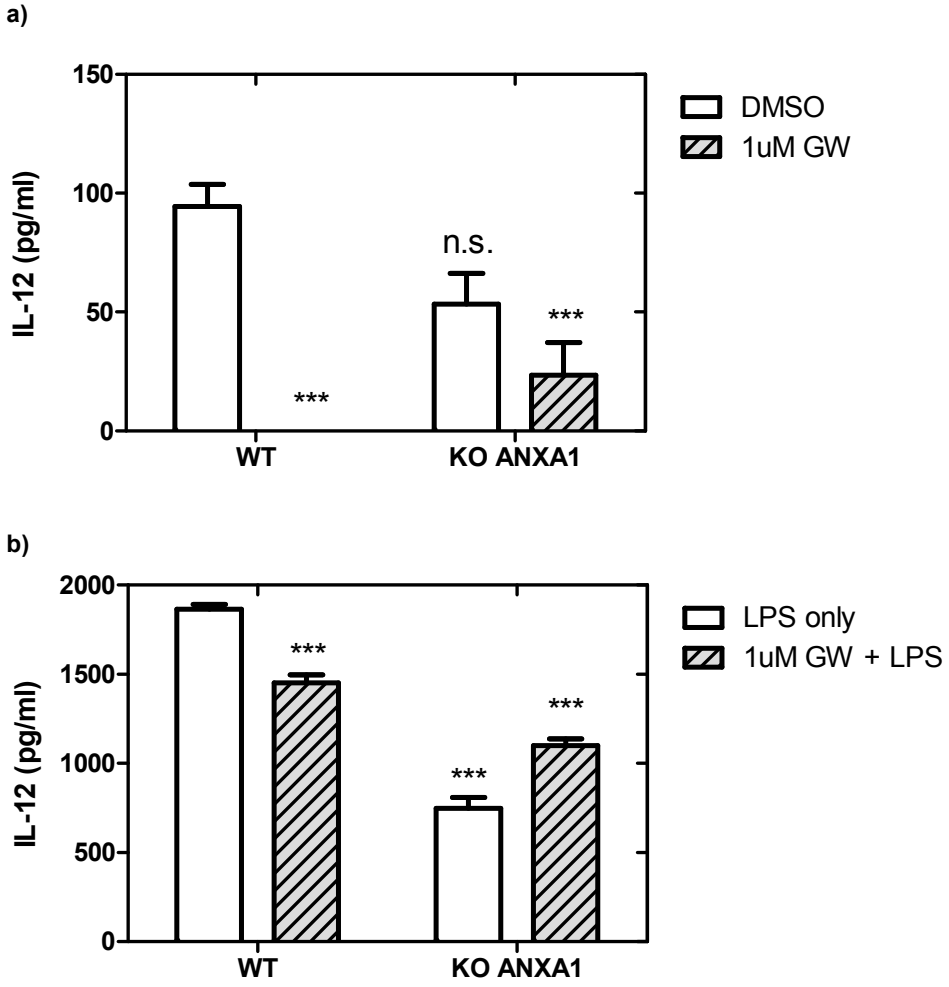


Figure 4.12. ANXA1 KO BMDM response to low dose of GW9662 treatment (1 μM). GW9662 was given as a pre-treatment 1 hour prior to addition of LPS to samples. ** denotes $p<0.01$ compared to WT. Results shown is a representative among three biological repeats (n=3).

As GW9662 is an inhibitor of PPAR-γ and PPAR-γ is an inhibitor of NF-κB, further analysis was required to visualize the effect of GW9662 on ANXA1 KO BMDM. To do so, the same method of analysis for percentage of inhibition of IL-12 done in PGJ_2 was employed. IL-12 production is enhanced at 1 μM GW9662 upon treatment with LPS (Figure 4.13). The result is consistent with the hypothesis that PPAR-γ is regulated by ANXA1, since inhibition of PPAR-γ in ANXA1 KO BMDM causes enhancement of IL-12 production. This result demonstrates that PPAR-γ regulates IL-12 production after stimulation with LPS, and ANXA1 regulates PPAR- γ.

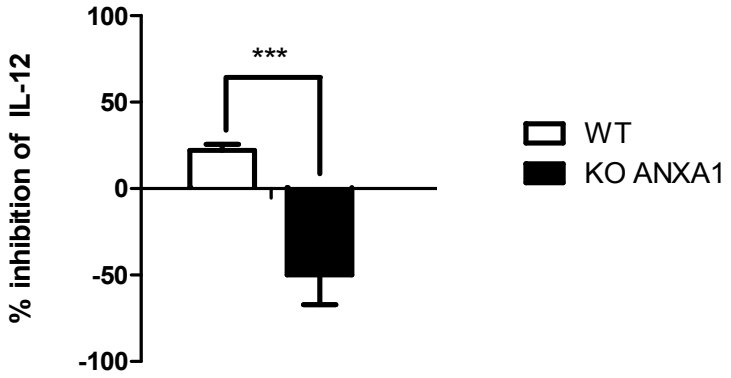


Figure 4.13. Percentage inhibition of IL-12 production after LPS stimulation with 1 μM GW9662 pre-treatment. *** denotes $p<0.05$ compared to WT. Results shown is a representative among three biological repeats (n=3).

4.3.5. Investigating a clinically relevant synthetic PPAR-γ ligand

Despite PGJ_2 showing a promising side in the likely involvement of PPAR-γ in deficiency of cytokine / chemokine production in ANXA1 KO macrophages at small doses of PGJ_2 (1 μM), it remains in question whether such observation is unique to the ligand PGJ_2. We investigated if other synthetically derived ligands used clinically may also be capable of showing that ANXA1 inhibit PPAR-γ activity during IL-12 production upon exposure to LPS. Troglitazone (commercially known as Rezulin) was once a popular PPAR- γ agonist in the anti-diabetes market until its sale was prohibited for liver toxicity (Penumetcha and Santanam, 2012).

Troglitazone by itself did not induce noticeable IL-12 production in both wild-type and ANXA1 KO macrophages (Figure 4.14). Clinically relevant doses of 10 μM troglitazone show significant inhibition of IL-12 production in wild-type but not ANXA1 KO BMDM when compared to production levels during LPS treatment alone. This result was consistent with observation made on PGJ_2 in this study. Therefore this result also supports the hypothesis that ANXA1 controls IL-12 production through the regulation of PPAR-γ.

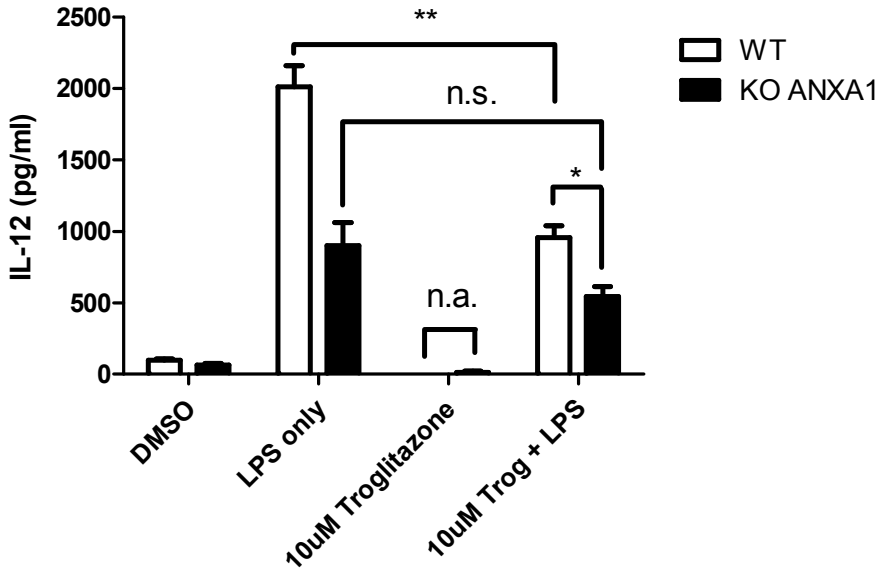


Figure 4.14. Wild-type and ANXA1 KO macrophage response to PPAR-γ troglitazone as a pre-treatment 1 hour prior to addition of LPS. * denotes $p<0.05$, ** denotes $p<0.01$ and "n.s." denotes "no significant difference", i.e. $p>0.05$. Results shown is a representative among three biological repeats (n=3).

4.4. Chapter 4 conclusion

Macrophage polarization is a good prognosticating tool in the study of macrophages which show impairment in several spectrums of cytokine / chemokine production pathways. This has been particularly useful to identify ANXA1 as a novel regulator of macrophage polarization.

This chapter demonstrated several key influences ANXA1 has on macrophage polarization. ANXA1 is a regulator of both NF-κB and IRF-3 transcription factors, which are important regulators of M1 polarization. Also, ANXA1 does not partake in IFN-γ activated STAT-1 pathway. Moreover, ANXA1 is a strong suppressor for M2 polarization in macrophages. Finally,

we showed that PPAR-γ is involved in the inhibition of IL-12 in ANXA1 KO macrophages. These results are summarized in Figure 4.15.

