ABSTRACT

Title of Document: **Activation of NLRP3 inflammasomes during complement-mediated phagocytosis by macrophages**

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The Complement System is an important component of innate immunity, whose activation has been associated with acute inflammation. Activation of complement leads to the generation of a Membrane Attack Complex (MAC) composed of C5b-C9 proteins. The function of the MAC has been primarily associated with target cell death through the polymerization and insertion of C9 on the surface of complement activating particles. Here, I provide evidence that MAC assembled on the surface of complement activating particles, can be transferred to host macrophages during the process of phagocytosis. This "bystander activation" onto macrophages causes potassium efflux and ROS production, which induces the assembly of the NLRP3 inflammasome, which in turn results in the activation of caspase-1 and the processing and secretion of IL-1β and IL-18 to regulate both innate and adaptive immunity. Inflammasome activation is not induced when macrophages phagocytize unopsonized particles or particles opsonized with serum deficient in one of the terminal complement components. The secretion of IL-1β and IL-18 by macrophages is dependent on NLRP3, ASC, and caspase-1, as macrophages deficient in any one of these components fail to secrete these

cytokines following complement-mediated phagocytosis. The phagocytosis of complement-opsonized particles increases leukocyte recruitment and promoted T_h17 biasing.

Leishmania major, an intracellular pathogen, follows a similar pattern, when infecting macrophages: Phagocytosis of the pathogen was accompanied by the activation of complement pathway resulting in "bystander activation" and NLRP3 inflammasome assembly. When macrophages are primed with IFNγ and IL-1β together prior infection, the killing of parasites is enhanced demonstrating that IL-1β can work in concert with IFNγ to activate macrophages and thus reduce the intracellular burden of the pathogen. This study demonstrates that the phagocytosis of complement-opsonized particles can induce inflammasome activation by a novel mechanism involving MAC-mediated "bystander activation" of host macrophages. This work provides another mechanism whereby complement activation can lead to acute inflammation and identifies a previously undescribed function of the MAC to activate inflammasomes on macrophages.

Activation of NLRP3 inflammasomes during complement-mediated phagocytosis by macrophages

By

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Dedication

This dissertation is dedicated to my parents Jagennathan Suresh and Jayashree Suresh. Thank you for all your wholehearted support throughout my life. I love you very much!

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I have to start by thanking my parents for all of the support they have provided over the years. I would also like to thank my brother, Rohit Suresh along with my grandmother Usha Sama Rao, and my uncles Pranesh Rao and Venkatesh Rao. Thank you all for all your jokes and sense of humor that made it possible for me to make it through my PhD in one piece.

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List of Abbreviations:

7AAD	7-Aminoactinomycin D
AF-649	Alexa Fluor 647
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
AA-Mϕ	Alternatively activated macrophages
BMMϕ	Bone marrow derived macrophages
C5,6,7,8,9	Complement components 5, 6, 7, 8, 9.
cAMP	Cyclic adenosine monophosphate
CARD	Caspase activation and recruitment domain
Casp-1	Caspase-1
CA-Mϕ	Classically activated macrophages
CL	Cutaneous leishmaniasis
CTL	Cytotoxic T-lymphocytes
DAMP	Danger associated Molecular Pattern
DC	Dendritic cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EGTA	Ethylene glycol tetraacetic acid
FcR	Fc (fragment crystallizable region) receptor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GPCRs	G-protein-coupled receptors
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
IFN	Interferon

Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IKK	IκB kinase
IL	Interleukin
IL-1β	Interleukin 1 beta
IL-12	Interleukin 12
IL-18	Interleukin 18
iNOS	Inducible nitric oxide synthase
IPAF	Ice protease-activating factor
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
LMW-HA	Low-molecular weight hyaluronic acid
LRR	Leucine-rich repeat
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex
MAC	Membrane attack complex
MAL	MyD88-adaptor like
M-CSF	Macrophage colony stimulating factor
MyD88	Myeloid differentiation primary response gene 88
NES	Nuclear export signal
NF-κB	Nuclear factor-κ B
NK Cells	Natural killer cells
NLR	Nucleotide-binding domain, Leucine-rich repeat containing Receptors
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric oxide
OVA	Ovalbumin

PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PMN	Polymorphonuclear neutrophil granulocytes
PRR	Pattern recognition receptors
qRT-PCR	Quantitative-real-time PCR
R-Mϕ	Regulatory macrophages
ROS	Reactive Oxygen species
SCM	Schneider's complete medium
SRBC	Sheep red blood cells
STAT	Signal transducers and activators of transcription
TCC	Terminal complement components
TCR	T-cell Receptor
TGFβ	Transforming growth factor β
Th	T-helper lymphocyte
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T-cell
T_h1	T helper cell 1
T_h2	T helper cell 2
T_h17	T helper cell 17

Chapter 1: Introduction

1.1 Innate Immunity

Innate immunity represents the first line of defense against pathogens. It consists of a broad spectrum of responses to different pathogens or environmental cues. Innate immunity is limited by the fact that these responses are not specific to particular antigens and do not confer long lasting memory. However, innate immunity acts quickly and provides vital resistance to the host until the adaptive immune response is properly activated.