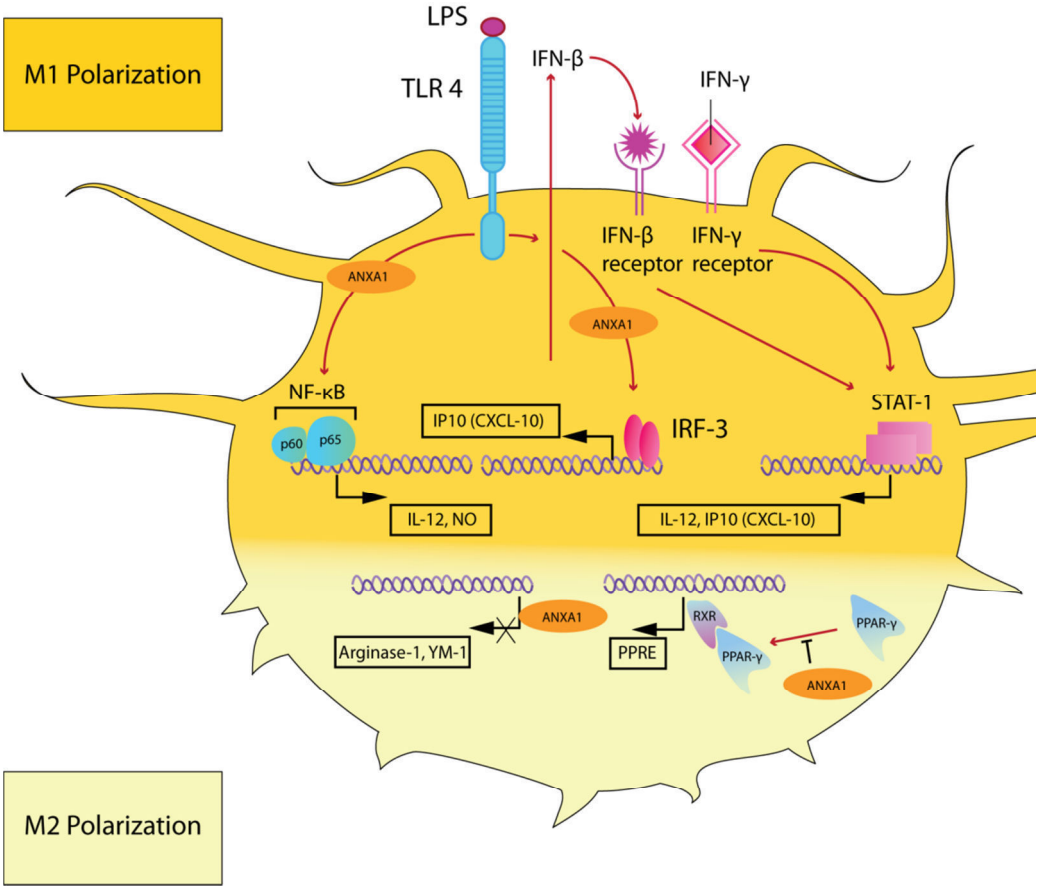


Figure 4.15. ANXA1 influences both M1 and M2 polarization. This diagram summarizes the overall picture of the involvement of ANXA1 in macrophage polarization. ANXA1 is important for activating transcription factors NF-κB and IRF-3, but is not associated with STAT-1 activation. Moreover, ANXA1 also influences M2 polarization, as absence of ANXA1 causes upregulation of expresion in M2 markers Arginase-1 and YM-1 (Infographic by Shu Shin La).

CHAPTER V: DISCUSSION

Sepsis is one of the most challenging bacteria-related conditions worldwide, and is the leading cause of death in patients with multi-organ failure

(Rittirsch *et al*., 2012). It is also the major cause of death in neonates younger than six months: an estimated one million neonates worldwide die in a year from sepsis (Wynn and Wong, 2010). In U.K., ~30% of adults admitted to intensive care unit meet the severe sepsis criteria (Padkin *et al*., 2003). In U.S. alone, more than 10 million patients were affected by sepsis between 1979 through 2000 (Martin *et al*., 2003) and annual total cost for the treatment of sepsis exceeds $16 billion annually (Angus *et al*., 2001).

Sepsis is a continuum beginning with a host-pathogen interaction that triggers and interplay between pro-inflammatory and anti-inflammatory mediators, and ending with an overly activated inflammatory response which then overwhelms the host (Rackow *et al*., 1991). TLR activation by microbial infection such as bacteria, viruses and fungi is a key component in the outcome of sepsis (Tsujimoto *et al*., 2008; Weighardt and Holzmann, 2007). Mice lacking key signaling adaptor proteins for TLR activation such as MyD88 $^{-/-}$ and TRIF $^{-/-}$ mice do not die of septic shock, purportedly due to reduced inflammatory burden and cytokine production during microbial invasion (Reim *et al*., 2011; Feng *et al*., 2011).

There are certain subsets of immune cells linked to sepsis: notably macrophages, a dominant sentinel found at initial sites of infection, a major source of many cytokines involved in immune response (Cavaillon, 1994; Holgate, 2000; Hume, 2000; Scull *et al*., 2010).

The innate immune system is evolutionarily conserved to provide first line of defense and host protection against invading microbial pathogens. Initially identified through Toll, a unique protein from fruit fly *Drosophila melanogaster* which showed participation in innate immune responses, homologues of Toll, termed Toll-like receptors (TLRs) found in mammals were also demonstrated to recognize PAMPs and elicit innate immune responses through induction of inflammatory cytokines (Akira *et al*., 2001). Since the discovery of TLRs, many studies have joined or segregated the intracellular signaling pathway of TLRs to the activation of NF-κB, a transcription factor extensively involved in the regulation of innate immunity and inflammation (Hoffmann, 2003; Medzhitov *et al*., 1997).

The most frequently activated form of NF-κB in TLR signalling is a heterodimer composed on RelA (p65) and p50 (Poltorak *et al*., 1998). This heterodimer is kept in its latent state through the interaction with the inhibitor,s IκB. Stimulation of TLR triggers the phosphorylation of Inhibitor of κB (IκB) by IκB kinase (IKK) complex signaling the degredation of IκB by poly-ubiquitination. NF-κB is released from inhibition by IκB, allowing for RelA and p50 heterodimer to begin transcription by translocating into the nucleus. The IKK complex therefore indirectly regulates NF-κB. The IKK complex is made of IKK-α and IKK-β which is important for the activation of NF-κB signaling, and a NF-κB essential modifier (NEMO or IKK-γ) which acts as a regulatory component of IKK complex (Medzhitov *et al*., 1997). We have recently shown that ANXA1 can interact with members of the NF-κB family, such as NF-kappa-B essential modulator (NEMO) or IKK-γ as well as

Receptor interacting protein 1 (RIP1), a signaling molecule important in the activation of NF-κB (Bist *et al*, 2011). Therefore, in this project, we placed emphasis on deciphering the roles of ANXA1 in innate immunity.

Activation of innate immunity is an important step towards antigen-specific acquired immunity development. Among pattern recognition receptors, TLRs are tasked to perform this specific role of activating innate immune response. Recognition of microbial pattern products by TLRs triggers TLR signaling pathways regulated by intracellular adaptors.

TLRs trigger downstream signaling cascade by recruiting specific combination of TIR domain-containing adaptors such as MyD88, MyD88 adaptor-like (MAL), TRIF and TRIF-related adaptor molecule (TRAM) (Takeda and Akira, 2005; Takeuchi *et al*., 1999). Among these adaptor proteins, MyD88 is the sole adaptor used by TLR5, TLR7 and TLR9. TLR2 and TLR4 also uses adaptor protein MyD88, although not as much as other adaptor proteins like Toll/Interleukin-1 receptor adaptor protein (TIRAP), TRIF and TRAM . (Takeuchi *et al*., 200; Ozinsky *et al*., 2000; Alexopoulou *et al*., 2001; Hayashi *et al*., 2001; Zhang *et al*., 2004; Yarovinsky *et al*., 2005; Hemmi *et al*., 2002). Only TLR3 specifically uses TRIF for signaling, and is independent of MyD88 signaling (Hayashi *et al*., 2001; Alexopoulou *et al*., 2001). Therefore activation of MyD88 is synonymous with TLR activation in all TLR except for TLR3, for activation of NF-κB, leading to the induction of inflammatory cytokine genes (Kawai and Akira, 2005). This leads to the notion of TLR signaling being largely segregated into two discrete pathways: the MyD88-dependent and the TRIF-dependent pathways. In this study, we focused on TLR agonists CpG DNA (CpG1826), poly (I:C) and LPS that

initiates TLR signal transduction through MyD88, TRIF- and both MyD88 and TRIF respectively. CpG 1826 activates TLR9 transduction of downstream signal solely through MyD88 adaptor protein. On the other hand, poly (I:C) ,a TLR3 agonist activates downstream pathway through TRIF adaptor protein. LPS is capable to sending downstream signals through both TLR 3 and 4, thus utilizing both MyD88 and TRIF adaptor proteins to relay downstream signals for activation of immune cell response.

The MyD88 adaptor protein is composed of a Toll/Interleukin-1 receptor (TIR) domain and a death domain. The death domain is used for interaction with Interleukin-1 receptor-associated kinase 1 (IRAK) family of protein kinases, namely IRAK1, IRAK2, IRAK4 and IRAK-M (Takeda and Akira, 2005). Function of IRAK2 remains unclear (Kawai and Akira, 2007). IRAK4 is initially activated by MyD88, which in turn phosphorylates IRAK1, forming a temporal complex between IRAK1, IRAK 4 and MyD88. IRAK-M is believed to inhibit release of IRAK1 and IRAK4 from MyD88, thus inhibiting signal transuction (Diebold *et al*., 2004). Alterantively , IRAK1 and IRAK4 are released from MyD88 upon their phosphorylation and interact with NEMO through another adaptor protein, TRAF6. This occurs by ubiquitination of NEMO at lysine 63 residue by TRAF6 (Heil *et al*., 2004, Heil *et al*., 2003), causing the recruitment of TAK1 and TAB (Heil *et al*., 2004), which then activates two downstream pathways, the NF-κB pathway and the MAPK pathway (Kawai and Akira, 2007). Our lab has previously published a report detailing the interaction of ANXA1 with IKK complex through NEMO, and that ANXA1 is important to the recruitment of RIP-1 to

the IKK complex, which is critical for activation of NF-κB (Meylan *et al*., 2004; Bist *et al*., 2011

NF-κB plays an important role in the regulation of variety of genes involved in inflammatory immune response (Ghosh *et al*., 1998). The active form of NF-κB is composed of NF-κB/Rel family members in homo- and hetero- dimers. In the cytoplasm, NF-κB exists as an inactive form that is bound to a family of inhibiting molecules I-kappa-B (IκB), consisting of three subunits, namely IκB-α, IκB-β and IκB-ε (Fumiko *et al*., 2000). Activation of NF-κB requires the phosphorylation of IκB, which is followed by the polyubiquitination degradation of IκB by 26S proteasome. The dissociation of IκB from NF-κB due to the phosphorylation of IκB allow NF-κB complex to be released and translocated into the nucleus, which cuases upregulation of myriad genes involved in pro-inflammatory cytokine response (Baeuerle and Henkel, 1994; Verma *et al*., 1995; Baeuerle and Baltimore, 1996). Due to the nature of inhibitory mechanism by IκB family which controls the activation of NF-κB, the activator of IκB complex, kinases responsible for IκB phosphorylation is an upstream epicentre for control of NF-κB activity, i.e. the IκB kinases or IKK complex. Our lab has shown that ANXA1 can positively regulate NF-κB activity in breast cancer cells. Therefore, we hypothesized that ANXA1 could also positively regulate NF-κB activity in macrophages stimulated with TLR agonists. However, through our initial studies, we determined that ANXA1 could regulate LPS but not CpG DNA-stimulated cytokine production, demonstrating that the MyD88-dependent pathway was not regulated by ANXA1. Therefore, as LPS also triggers the MyD88-independent pathway, or the TRIF-dependent pathway, we determined if ANXA1 could activate TRIF

signaling to regulate NF-κB activation. IRF-3 is also dependent on NEMO (IKK-γ) for TLR3 mediated immunity (Audry *et al*., 2011), and we hypothesized that ANXA1 may regulate NF-κB through TRIF. Indeed, ANXA1 regulated NF-κB p65 nuclear translocation in response to poly(I:C) but not LPS, indicating that TRIF-dependent NF-κB activation, and not MYD88-dependent NF-κB activation is regulated by ANXA1.

The adaptor protein TRIF links TLR3 and TLR4 to IRF-3, an anti-viral transcription factor, through TBK-1 recruitment and phosphorylation of IRF-3 by TBK1. This induces IRF-3 to dimerize and translocate into the nucleus, triggering transcription of type 1 interferons (IFN-α and IFN-β) (Sugimoto *et al*., 2004). Another adaptor protein that links TRIF and TBK1 is TRAF3. TRAF3 deficient mice fail to induce IFN-β response when stimulated with TLR3 and TLR4 ligands (Kawai *et al*., 1999; Theofilopoulos *et al*., 2005). Therefore TLR3 and TLR4 use the TRIF pathway to induce INF-β to develop an antiviral state. This activation of TRIF dependent pathway is independent of MyD88 pathway, as evidenced by the impairment of TLR3 and TLR4 ligand induced activation only observed in TRIF deficient mice but not in MyD88 deficient mice (Alexopoulou *et al*., 2001; Yarovinsky *et al*., 2003). We demonstrate that ANXA1 is required for IRF-3 to translocate into the nucleus to initiate macrophage activation after stimulation with LPS or poly (I:C), and other data from our lab also show that ANXA1 can control IRF-3 phosphorylation, in ANXA1 deficient macrophages which exhibit impaired phosphorylation and dimerization of IRF-3. In addition, surface activation markers MHCII and CD86 on macrophages from ANXA1 KO mice show an attenuated activation profile after LPS or poly (I:C) treatment . This data

implies that ANXA1 can activate IRF-3 in the MyD88-independent or TRIF-dependent pathway, which is possibly responsible for the impairment of IL-12 and IP-10 production in ANXA1 KO macrophages.

The TRIF adaptor protein can also activate NF-κB, but as a late phase response. TRIF directly binds to TRAF6, purportedly linking MyD88 and TRIF in early response (Lund *et al*., 2003; Coban *et al*., 2005), but a detailed study that followed TRAF6 activity concluded that TRAF6 is dispensible for TRIF-dependent NF-κB activation (Gohda *et al*., 2004). Therefore the activation of NF-κB by TRIF is more likely through an auto-feedback loop derived from the production of type-I antiviral response (Du *et al*., 2007; Pfeffer, 2011). It may be possible that ANXA1 plays a role in the cross-talk between the MyD88 and TRIF pathways, but this needs to be investigated further.

Therefore, the TLR agonists chosen in this study stimulate two distinct signaling mechanisms: MyD88 dependent and TRIF dependent pathways. This draws a clear division for the role of ANXA1 in TLR agonist-induced cytokine production. IL-12 / IP-10 production was only significantly inhibited in the ANXA1 KO when LPS and poly (I:C) treatment was given, but not after exposure to CpG 1826. This highlights the importance of ANXA1 in TRIF-dependent pathway, but not TLR2 activated MYD88-dependent pathway. The impairment of TLR 3 and 4 dependent IL-12 / IP-10 production is not a unique phenotype to ANXA1 KO macrophages- it was previously reported that macrophages from MyD88 deficient mice are incapable of producing cytokines in response to TLR3 and TLR4 agonists.(Yamamoto *et al*., 2003). IL-12 production is completely abrogated in MyD88-deficient mice

administered with LPS, and they do not undergo endotoxin shock (Kawai *et al*., 1999). This highlights the importance of MyD88 as an adaptor protein in cytokine production, in particular IL-12. On the other hand, IP-10 levels are shown to be impaired in TRIF-deficient mice compared to wild-type mice, indicating the relevance of TRIF adaptor protein in IP-10 production during TLR dependent activation (Yamamoto *et al*., 2002). As both IL-12 and IP-10 levels are impaired, the result implicates ANXA1 in both MyD88 dependent and TRIF dependent pathways. It is however, more akin to what was observed in TRIF knockout mice, where both TLR3 and TLR4 mediated responses were impaired, once again suggesting that ANXA1 is important for TRIF-dependent IL-12 and IP-10 production.

This study has demonstrated that although IL-12 production was impaired in ANXA1 KO PM when compared to WT after treatment with LPS, there was no significant difference in IL-6 production between WT PM and ANXA1 KO PM after treatment with LPS. This may be due to the specificity of IL-6 to the MyD88 pathway, i.e. MyD88 deficient mice is unresponsive to LPS and does not produce IL-6 (Kawai et al., 1999). In contrast, IL-12 is a cytokine produce in synergy with both MyD88 and TRIF pathway, i.e. TRIF pathway can also trigger IL-12 production (Krummen et al., 2010). As this study proposes that ANXA1 KO PM exhibit impaired TRIF dependent response, therefore the impairment of IL-12 production is a TRIF specific pathway impairment, compared to IL-6 that is solely MyD88 dependent in production.

Yang *et al*., (2009) and Yona *et al*., (2006) investigated IL-6 and TNF-α levels in ANXA1 KO macrophages after LPS stimulation, and both

cytokines were shown to be increased compared to wild-type macrophages. However, in this study the TNF-α mRNA expression was observed to be higher in ANXA1 KO than wild-type macrophages, yet no difference was observed for IL-6 level between wild-type and ANXA1 KO macrophages. The result suggests that MyD88 pathway may be intact and that IL-12 impairment may be due to impairment of pathways independent of MyD88, since IL-6 is a MyD88 dependent cytokine (Kawai *et al*., 1999). Significant IL-6 production was observed after treatment with poly (I:C) or LPS. The result is similar with findings from other reports (Meng and Lowell, 1997; Bae *et al*., 2010). Interestingly CpG 1826 did not cause noticeable IL-6 production, consistent with other studies based on CpG 1826 (Yasuda *et al*., 2004).

The importance of finding that ANXA1 regulates TRIF pathway is particularly evident when it is juxtaposed with the results of a study that revealed TRIF pathway activation to be responsible for 74.4% of the LPS-induced transcriptome in murine macrophages (Björkbacka *et al*., 2004). Moreover, steady state neutrophil homeostasis is found to be dependent on TLR4 and TRIF signaling pathway (Bugl *et al*., 2012), hinting on the potential interaction that remains to be discovered between ANXA1 and TRIF in neutrophils, since ANXA1 is found to be highly expressed in neutrophils and is dependent on ANXA1 for neutrophil extravasation (Lim *et al*., 1998). Further, TRIF pathway activation was also identified to be essential for differentiation of dendritic cells and mobilization to the lymph nodes during bacterial infection studies in mice (Cheng *et al*., 2010). More importantly, identifying new regulators of TRIF pathway can directly impact sepsis research, as patients who are suffering from gram-negative bacterial sepsis

were found have upregulated in the expression of TRIF-dependent genes such as IFN-β, CCL5 and IP-10 (Shalova *et al*., 2012).

IRF-3 requires phosphorylation by IKK-ε of the IKK complex and TBK1 (Fitzgerald *et al*., 2003; Nomura *et al*., 2000) in order to translocate into the nucleus. In human macrophages, TBK1 and IKK-ε are involved in directing LPS induced IFN-β transcription and activation (Solis *et al*., 2007). Our lab has unpublished data that ANXA1 KO macrophages show delayed kinetics in TBK1 phosphorylation and activation when compared to WT (unpublished data). TBK1 may be responsible for the impaired IRF-3 nuclear localization in ANXA1 KO PM. TBK1 is important in NF-κB signaling as macrophages from TBK1 -/- mice exhibit reduced NF-κB directed transcription caused by ablation of IFN-β production. Interestingly, ANXA1 KO mice exhibit similar phenotype as TBK1 KO mice in terms of LPS induced lethality (Marchilk *et al*., 2010). It therefore suggests a direct role of TBK1 in the regulation of TRIF dependent pathway through ANXA1.