Apart from anatomical barriers like skin and the mucous membranes which provide nonspecific physical barricades to the invading pathogen, there are two main components of innate immunity: (i) humoral factors (e.g. the complement system, and the coagulation pathway, etc.) and (ii) cellular components (e.g. macrophages and neutrophils). Activation of humoral factors in response to a pathogens is instantaneous. The coagulation cascade can induce blood clotting to limit the entry of pathogens. Complement components bind to pathogen, thus tagging them to be taken up by phagocytes. Complement can also form membrane attack complexes (MAC) on the surface to lyse pathogens. The activated components from the coagulation and complement cascades induce the infiltration of leukocytes including neutrophils, macrophages and dendritic cells (DC), which leads to inflammation. The cellular components of the innate immune system are adept at actively taking up pathogens through the process of phagocytosis, for

destruction in the phagolysosome and subsequent processing for antigen presentation, hence they are termed professional phagocytes.

One critical role of the innate immune system, apart from first response to infectious pathogens, is to identify and discriminate healthy and damaged tissues. Sensing tissue stress helps the immune system to simultaneously induce inflammation and tissue repair thereby maintaining homeostasis. It is now apparent that there are a variety of Pattern Recognition Receptors (PRRs) on innate immune cells that serve to identify Pathogen Associated Molecular Patterns (PAMPs) and/or Danger Associated Molecular Patterns (DAMPs)[1]. While PAMPs are derived from microorganisms, DAMPs are cell-derived in response to trauma and tissue damage that occurs either in the absence or presence of pathogenic infection. DAMPs constitute a large family of endogenous molecules that include alarmins, heat shock proteins, extracellular ATP, low molecular weight hyaluronic acid, HMGB1, uric acid etc. Exposure to these molecules triggers the innate immune system and directs its activity towards resolution of danger and tissue distress. The innate immune cells then initiate the adaptive immune responses; which can include cell-mediated immunity or humoral immunity mediated by T or B cells, respectively. This adaptive response enables the host to "remember" the pathogen, which ensures that any secondary infections will lead to a stronger immune response and the rapid clearance of the pathogen.

Janeway suggested that PRRs were important for the initiation of adaptive immunity[2]. The seminal work that identified the first PRR was provided by Le Maître and Hoffman when they demonstrated that mutant flies lacking a functional

Toll protein were susceptible to fungal infections[3]. This initiated an avalanche of studies to identify PRRs and understand how innate immunity could initiate and shape adaptive immune responses [4]. Since then, many surface and cytosolic PRRs have been identified in higher vertebrates and throughout evolution, and we are beginning to understand how signaling through these receptors allows for the initiation of adaptive immune responses. We have also come to realize that the activation of these PRRs can lead to inflammatory immunopathology. Therefore studies to understand how PRR signaling can be modified or interrupted are providing new hope for the development of therapeutics to treat inflammatory diseases[5].

There are two major consequences of ligating PRRs on immune cells. The first is to signal danger to the cells and to initiate a cascade of responses that can help to direct host defense responses. Most of these responses are in the form of cytokines or antimicrobial compounds that are produced by leukocytes in response to PRR stimulation. The second important consequence is to induce the competency of select cells to present antigen to T cells. The presentation of antigen by antigen presenting cells (APCs) is necessary to initiate adaptive immune responses. PRR stimulation induces DC maturation to stabilize MHC molecules on their surface and facilitates antigen presentation by inducing the expression of co-stimulatory molecules on APCs to induce T cell proliferation and differentiation. Thus, the ligation of PRRs can benefit the host in two ways. First it can initiate innate immune responses to kill pathogens directly, and if those responses are not sufficient to clear

the pathogen it can initiate antigen-specific adaptive immune responses to provide the next layer of protection[6].