After confirming that ANXA1 is required for cytokine production in response to LPS and poly (I:C), it was important to determine surface activation markers of macrophages after TLR agonist stimulation to determine the capacity of ANXA1 KO macrophages to activate adaptive immune response. MHCII and CD86 were chosen as activation markers for macrophages. MHCII interacts with the T-cell receptor (TCR) of the $CD4^+$ T-helper cells while CD86 binds and prime the T cells against presented antigens (Blander, 2008). The role of ANXA1 as a regulator of T cell activation was established previously (D'Acquisto, 2007). Our study showed that a substantial proportion of ANXA1 KO macrophages are not activated

after stimulation when compared to WT macrophages, demonstrating that ANXA1 is required for proper macrophage activation in response to TLR stimulation.

Furthermore, macrophages secrete cytokines which affect B cell activation. Although B cells do possess TLRs and are capable of being activated directly by TLR agonists such as LPS and CpG (Kim *et al*., 2012), B cells are, in most cases, downstream of macrophage activation during initial stages of pathogen invasion (Cerutti *et al.,* 2012). Upon analysis of B cell activation by TLR agonists, our data showed that a subset population in ANXA1 KO B cells are either not capable of being activated or is not activated enough to express surface markers such as CD69. This is interesting as B cells from MyD88-deficient mice also exhibited similar unresponsiveness to LPS response (Kawai *et al*., 1999). The only other study that investigated B-cells in relation to ANXA1 was a study for non-Hodgkin's lymphomas, and it was shown that ANXA1 expression is completely abrogated in these lymphomas, when ANXA1 is expressed in healthy B cells (Vishwanatha *et al.,* 2004).

Exposure to live pathogens provide a better understanding to whether our results are agonist-specific observations or if defects are also persistent in live sepsis. Live *Escherichia coli (E. coli*) DH5α bacteria were cultured with macrophages overnight. While MyD88-dependent cytokines IL-12 and IL-6 production were not observed to be different between ANXA1 KO and wild-type macrophages, the TRIF dependent chemokines IP-10 is regulated by ANXA1. This once again highlights impairment in the TRIF-dependent pathway which is activated by TLR4. These results provide more clinical

relevance to the results obtained, associating ANXA1 with clinical sepsis. Damazo *et al.,* previously demonstrated that ANXA1 is negatively involved in sepsis, where ANXA1 KO mice exhibit greater lethality to LPS (Damazo *et al*., 2005), and TNF levels were found to be enhanced. However, it was previously not known if the TRIF-dependent cytokines were affected by LPS. Our results here extend the studies above, demonstrating that ANXA1 can positively regulate TRIF-dependent cytokine production such as IP-10, in response to LPS.

To examine the relevance of ANXA1 in TRIF dependent activity *in vivo*, TRIF dependent cytokine and chemokine response was investigated after intraperitoneal injection of poly (I:C) in mice. Not surprisingly, there is a drastic down regulation of TRIF dependent genes such as IFN-β and IP-10, and this phenotype is consistent with TRIF knockout mice (Yamamoto *et al*., 2003). IFN-β regulation is directly dependent on IRF-3 activation, and when taken into discussion together with the evidence that ANXA1 KO has impaired nuclear localization of IRF-3, further supports the notion that ANXA1 is directly involved in regulating the TRIF pathway through activation and translocation of IRF-3. Therefore, our *in vivo* study also supports the conclusion that ANXA1 regulates TRIF dependent pathway.

The study has demonstrated that the serum levels of IFN-β in ANXA1 KO mice after intraperitoneal injection of poly (I:C) is significantly inhibited at 4 h when compared WT mice, but not IFN-α. IRF-3 activates the transcription of IFN-β but not IFN-α, while regulation of IFN- α is mainly through IRF-7 (Conzelmann, 2005). This result further establishes the role of ANXA1 as a regulator specifically for the TRIF/IRF-3 pathway.

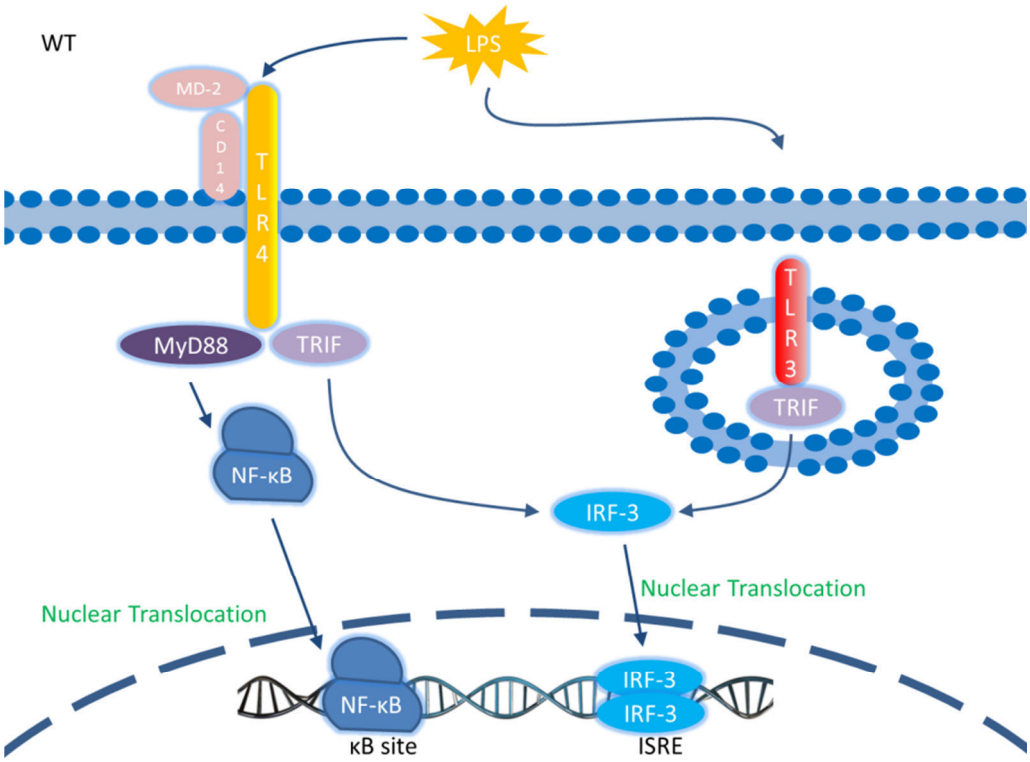
TRIF dependent pathway is activated through the recognition of viral RNA by TLR3. Since increased IP-10 production is a marker for better therapeutic outcome during viral infection (Lagging *et al.,* 2006), *in vivo* studies in ANXA1 KO mice should likely give a better survival outcome during viral burden compared to wild-type mice. There is currently no literature on ANXA1 KO macrophages or plasmacytoid dendritic cells co-cultured with virus. As ANXA1 KO macrophages show impaired TRIF response, the data strongly suggests that ANXA1 is an important regulator of anti-viral response mediated through TRIF pathway. It may also be possible that ANXA1 plays a regulatory role in suppressing anti-viral response through another anti-viral pathway, independent of TRIF adaptor protein activity, such as RIG-I.

Nuclear localization of NF-κB is considered a hallmark of NF-κB activation. In WT PM, nuclear localization of NF-κB is observed after 1h of stimulation with LPS (Figure 5.1 a). LPS induces signal transduction to activate NF-κB through the activation of TLR4 and the subsequent downstream signalling pathway induced by the adaptor protein MyD88. However, in ANXA1 KO PM, nuclear localization of NF-κB is impaired after stimulation with LPS for 1 h. This is consistent with the previous report from our lab that demonstrated the binding of ANXA1 to IKK-g (NEMO) and requirement of ANXA1 for activation of the phosphorylation of IkB by IKK complex (Bist et al., 2011). Therefore ANXA1 KO PM exhibits an impairment of NF-κB activation and nuclear localization due to the absence of ANXA1 to bind and activate IKK-g to drive downstream activation of NF-κB (Figure 5.1 b).

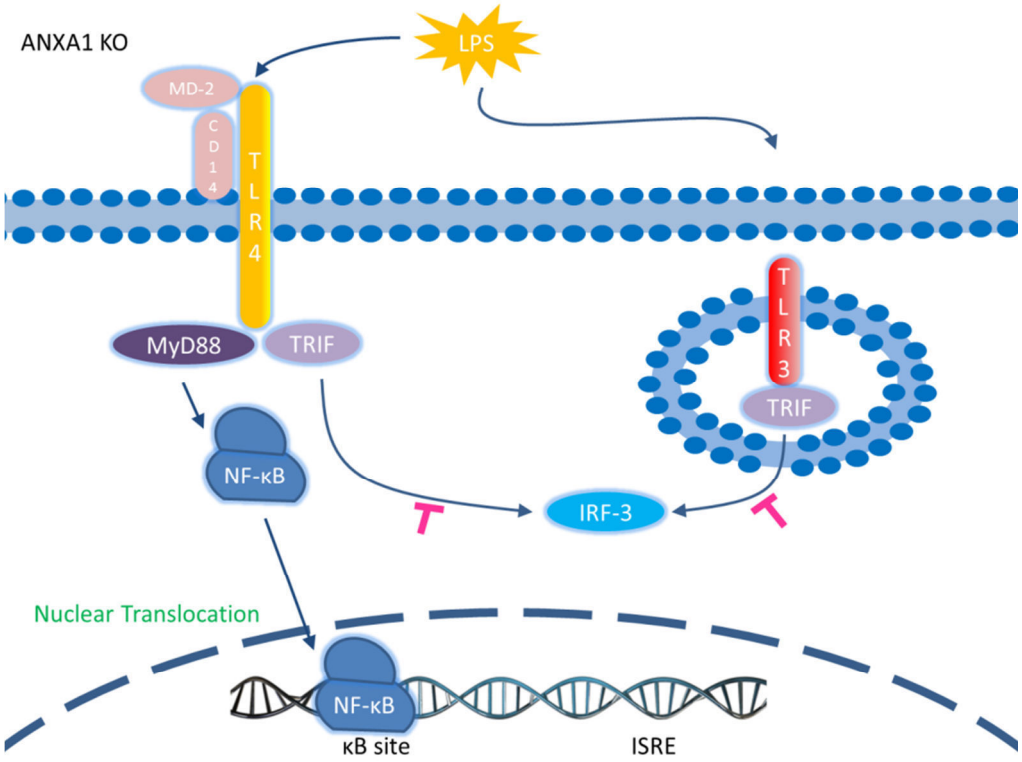
This study has also dissected the TLR pathway by investigating TRIF specific TLR3 agonist poly (I:C). In WT PM, stimulation of endosomal TLR3 with poly (I:C) triggers TRIF dependent cascade that activates IRF-3 nuclear localization, an important step in the activation of transcription factor IRF-3 (Figure 5.1 c). LPS is also capable of activating TRIF dependent IRF-3 nuclear translocation and activation, through the TRIF adaptor protein that can bind and be activated directly by TLR4.