1.1.1 Macrophages

An important component of the innate immune system is the macrophage. Macrophages are important phagocytes in the host as they serve two general functions. The first is the maintenance of homeostasis, which involves the regulation of iron metabolism, the clearing of apoptotic cells, and tissue regeneration. The second general function is host defense. As an innate immune effector cell, macrophages are involved in the phagocytosis of foreign particles, and the elimination of pathogens[7]. During inflammation, macrophages are primarily derived from precursor monocytes that are derived from the hematopoetic stem cells in the bone marrow and continuously circulate in the bloodstream. These cells constantly replenish tissue macrophages after exiting the bloodstream to reside in the tissue and becoming resident cells. Based on the anatomical site, the macrophages in different tissues are termed osteoclasts (bone), microglia (CNS), Kupffer cells (liver), histiocytes (connective tissue), and alveolar macrophage (lung). These tissue specific macrophages have their own specialized functions in maintaining homeostasis and providing the first line to defense against pathogens.

Macrophages express PRRs that help in identifying DAMPs and PAMPs. They are generally of two types: cell surface receptors such as toll-like receptors (TLRs), mannose receptor and other lectin receptors; and cytosolic receptors, including NOD-like Receptors (NLRs) and RNA helicases. The presence of multiple PRRs helps macrophages to identify a broad range of pathogens. The

ligation of these receptors enables macrophage activation. These receptors activate transcription factors such as NF-κB and IRFs that are involved in the synthesis of many proinflammatory cytokines such as IL-1β, TNF, IL-12, IL-6, and IFNγ etc. that play an important role in anti-microbial defense[8].

Macrophage activation shows remarkable plasticity as their phenotype is regulated by the sum of all environmental signals, with dynamic changes occurring over time. Macrophages can be broadly classified according to their fundamental roles in maintaining different aspects of homeostasis. Classically activated macrophages (CA-Mϕ) are activated by a combination of IFNγ and TNF. They have been shown to be important to eliminate intracellular pathogens like *Leishmania* spp. and thus are an important component of the delayed type hypersensitivity reaction. Although they are considered important in host defense against pathogens, their actions need to be regulated as their indiscriminate microbicidal properties can also lead tissue damage to the host that can cause autoimmune diseases[9]. Regulatory macrophages, (R-Mϕ), a subset of macrophages that were first described in this lab[10], in contrast to classically activated macrophages, are characterized by high production of the anti-inflammatory cytokine IL-10 and decreased production of IL-12[11]. R-Mϕ are generated by two signals. The first signal (immune complexes, prostaglandin E2, adenosine, apoptotic cells, etc.) does not significantly induce cytokines on its own, but in combination with a second stimulus like TLR ligands, can subvert macrophage activation to produce anti-inflammatory cytokines. R-Mϕ are thought to be present during the initial innate immune response to control inflammation and also during

late stages of the adaptive immune response to limit cytotoxicity. Since they are immuno-suppressive in nature, R-Mϕ can be exploited by different pathogens. Viruses can use antibodies to enhance their ability to infect while inducing the production of anti-inflammatory cytokines[12]. *Leishmania* spp. and *Bacillus anthracis* have been shown to induce the production of these Mϕ subsets to establish an infection[13]. Wound healing macrophages or alternatively activated macrophages (AA-Mϕ) form a third type of macrophages that are derived by IL-4 and/or IL-13 activation[14]. IL-4 is generated by basophils early during an immune response and later by T_h2 cells. AA-Mϕ have been shown to be involved in the generation of extracellular matrix by stimulating arginase activity[15] important in clearing helminthes and nematodes. AA-Mϕ have been reported to be more permissive to intracellular pathogens like *M. tuberculosis*, *L. major*, and *F. tularensis*, possibly because they fail to produce nitric oxide[16,17]. All three populations of activated macrophages govern critical functions in the host, and consequently all of their activation responses must be tightly controlled to avoid potential complications.

1.1.2 Inflammasome

The inflammasome is a term used to describe a molecular platform that activates inflammatory caspases leading to the maturation and secretion of important cytokines like IL-1β and IL-18[18]. Inflammasomes are multi-protein oligomers composed of leucine-rich repeat containing receptors (NLRs), adaptor molecules like apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1. Sometimes human capase-5, and mouse caspase-11 are found to be

recruited to NLRP1 inflammasomes[19]. They are termed inflammasomes in analogy to another caspase activating platform found on cells that controls apoptosis and is known as the apoptosome[20]. There are three prototypical inflammasomes: NLRP1inflammasomes, NLRP3 inflammasomes and NLRC4 inflammasomes[21]. Caspase-1 is a cysteine protease that is normally present as an inactive precursor in the cytosol, known as a procaspase. Once the inflammasome is activated, multiple procaspase-1 molecules are brought close to each other and in an autocatalytic reaction are cleaved into mature enzymes. Activation of caspase-1 occurs downstream of the signaling of several members of the NLR family of cytosolic receptors (Figure 1).

1.1.3 NLRs

NLRs are composed of C terminal leucine-rich repeats (LRRs) that bind to ligands, a central NACHT domain that results in NLR oligomerisation, and an N terminal effector domain (CARD or PYD). The NLRs can be subdivided into many subfamilies based on their structure; NLRA (CCTIIA), NLRB (NAIP), NLRC (NOD1, NOD2, IPAF etc), NLRP (14 NLRPs have been identified to date) and NLRX (NOD9)[22]. Their tissue distribution is varied. For example, while NLRP1 is widely expressed in many tissues, NLRP3 is expressed in immune cells and epithelial cells. NLRP4, 5, 7, 8, 10, and 11 are expressed in germ cells. IPAF and NAIP are expressed in brain, spleen, liver lung and other macrophage rich tissues. Their expression can be up-regulated by ligation of TLRs thus demonstrating the capability of NLRs to synergize with TLRs during an immune response[23].

The basic mechanisms involved in the activation of caspase-1 are quite similar for the subfamilies, but they might require additional molecules; for example NLRP1 and NLRP3 inflammasomes require ASC to interact with the N terminal PYD domain of the NLRs and recruit caspase-1. The NLRP1 inflammasome can also interact with caspase-5 through its C terminal CARD domain. IPAF can directly interact with caspase-1 through its N terminal CARD domain. All of NLRs require ATP to bind to the NACHT domain to initiate oligomerization thus forming a "donut shaped" platform[24] for caspase-1 to be activated.

The importance of caspase-1 in innate immunity is shown by caspase-1 deficient mice that have a defect in the maturation of IL-1β and IL-18 and are more resistant to endotoxic shock[25]. Caspase-1 knockout mice are more permissive to Listeria infections during the initial phase of the infection[26]. IL-18 is best known as a stimulator of NK cell activity, inducing IFNγ from splenocytes along with IL-12 and up-regulating adhesion molecules The proinflammatory properties of IL-1β include up-regulation of adhesion molecules, influencing leukocyte recruitment by inducing chemokines, induction of fever, reducing pain threshold, vasodilation and hypotension[27].

1.1.4. NLRP1 Inflammasome

The NLRP1 inflammasome contains molecules of NLRP1, ASC, caspase-1 and sometimes caspase-5. The NLRP1 inflammasome has been implicated in anthrax caused by *Bacillus anthracis*[28] infection. Anthrax lethal toxin is one of the major virulence factors that can cause systemic infections, possibly through the

activation of NLRP1 inflammasome[29]. Polymorphisms in *NLRP1b* have been identified and shown to be required for mouse macrophage susceptibility to anthrax lethal toxin[30]. Although the exact mechanism of NLRP1 activation has not been shown, binding, uptake, endosome acidification and final translocation of the lethal factor have been shown to be important in caspases-1 activation. NLRP1 can associate with ASC through its PYD domain in the N terminal and can recruit caspase-1 through the card domain leading to the activation of caspase-1. Through its C terminal CARD it can either bond to a second molecule of caspase-1 or recruit caspase-5. The activation of these caspases is known to be required for IL-1β processing and release. Their activation is also known to induce pyroptotic cells death in macrophages.

1.1.5. NLRC4 inflammasome

NLRC4 (IPAF) has been shown to be required for inflammasome activation upon interacting with *Salmonella typhimurium*[31]. NLRC4 deficient mice were found to be resistant to IL-1β release by *S. typhimurium*- and host cell death. Bacterial flagellin is reported to be the ligand for NLRC4 as it has been shown that purified flagellin can induce caspase-1 activation when introduced directly into the cytosol. NLRC4 can directly activate caspase-1 through its N terminal CARD domain. Although the role of ASC is unclear, it is proposed to stabilize the caspase-1 interaction with NLRC4[32]. Flagellin-dependent caspases-1 activation requires a type three-secretion system in bacteria as it has been shown that *Pseudomonas aeruginosa* also activates NLRC4, and mutations in its type three-secretion system abrogate the release of IL-1β.