In ANXA1 KO PM, the nuclear translocation of IRF-3 is impaired after treatment with poly (I:C). This is consistent with the our lab data that ANXA1 KO PM has inhibited TBK1 phosphorylation, which is required for the phosphorylation and dimerization of IRF-3 and its nuclear translocation (unpublished data). NF-κB nuclear translocation is unaffected, likely through the signalling adaptor protein TRAF6. TRAF6 -/- mouse embryonic fibroblasts (MEFs) exhibit completely abolished poly (I:C) induced NF-κB activation (Jiang et al., 2004). Therefore, this study suggests that signal transduction from TRIF is impaired but not MyD88 or TRAF6 (Figure 5.1 d).

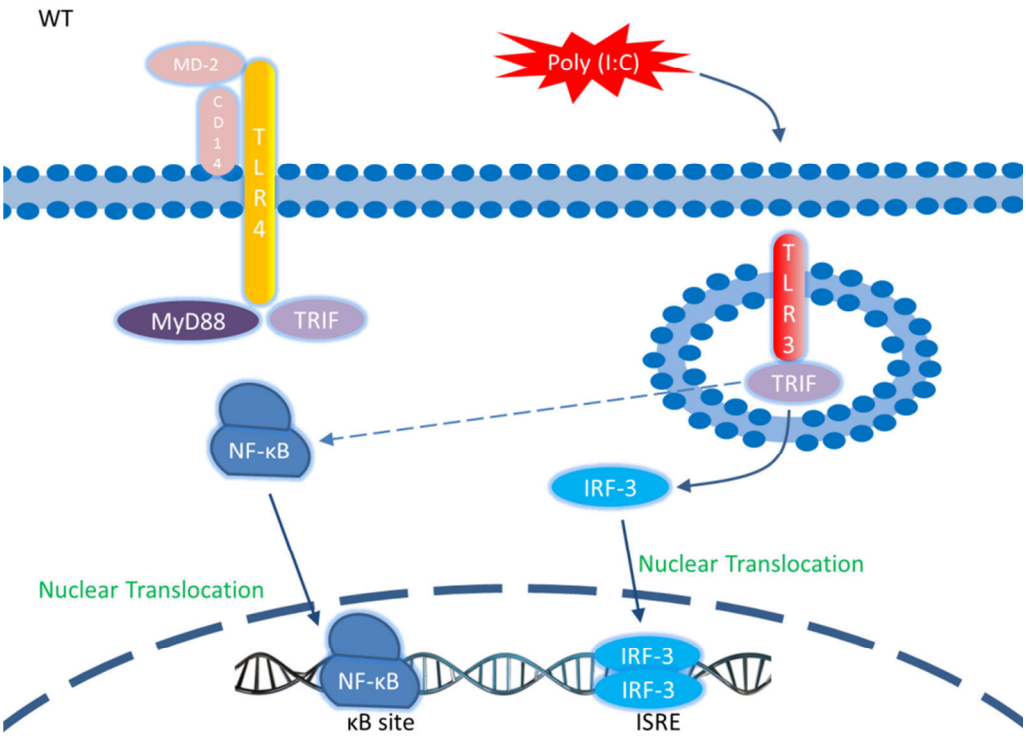
a)


WT
LPS
MD-2
CD14
TLR4
MyD88
TRIF
TLR3
TRIF
NF-κB
IRF-3
Nuclear Translocation
Nuclear Translocation
NF-κB
IRF-3
IRF-3
κB site
ISRE

b)


ANXA1 KO
LPS
MD-2
CD14
TLR4
MyD88
TRIF
TLR3
TRIF
NF-κB
IRF-3
Nuclear Translocation
NF-κB
κB site
ISRE

c)


WT
MD-2
CD14
TLR4
Poly (I:C)
MyD88
TRIF
TLR3
TRIF
NF-κB
IRF-3
Nuclear Translocation
Nuclear Translocation
NF-κB
IRF-3
IRF-3
κB site
ISRE

d)

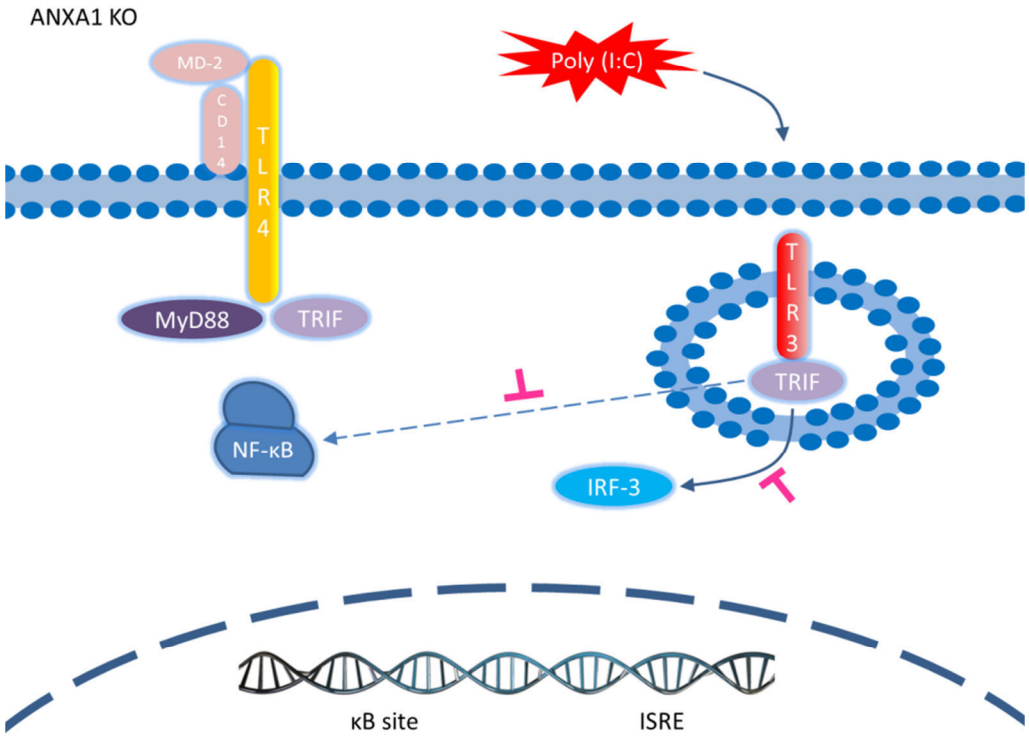

ANXA1 KO
MD-2
CD14
TLR4
Poly (I:C)
MyD88
TRIF
TLR3
TRIF
NF-κB
IRF-3
κB site
ISRE

Figure 5.1. Proposed mechanism behind ANXA1 regulating nuclear translocation of NF-κB and IRF-3, after stimulation with LPS or poly (I:C). a) Proposed mechanism for transcription of NF-κB and IRF-3 in WT PM after LPS stimulation. b) Proposed mechanism for transcription of NF-κB and IRF-3 in ANXA1 KO PM after LPS stimulation. c) Proposed mechanism for transcription of NF-κB and IRF-3 in WT PM after poly (I:C) stimulation. d) Proposed mechanism for transcription of NF-κB and IRF-3 in ANXA1 KO PM after poly (I:C) stimulation.

Macrophage polarization studies require a naïve subset of macrophages that are not specifically skewed towards any particular phenotype. PM were generated using elicitation of macrophages to peritoneal cavity with thioglycollate injection, and this may affect macrophage polarization study, even if they are not activated macrophages. The use of BMDM resolves this issue as macrophages are grown directly on tissue culture dish from its progenitor monocytes and therefore does not undergo any form of stimulation that may skew the macrophages permanently towards a particular phenotype. Interestingly, our data shows that ANXA1 KO bone marrow derived macrophages show similar defects in cytokine production after LPS exposure. This indicates that the impairment of TLR- dependent pro-inflammatory response in macrophages in absence of ANXA1 is not simply an observation specific to activated or thioglycollate elicited peritoneal macrophages, but a defect which impairs macrophage potential at pre-monocytic lineage level. In addition, this data suggest that the regulation of cytokine production by ANXA1 is not due to other immune cell such as T-cells or B-cells which is known to modulate macrophage response by altering its activation after thioglycollate elicitation.

Macrophage polarization is the classification of diverse macrophage phenotypes into two distinct dipolar states mirroring the T helper Type I (Th1)-T helper type 2 (Th2) polarization. Classically activated macrophages are classified as M1 on one end of the spectrum, while alternatively activated macrophages are classified as M2, representing the other end of spectrum of macrophage gene expression profile (Mantovani *et al*., 2002; Mantovani *et al*., 2008; Mosser *et al*., 2008). M1 macrophages are generally considered potent effector cells that produce copious amounts of pro-inflammatory cytokines. In contrast, M2 macrophages are able to tune inflammatory responses, adaptive Th2 immunity and promote tissue repair (Mantovani *et al*., 2002)

TLR engagement leads to activation of NF-κB and inflammatory mediators associated with M1 macrophages (Bonizzi *et al*., 2004). Alternatively activated (M2) macrophages show defective NF-κB activation in response to different pro-inflammatory signals (Sica *et al*., 2000; Sica *et al*., 2006; Saccani *et al*., 2006). The defective NF-κB activation in M2 macrophages correlates with impaired expression of NF-κB dependent inflammatory function such as TNF-α and IL-12 (Mantovani *et al*., 2002). Indeed, it may be possible that ANXA1 deficient macrophages are more skewed towards an M2 phenotype which release less pro-inflammatory cytokines.

Our study has examined the effect of ANXA1 on three major transcription activation pathways (MyD88/NF-κB, TRIF/IRF-3, IFN-γ/STAT-1), through the use of ANXA1 KO macrophages. The NF-κB inhibitor BAY-11 (which acts through inhibition of IκB kinase) was shown to affect IL-12 production only in wild-type macrophages, but not ANXA1 KO macrophages.

This gave an indication that ANXA1 KO macrophages were either resistant to NF-κB inhibition, or that ANXA1-regulated IL-12 production is NF-κB dependent. This may seem to be contradicting several articles that suggest ANXA1 is an inhibitor of NF-κB activity (Wang *et al.,* 2011; Zhang *et al.,* 2010), as these studies correlated an increase in ANXA1 with the anti-inflammatory activity of ANXA1. However, there are new studies that suggest ANXA1 may not be a completely anti-inflammatory molecule. In a recent study, cleaved N-terminus of ANXA1 was demonstrated to play an anti-inflammatory role while C-terminus end of truncated ANXA1 played a pro-inflammatory role which activated ERK activity and up regulated ICAM-1 clustering around adherent neutrophils and anchor them to the endothelium, thus promoting transmigration (Williams *et al.,* 2010). Our result therefore supports a more homeostatic role ANXA1 plays at endogenous level than what was previously perceived of ANXA1, and that it is not an "anti-inflammatory bullet". In other words, a high concentration of ANXA1 induced by anti-inflammatory drugs may inhibit NF-κB, while a low homeostatic concentration of ANXA1 may regulate proper cytokine production during inflammation.

We also investigated the nitric oxide production level of macrophages, as they are a major source of inflammatory nitric oxide producer during inflammatory processes. We demonstrated that nitric oxide levels in ANXA1 KO macrophages are significantly attenuated in both poly (I:C) and LPS stimulation, indicating that ANXA1 regulates NO production. As M1 polarization also involves the extent of NO production, this shows that ANXA1 may affect M1 polarization in more ways than one, and it also

presents novel data on nitric oxide regulation in ANXA1 KO macrophages, which is consistent with the other studies which overexpressed ANXA1 and showed a concomitant increase in iNOS expression level (Roviezzo *et al.,* 2002; Smyth *et al.,* 2006).

STAT-1 is an important element in IRF-3 signaling. Canonical IRF-3/STAT-1 signaling pathway is critical for skewing macrophage polarization to M1 (Sica *et al*., 2007), and STAT-1 plays a role in IRF-3 mediated IP-10 production (Kopydlowski *et al*., 1999; Toschchakov *et al*., 2002). Our study investigated whether STAT-1 activation status in ANXA1 KO and WT macrophages by measuring the phosphorylation of STAT-1. It was observed that the phosphorylation of STAT-1 is identical in both WT and ANXA1 KO macrophages, suggesting that STAT-1 is not regulated by ANXA1. To further establish that ANXA1 regulates TLR activation independently from STAT-1 dependent pathway, we examined the effect of IFN-γ stimulation, as it is an important ligand for M1 polarization through STAT-1 activation. Upon stimulation with IFN-γ, no difference in IL-12 production was observed between WT and ANXA1 KO macrophages. Hence, IFN-γ dependent STAT-1 activation is not regulated by ANXA1. This result presents strong evidence that the specificity of action by ANXA1 is TLR dependent, and not IFN-γ or STAT-1 dependent. This however, does not mean ANXA1 is not related with the STAT family. N-terminal cleaved peptide of ANXA1 (Ac2-26) released by apoptotic cells has been shown to activate STAT-3 through formyl peptide receptor (Pupjalis *et al.,* 2011).

We also investigated if ANXA1 can affect M2 polarization. The determination of M2 polarization is complicated as there are not many M2

specific markers available to confirm the existence of M2 macrophages. YM1, FIZZ1 and arginase-1 (Arg1) were chosen as they are key markers for M2 polarization (Kurowska-stolarska *et al*., 2009; Arranz *et al*., 2012). In this study, ANXA1 KO macrophages were shown to be more M2 polarized as they expressed markedly higher levels of Arg1 and FIZZ-1 mRNA expression levels, indicating that ANXA1 either enhanced M1 polarization or inhibited M2 polarization. The Arg1 PCR data complement the results with NO production, where ANXA1 KO macrophages exhibit lower NO production during LPS treatment. A similar observation was reported in M2 polarized macrophages of Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP) deficient mice (*ship* -/-), where the ship-/- macrophages exhibited lower NO production and enhanced Arg1 expression (Rauh *et al*., 2005). $IL12^{low}$, $IL10^{high}$ expressing macrophages are hallmarks of M2 polarized macrophages (Gordon *et al*., 2003; Mantovani *et al*., 2003; Mantovani *et al*., 2005; Mosser *et al*., 2003). Our data show that ANXA1 KO macrophages indeed exhibit low levels of IL-12 production suggesting that ANXA1 may be a modulator of macrophage polarization. It is possible that ANXA1 regulates M2 polarization which is capable of inhibiting M1 polarization (Briken and Mosser, 2011). Further study can be done to elucidate the exact role of ANXA1 in M2 polarization.

The significance of ANXA1 affecting macrophage polarization is far-reaching as macrophages mediate a diverse spectrum of physiological and pathological disease outcome. Physiologically, M1 polarized macrophages can mediate tissue damage and inflammatory responses (Gordon and Martinez, 2010; Biswas and Mantovani, 2010). On the other hand, M2 phenotype

macrophages are important for tissue damage recovery, as patients with severe burns have up regulated M2 polarized macrophages, and absence of M2 macrophages causes substantially increased formation of scar tissues in wounds (Lucas *et al*., 2010; Cairo *et al*., 2011). Pathologically, M1 macrophages are generally considered the key element responsible for resisting intracellular pathogens, such as *Listeria monocytogenes* (Shanghnessy *et al*., 2010), *Salmonella typhi*, *Salmonella typhimurium* (Jouanguy *et al*., 1999). Moreover, M1 macrophages are also important in resisting early phases of infection with *Mycobacterium tuberculosis* (Chaon-Salinas *et al*., 2005), *Mycobacterium ulcerans* and *Mycobacterium avium* (Kiszewski *et al*., 2006; Murphy *et al*., 2006). With respect to M2 macrophages, there are parasites that favour M2 polarized macrophages. In experimental and human parasite infections, macrophages generally undergo M2 polarization switching (Noel *et al*., 2004; Babu *et al*., 2005). For example, late phases of *Taenia crassiceps* infection switches macrophages to M2 state (Murray and Wyunn, 2011; Brys *et al*., 2005) and similar observations are also reported in other parasitic infections such as *Schistosoma mansoni* and *Trypanoma conglense* infections (Pearce and MacDonald, 2002). M2 polarization affects disease outcome in viral infections and is important for reducing inflammation and epithelial damage in lungs (Shirey *et al*., 2010).

The clinical relevance of ANXA1 being a regulator of macrophage polarization should be investigated. While still in its infancy, therapeutic macrophage targeting exists. Macrophage control in therapeutic settings are done through the use of PPAR-γ agonists, as several studies have established PPAR-γ to be an important regulator of M2 polarization (Lu *et al*., 2011;

Stienstra *et al*., 2008; Charo, 2007). Since this study has evidence to suggest ANXA1 as a regulator of M2 macrophage polarization, we hypothesized that ANXA1 could regulate PPAR-γ induced cytokine production and that PPAR-γ is involved in controlling the macrophage polarization phenotype observed in ANXA1 KO.

PPAR-γ plays an important role in the differentiation, activation and regulation of inflammatory activities of macrophages (Yessoufou *et al*., 2010; Nagy *et al*., 1998; Ricote *et al*., 1998; Ricote *et al*., 1999; Tontouoz *et al*., 1998; Jiang *et al*., 1998). The role of PPAR-γ in clinical disease is diverse. Patients with mutations in PPAR-γ gene in the ligand binding domains manifested clinical syndromes such as severe insulin resistance, diabetes and hypertension (Barroso *et al*., 1999). Interestingly, macrophage foam cells express PPAR- γ in atherosclerotic plaques (Marx *et al*., 1998; Ricote *et al*., 1998). Thiazolidineones, a class of anti-diabetic drugs have high affinity for PPAR-γ, and were developed for the treatment of type-2 diabetes as they were capable of lowering blood glucose levels (Lehman *et al*., 1995), significantly reducing vascular inflammation in non-diabetic patients (Meisner *et al*., 2006), and are frequently prescribed to patients with type 2 diabetes (Gerstein *et al*., 2006; Kahn *et al*., 2006). However, a few of these drugs were taken off the market due to cardiovascular side-effects and complications (Penumetcha and Santanam, 2012). PPAR-γ is also implicated in chronic autoimmune diseases. For example, PPAR-γ is up-regulated in patients with rheumatoid arthritis (Palma *et al*., 2012). It is also clinically relevant in other chronic diseases, in particular Alzheimer's disease (AD), where PPAR-γ activation reduces AD

risk by as much as 80% (Heneka *et al*., 2001; Kielian and Drew, 2003; Laridreth and Heneka, 2001; Mandreka-lolucci *et al*., 2012).