1.1.6. NLRP3 Inflammasome

NLRP3 is the most well characterized inflammasome and it consists of 3 proteins; NLRP3, ASC and caspase-1. The NLRP3 inflammasome has been implicated in responding to environmental stress and danger. Auto-inflammatory diseases like familial cold induced auto-inflammatory syndrome and Muckle Wells syndrome have been linked to mutations in the NLRP3 gene. There are many NLRP3 activators that include both exogenous and endogenous danger signals[33]. Since all these activators lack obvious similarities in their structure or function, it is hypothesized that they can activate a common endogenous molecule that can serve as a NLRP3 ligand[21]. Inspite of these observations, the precise mechanism of NLRP3 inflammasome activation is not known. Three models of NLRP3 activation have been proposed: (i) pore formation inducing potassium efflux and membrane permeabilization which induce direct interaction of an agonist with NLRP3; (ii) phagocytosis of crystalline particles can cause lysosomal damage triggering release of cathepsins from lysosome enabling the activation; and (iii) NLRP3 agonist induced K+ efflux can trigger the activation of the inflammasome. As shown in Figure 1, upon activation, NLRP3 oligomerizes to form a platform for ASC interaction through the PYD domains. The CARD domain on ASC then interacts with procaspase-1. Procaspase-1 clustering leads to the autocatalytic reaction leading to caspase-1 activation. Caspase-1 then cleaves its substrates IL-1β and IL-18. Caspase-1 along with IL-1β and IL-18 are secreted into the extracellular space through a non-classical mechanism. Secretory lysosomes, micro-vesicle shedding

and the presence of dedicated transporters are implicated in the secretion of inflammasome-activated cytokines[34].

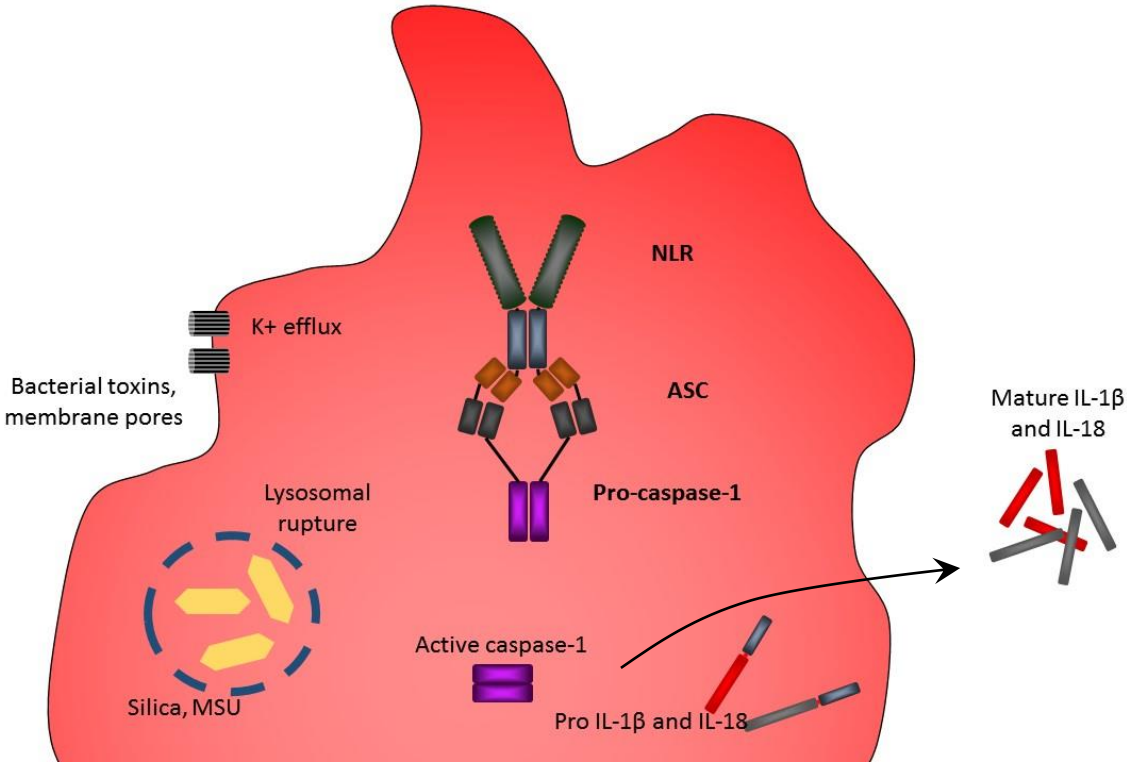

Figure 1: The Activation of NLRP3 Inflammasome:

TLR Primed BMMϕ expresses NLRP3 inflammasome and proIL-1β protein. When macrophages are exposed to danger signals like extracellular ATP or bacterial toxins or phagocytosis of crystalline particles like monosodium urate or silica, the membrane potential of macrophages is perturbed. This causes loss of K+ ions from the cell triggering the assembly of NLRP3 inflammasome which is composed of NLRP3, ASC and pro-caspase-1 in a specific area of the cell and form a speck. They serve as platforms for the generation of activated caspase-1. Activated caspase-1 then promotes the maturation and secretion of mature IL-1β and IL-18.