The endogenous and natural ligand of PPAR-γ is PGJ_2, a downstream metabolite of the arachadonic acid pathway of inflammation and is known for its regulatory role of IL-12 inhibition (Azuma *et al*., 2001). PGJ_2 is a prostaglandin produced through the enzymatic cleavage of membrane phospholipids by phospholipase A2 (PLA_2) at the second carbon group of glycerol to produce arachidonic acid, which is further processed endogenously to form prostaglandins.

Our data show that PGJ_2 induces small amounts of IL-12 at low doses, but inhibits IL-12 production after treatment with LPS in WT macrophages, similar to other studies (Drew and Chavis, 2001).

Endogenous level of prostaglandins such as PGJ_2 is dictated by PLA_2 activity. ANXA1 inhibits PLA_2 and subsequently inhibits prostaglandin production (Flower and Blackwell, 1979). As ANXA1 inhibits the production of prostaglandins, PPAR-γ activity in the cell during homeostasis is low and therefore PPAR-γ does not inhibit NF-κB activity and IL-12 production in a WT BMDM (figure 5.2 a).

In our experiments, WT BMDM is capable of producing high levels of IL-12 after stimulation with LPS. However in ANXA1 KO BMDM, IL-12 production is impaired after stimulation with LPS when compared to WT BMDM.

In ANXA1 KO cells, PLA_2 activity is uninhibited, thereby causing the endogenous levels of prostaglandins such as PGJ_2 to be high. It is proposed that these endogenous PGJ_2 act as a ligand to activate PPAR-γ, thus increasing PPAR-γ activity in the cell, leading to the inhibition of NF-κB activity and IL-12 production (Figure 5.2 a). It was observed in this study that exogenous pre-treatment of PGJ_2 prior to LPS treatment does not show inhibit IL-12 production in ANXA1 KO. It is proposed that exogenous addition of PGJ_2 does not alter the level of PPAR-γ as endogenous PGJ_2 level is already high (Figure 5.2 b).

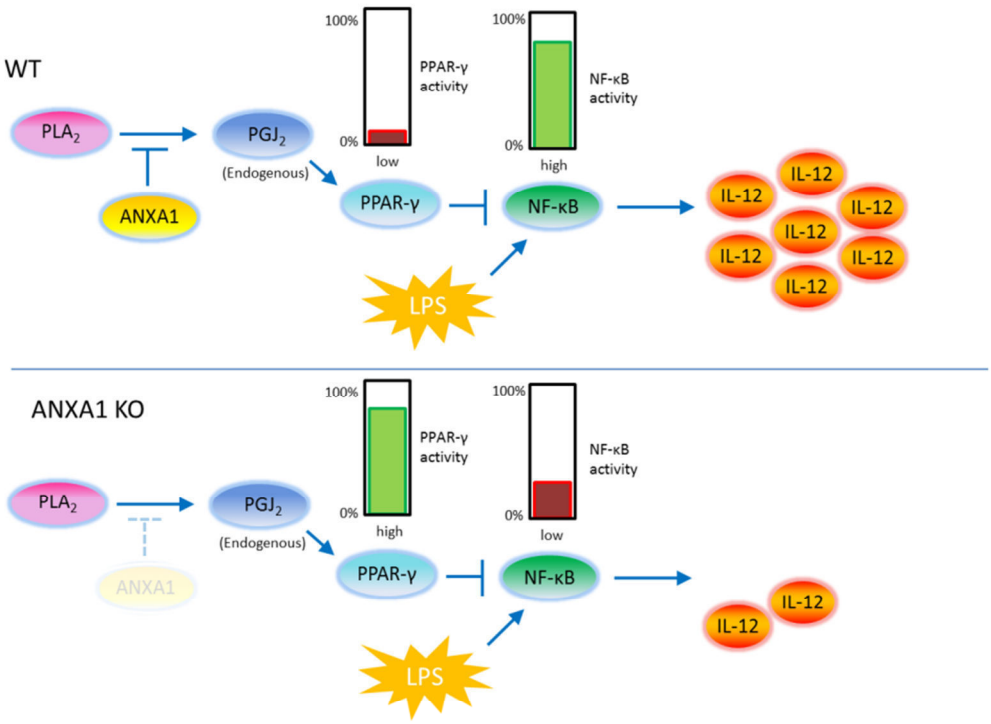
To support these claims, GW9662, a PPAR-g specific inhibitor was used to examine whether ANXA1 KO BMDM exhibit inhibited IL-12 production due to increased endogenous PPAR-γ activity. In line with our hypothesis, pre-treatment with GW9662 before LPS stimulation substantially reversed the inhibition of IL-12 production observed in ANXA1 KO BMDM when compared to WT BMDM. This observation is due to the inhibition of PGJ_2 activity which is high in ANXA1 KO, causing NF-κB activity to be enhanced, leading to a reversal of inhibition and therefore increased production of IL-12 compared to WT BMDM (Figure 5.2 c). Taken together, ANXA1 may regulate endogenous PPAR-γ activity, but further experiments to quantify PPAR-γ activity level in ANXA1 KO BMDM is required to validate the hypothesis. A possible experiment would be to measure the PPAR-γ promoter activity level in ANXA1 KO BMDM after treatment with LPS and compare with WT BMDM, by using a luciferase reporter assay.

Further study needs to examine whether ANXA1 affects both PPAR-g dependent and PPAR-g independent activity of PGJ_2 (Hortelano *et al*., 2000;

Tsubouchi *et al*., 2001; Petrovaet *et al*., 1999; Vaidya *et al*., 1999). This is because a study has shown that PGJ_2 has a unique ability to inhibit IL-12 production through the inhibition and modification of IKK2 subunit of the IKK complex, preventing the phosphorylation of the inhibitory IκB proteins (Rossi *et al*., 2000).

In conclusion, this thesis has shown that ANXA1 is able to regulate IL-12/IP-10 production through TRIF dependent pathways by controlling NF-κB and IRF-3 translocation to the nucleus upon activation. ANXA1 is required for proper M1 polarization and suppression of M2 polarization, and PPAR-γ activity in the production of IL-12 after LPS stimulation is regulated by ANXA1.

a)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PLA2
PGJ2
(Endogenous)
ANXA1
100%
0%
low
PPAR-γ activity
100%
0%
high
NF-κB activity
PPAR-γ
NF-κB
LPS
IL-12
IL-12
IL-12
IL-12
IL-12
IL-12
ANXA1 KO
PLA2
PGJ2
(Endogenous)
ANXA1
100%
0%
high
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0%
low
NF-κB activity
PPAR-γ
NF-κB
LPS
IL-12
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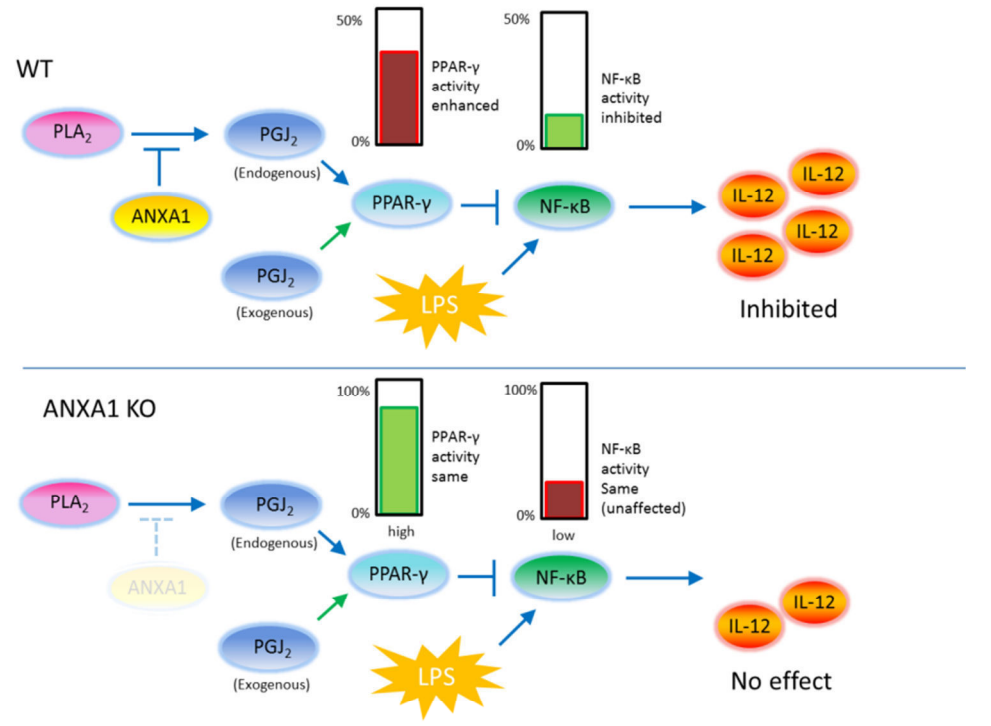
b)


WT
PLA2
PGJ2
(Endogenous)
ANXA1
PGJ2
(Exogenous)
50%
0%
PPAR-γ activity enhanced
50%
0%
NF-κB activity inhibited
PPAR-γ
NF-κB
LPS
IL-12
IL-12
IL-12
IL-12
IL-12
IL-12
Inhibited
ANXA1 KO
PLA2
PGJ2
(Endogenous)
ANXA1
PGJ2
(Exogenous)
100%
0%
high
PPAR-γ activity same
100%
0%
low
NF-κB activity Same (unaffected)
PPAR-γ
NF-κB
LPS
IL-12
IL-12
No effect

c)

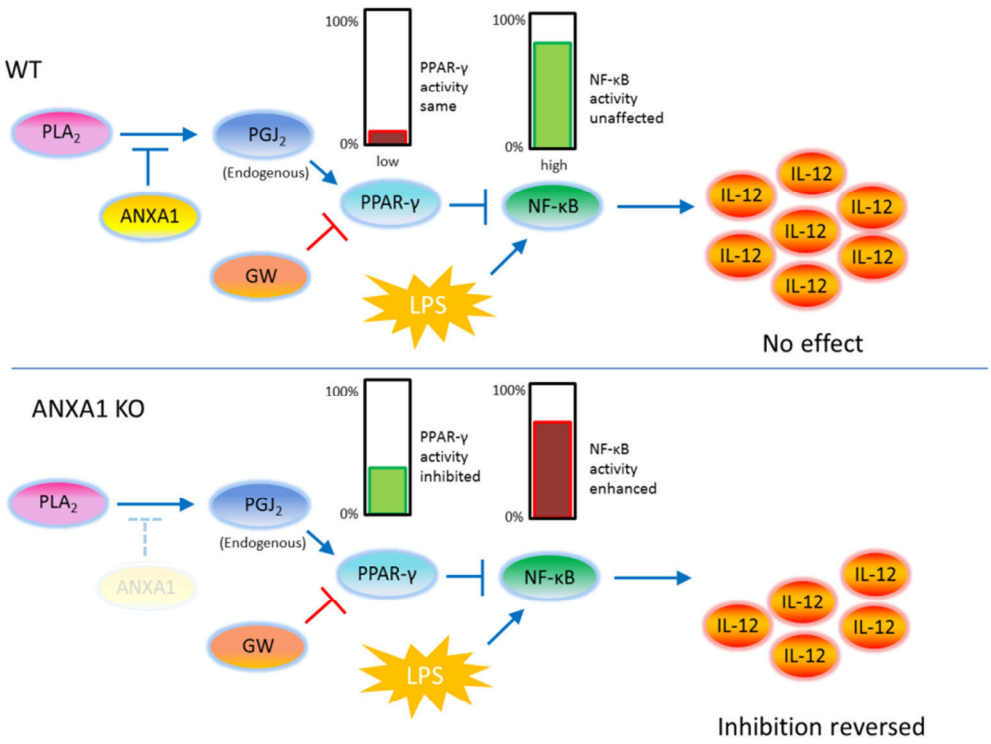


Figure 5.2. Proposed mechanism for the regulation of ANXA1 by PPAR-γ with and without pre-treatment with PPAR-γ agonist PGJ_2 and PPAR-γ inhibitor GW9662. a) Proposed mechanism for endogenous control of PPAR-γ by ANXA1 after LPS stimulation. b) Proposed mechanism during exogenous pre-treatment of PGJ_2 before LPS stimulation. c) Proposed mechanism during pre-treatment of GW9662 before LPS stimulation. Infographic by Shu Shin La.

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7. APPENDICES

ANXA1 interacting proteins					
Interacting Partner	Description of its known functional role (summarized from UniProt)	Species of the two interacting molecules	Interaction Detection Method	Protein Database Source	References
ACTB (Cytoplasmic beta-Actin)	Key molecule in cell motility that is ubiquitously expressed in all eukaryotic cells. Often used as a loading control in protein assays	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
Alpha-enolase	Multifunctional enzyme involved in glycolysis, plays a part in growth control, hypoxia tolerance and allergic response	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
ATP5A1 (ATP synthase subunit alpha, mitochondrial)	Part of mitochondrial membrane ATP synthase complex	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
CD4 (T-cell surface glycoprotein CD4)	Accessory protein for MHC class II antigen/ T-cell receptor interaction	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
DDX3X (ATP-dependent RNA helicase)	RNA helicase	Human - Human	Molecular Sieving	IntAct	Ewing *et al.,* 2007
DHRS2 (Dehydrogenase/reductase SDR family member 2)	An NADPH-dependent dicarbonyl reductase	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004

DLG3 (Disks large homolog 3)	Defects in DLG3 are the cause of mental retardation X-linked type 90.	Human - Human	Anti-bait immunoprecipitation	IntAct, GRID	Ewing *et al.,* 2007
EEF1B2 (Elongation Factor1-beta)	Responsible for delivery of tRNAs to ribosome	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
EGFR (Epidermal growth factor receptor)	Recruits adapter proteins like GRB2 and activate RAS-RAF pathway, PI3 kinase-AKT pathway, PLC gamma-PKC pathway, and STAT modules	Human - Human	Colocalization by Immunostaining	MINT, HPRD	Radke *et al.,* 2004
EIF3E (Eukaryotic initiation factor 3 subunit E)	Part of eIF-3 complex that stimulates mRNA recruitment into ribosome subunit	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
EIF4A2 (Eukaryotic initiation factor 4A-II)	RNA helicase required for mRNA binding to ribosome	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
FARSB (Phenylalanine-tRNA ligase beta subunit)	tRNA carrier for the amino acid phenylalanine during translation	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
GAPDH (Glyceride-3-phosphatase dehydrogenase)	glyceraldehyde-3-phosphate dehydrogenase	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
GNAI2 (Guanine nucleotide-binding protein G subunit alpha-2)	Transmembrane signaling system involved in the regulation of adenylate cyclase	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004

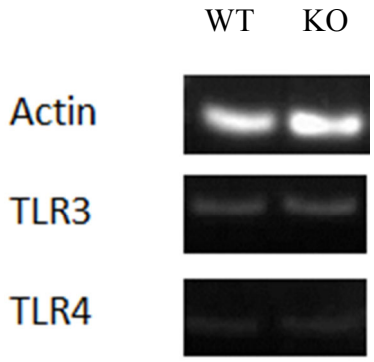
HNRPH1 (Heterogeneous nuclear ribonucleoprotein H)	A component of hnRNP complex which provides substrate for pre-mRNA processing	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
HSPA8 (Heat shock cognate 71 kDa protein)	Repressor of transcriptional activation. Inhibits Smad-mediated transcription. Also acts as a chaperone	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
KPNB1 (Importain subunit beta-1)	Functions in nuclear protein import. Servces itself as an NLS receptor	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
LCK (Tyrosine-protein kinase)	Selection and maturation of developing T-cells	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
LRPPRC (Leucine-rich PPR motif-containing protein, mitrochondrial)	Binds to poly (A) mRNA in mitochondria	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
MME (Neprilysin)	Destruction of opiod peptides and degradation of atrial natriuretic factor. Also possess elastase activity under UV-induction	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
MYL12A (Myosin regulatory light chain 12A)	Myosin regulatory subunit	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
NCL (Nucleolin)	Induces chromatin condensation by binding to histone H1	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004

NFKBIA (NF-κB inhibitor alpha)	Inhibits activity of dimeric NF-kappa-B/REL complexes by masking nuclear localization signals	Human - Human	Tandem Affiinity Purification	IntAct	Bouwmeester *et al.,* 2004
PHB (Prohibitin)	Inhibits DNA synthesis; plays a role in regulating proliferation	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
PPM1B (Protein phosphotase 1B)	Dephosphorylates CDK2 and CDK6 *in vitro*	Human - Human	Anti-bait immunoprecipitation	IntAct, GRID	Ewing *et al.,* 2007
RARA (Retinoic acid receptor alpha)	Receptor for retinoid acid	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
RPL7 (60S ribosomal protein L7)	Binds to G-rich structures in 28S rRNA and in mRNAs; Plays a regulatory role in translation and inhibits cell-free translation of mRNAs	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
RPL7A (60S ribosomal protein L7-premature form)	Binds to G-rich structures in 28S rRNA and in mRNAs; Plays a regulatory role in translation and inhibits cell-free translation of mRNAs	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
RPS18 (40S ribosomal protein S18)	Forms the head of the 40S ribosomal protein complex	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
RPS19 (40S ribosomal protein S19)	Required for pre-rRNA processing and maturation of 40S ribosomal subunits	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
S100A11 (Protein S100-A11)	Differentiation and cornification of kertinocytes	Human - Human	two-hybrid	HPRD	Bianchi *et al.,* 2003

SSRP1 (FACT complex subunit 1)	Component of the FACT complex, a nucleosome reorganizer	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
TNPO1 (Transportin-1 / Importin beta-2)	Nuclear transport receptor	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
TRPM7 (Transient receptor potential cation channel subfamily M member 7)	Essential ion channel and serine/threonine-protein kinase. Permeable to calcium and magnesium. Phosphorylates ANXA1 at serine residue position 5.	Human - Human	two-hybrid	HPRD	Dorokov *et al.,* 2004
TUBB (Tubulin)	Major constituent of microtubles	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
VDAC3 (Voltage-dependent anion-selective channel protein 1)	Forms a channel through the mitochondrial outer membrane that allows diffusion of small hyrophillic molecules	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
VDAC3 (Voltage-dependent anion-selective channel protein 2)	Forms a channel through the mitochondrial outer membrane that allows diffusion of small hyrophillic molecules	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
VDAC3 (Voltage-dependent anion-selective channel protein 3)	Forms a channel through the mitochondrial outer membrane that allows diffusion of small hyrophillic molecules	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004
VIM (Vimentin)	Intermediate filaments found in various non-epithelial cells	Human - Human	Molecular Sieving	IntAct	Bernhard *et al.,* 2004

YWHAZ (14-3-3 protein zeta/delta)	Adapter protein affecting BAX, p53 and AKT1 pathways	Human - Human	GST pull-down	MINT	Meek *et al.,* 2004

Appendix A. Consolidated results for ANXA1 interacting proteins from databanks. The table includes interactions chosen using protein database browsing service by Proteomics Standards Initiative (www.ebi.ac.uk). Proteins with no known function are not included. All interactions tabulated herein are based on "Evidence at protein level" type of evidence, which rates among the highest in UniprotKB database classification of bioinformatics data, i.e. according to UniprotKb definition: "…(the) evidences are clear experimental evidences for the existence of protein (interaction)" (Source: UniprotKB).



Appendix B. TLR3 and TLR4 mRNA expression in WT and ANXA1 KO PM. No difference in TLR3 and TLR4 expression is observed between WT and ANXA1 KO PM.