1.2. Complement System

The complement system is a serine protease enzymatic cascade composed of a group of soluble plasma proteins, which can become activated to bind to foreign surfaces. The fact that the basic components of the complement system are found in many species with conserved biochemical properties signifies the importance of the system in the host. There are three distinct pathways of complement activation, called the Classical, the Alternative, and the Lectin pathways. Antibody bound to an antigen can activate the Classical complement pathway. Some foreign surfaces can directly activate the Alternative complement pathway and the recognition of microbial polysaccharides on cell surfaces can activate the Lectin pathway. The three different pathways converge by activating C3 and later C5, leading to the formation of Membrane Attack Complex (MAC), which is assembled by the sequential addition of late complement components C5-C9. For a time after the discovery of complement, the formation of MAC was believed to be the only function of the complement system[35]. Now it is known that complement has many important biological functions in regulating the immune response including activation and recruitment of leukocytes via anaphylatoxins; opsonization of pathogens to promote phagocytosis; and clearance of immune complexes and apoptotic cells from circulation[36]. Patients with complement component deficiencies are usually given prophylactic antibiotics as complement plays an important role in host defense to various pathogens

1.2.1 Complement Activation

There are three major pathways of complement activation, classical, alternative and lectin, which converge at the level of C3. All three pathways thereafter share a common sequence through the late components C5, C6, C7, C8 and C9, leading to the generation of the MAC. As shown in Figure 2 the classical pathway is initiated by C1 when it recognizes antibody bound antigen. Being a multimeric complex with serine esterase activity C1 cleaves C4. The resulting C4b fragment has a binding site for C2, which allows C1 to cleave the zymogen C2. Cleaved C2 (a serine protease) proteolytically activates C3 and then C5 though the formation of C3 and C5 convertases. The phylogenetically older alternative pathway is activated by the constant ticking over of C3 to C3b. Using fragments of C3 and factor B it forms the bimolecular enzyme C3bBb after being activated by factor D which circulates as an active serine esterase. However, the pathway is normally restricted because the inhibitor factor H prevents the binding of factor B with C3b, and factor D is only able to cleave B that is bound to C3b. The ability of factor H to discriminate between host and microbial carbohydrate determinants provides the specificity for activation of the alternative pathway. The lectin pathway is initiated when mannose binding lectin bids to polysaccharies on bacterial surfaces (Figure 2).

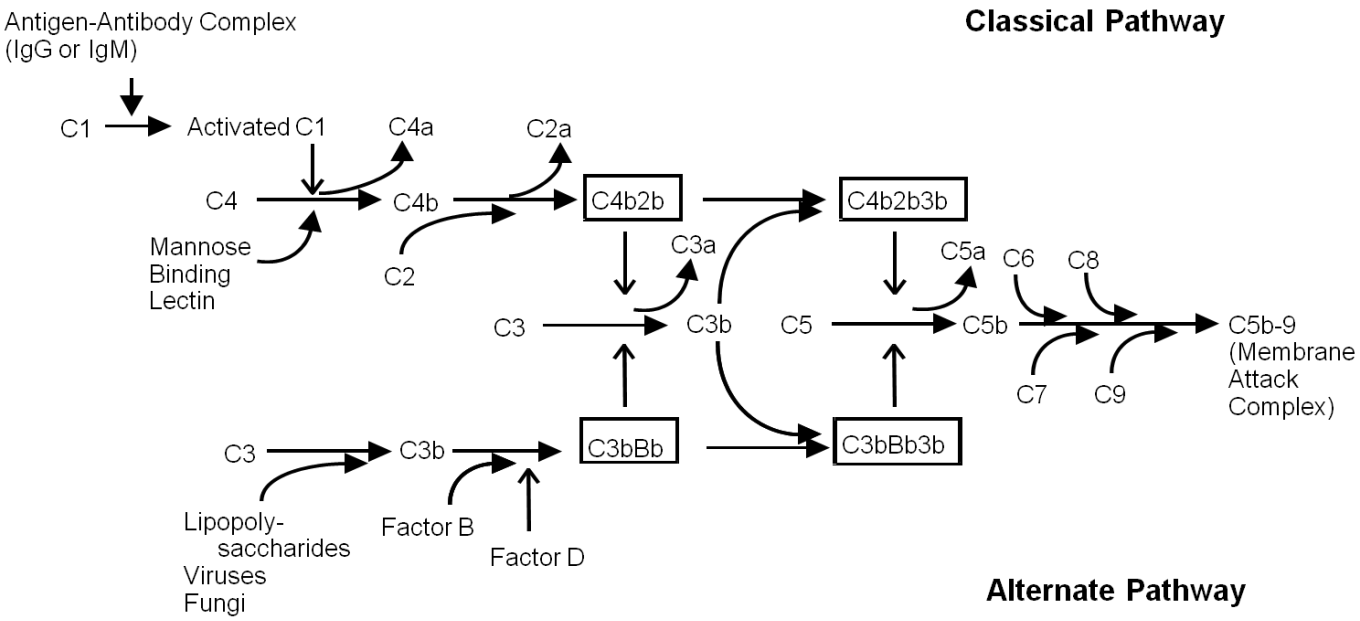

Figure 2: Complement activation:

The three major pathways of complement activation are shown (lectin pathway is initiated by mannose binding lectin). These pathways lead to the formation of C3 convertases (C4b2b represents the classical/lectin pathway C3 convertase, and C3bBb the alternative pathway C3 convertase), which activate the central component C3. The C3b fragment can bind to the existing convertases and thus form the C5 convertases of the classical/lectin pathway (C4b2b3b) or the alternative pathway (C3bBbC3b). C5 convertase is essential for the activation of the common terminal pathway, which leads to the generation of the lytic membrane attack complex composed of C5b, C6, C7, C8, and C9. The C3b fragment is also a major opsonin, mediating phagocytosis via C3-fragment receptors on phagocytes. The generated C3a and C5a are potent anaphylatoxins that are involved in the activation of various cell types and mediate inflammatory processes.

1.2.2 Activation of the Late Components

The activation of the late components is initiated by a C5 convertase which results in the formation of the "active" form of C5 termed C5b. It is a short lived molecule still associated with the convertase which then binds to C6. The resulting C5b6 complex is stable and can initiate the formation of the MAC on its own. C5b6 can reversibly associate itself with membrane and other hydrophobic surfaces but the binding of C7 yields a stable trimolecular complex that exposes for a few milliseconds hydrophobic domains on the complex that is bound to the membrane that can associate with C8. The addition of C7 to C5b6 forms C5b67[37]. If C5b67 collides with a lipid membrane during this short interval, the C5b67 inserts in the membrane; if not, the C5b67 rapidly decays in the fluid phase. A domain on membrane bound C5b67 provides a binding site for C8. Although lysis of cells can be detected at this stage, it is substantially enhanced by the addition of C9 which forms the characteristic pore that results in lysis[38].

Complement activation can be separated into the activation step that ends with the formation of the C5 convertase and the effector step involving the late components. Only the latter effector step is essential for the lysis of target cells. More than 40 years ago, it was demonstrated that it was possible to lyse un-sensitized cells (cells lacking activating convertases) as experimentally shown by "reactive lysis" experiments pioneered by Lachmann [39]. The modulation of what is now referred to as "bystander activation" was shown to depend on many fluid phase factors like the presence of poly-cations and pH. We propose that during inflammation this phenomenon of innocent bystander activation of the MAC on

cells other than the target could have important consequences in modulating the immune/inflammatory processes[40].

1.2.3 Lytic and sublytic MAC

Complement was first recognized because of its capacity, in conjunction with antibody to lyse cells, in particular, bacteria. Complement mediated lysis is an important part of the response to infectious organisms, utilized as a defense not only in the early stages of infection, but also once adaptive immunity has developed. The key molecule in the MAC is C9 which inserts through the cell membrane and is then able to polymerize to form the "pore" of the MAC. When C9 is present in low copy numbers these pores form rigid, hollow protein-lined channels[41]. Erythrocytes were among the first cells to be investigated with regard to the lytic mechanism of complement action, and they are still used extensively today. It has been shown that a single functional MAC in the membrane of a metabolically inert, aged erythrocyte is sufficient to lyse the cell by colloid osmosis. Not surprisingly, the efficiency with which lysis occurs depends on the number of MACs in the cell membrane, and on the composition of the extracellular fluid. This simple picture of osmotic cell lysis does not extend to analyses of MAC-mediated killing of nucleated cells[42]. More subtle cell-damaging effects of the MAC would therefore not be detected in this model, resulting in the popular view of the MAC as a moiety which rapidly and efficiently kills cells. Unlike in erythrocytes, several channels are required to lyse a metabolically active nucleated cell or bacteria. The multichannel requirement for nucleated cell lysis has been attributed to a defense response of the cell though ion pumps to stabilize membrane polarization and to

promote elimination of terminal complement complex (TCC) from the PM by endocytosis or by endocytosis and vesiculation. Complement- mediated lysis of nucleated cells displays "multi-hit" kinetics, implying a requirement for many MACs on the cell surface, and factors other than colloid osmotic dysregulation, notably the presence of calcium in the extracellular fluid, influence the efficiency of killing. Host cells are also endowed with abundant complement regulatory proteins like CD59, CD55 and CD46. Nucleated cells are thus more difficult to kill with antibody and complement than are metabolically inert targets like aged erythrocytes or liposomes[43,44].

The MAC however has been implicated in a variety of other non-lytic effects that differ according to the nature of the target cell and the system interrogated. Especially when formed on phagocytes (neutrophils and macrophages), metabolically active cell types that are intrinsically resistant to complement-mediated lysis, the MAC induces profound activation with the production and release of inflammatory mediators such as prostaglandins, thromboxanes, leukotrienes and reactive oxygen species[45]. These processes aid in the activation of the immune system by stimulating the inflammatory response and arming the host immune system to deal with invading organisms or danger signals.

1.2.4 The Role of MAC in Inflammatory diseases

The MAC can be implicated in a disease process based on the demonstration of increased consumption of terminal complement components or production of terminal complement complexes (SC5b-9 or MAC) in biological fluids, localization of the MAC in diseased tissue or abrogation of the disease by inhibition

of MAC-producing capacity. The availability of antibodies specific to neo-antigens of terminal complement complexes has made their measurement in biological fluids and localization in diseased tissue a relatively simple process and has stimulated an interest in TCCs, reflected in a large number of publications, and an increasingly long list of diseases in which TCCs are implicated[40].

The first disease in which the MAC was implicated was myasthenia gravis, an autoimmune disease characterized by the loss of acetylcholine receptors from the motor end plate, resulting in extreme muscle fatigability[46]. Anti-C9 antibodies were used to localize the MAC at the end plate, and a role of the MAC in acetylcholine receptor loss was proposed A requirement for terminal complement components, specifically C6, has been demonstrated in a model of myasthenia gravis, experimental myasthenia induced in rats by inoculation with antibodies to the acetylcholine receptor, thus demonstrating that, at least in this model, MAC formation is essential for disease expression. It has been suggested that the MAC causes 'focal lysis' of the junctional membrane resulting in loss of membrane and acetylcholine receptors

Multiple sclerosis is a common neurological disease characterized by widespread demyelination within the central nervous system. The pathogenesis of the disease is obscure, though many factors have at various times been implicated. The first evidence suggestive of a role of the MAC came from the demonstration that the concentration of the terminal complement component C9 was reduced in cerebrospinal fluid from patients with this disease[47], implying its utilization in MAC formation. Non-lethal effects of the MAC demonstrated at the cellular level

in vitro may therefore be, at least in part, responsible for the relapsing and remitting course of the clinical disease.

The C5b-9 MAC has also been recently reported to mediate mesangial cell apoptosis in experimental glomerulonephritis. Separating the role of the C5b-9 membrane attack complex from earlier complement components by examining the effects of cobra venom factor (which depletes all complement components and prevents the generation of the anaphylotoxins C3a and C5a, as well C5b-9, etc.) and isolated C6 deficiency by using C6-deficient PVG rats[48] (which can generate C3a and C5a but are unable to form the C5b-9 membrane attack complexes), it was found that complement is the principal inducer of endothelial cell apoptosis in antibody-mediated glomerulonephritis. This effect was mediated primarily by sublytic C5b-9[49].

1.3 Adaptive Immunity

The adaptive immune system is composed of lymphocytes that are important for prevention and elimination of pathogen growth. They consist primarily of T cell and B cells. T cells are endowed with T cell receptors and B cells possess the ability to generate and secrete antibody. Two of the defining features of the adaptive immune system are specificity and immunological memory. Specificity in the adaptive immune system is achieved by a combination of DNA recombination and somatic hypermutation. Both these processes confer diversity of receptors to the cells of the adaptive immune system. Due to this, unlike the innate immune cells which can only recognize molecular patterns, the adaptive immune system can recognize antigens and respond appropriately. Immunological

memory is conferred to cells of the adaptive immune system though the process of clonal expansion and maintenance of memory cells. If a T or B cell becomes activated by binding to foreign molecules, it starts replicating, generating a huge colony of cells that specifically recognizes a particular epitope of an antigen. Immunological memory forms the basis for the strategy of vaccinations which are given to children.

There are two major phases of the adaptive immune response to a pathogen: the recognition phase and the effector phase. The recognition phase consists of the receptors of a T or B cell binding to the antigen. T cells bind to a peptide derived after processing of the antigen and presentation on MHC molecules on antigen presenting cells (APCs) such as dendritic cells or macrophages. The T cell receptor (TCR) binds to the peptide MHC complex which constitutes the first of two signals needed for T cell activation. The second signal, called costimulation, is also expressed on APCs after the activation through PRRs. These costimulatory molecules are typically CD80 and/or CD86. The first and the second signals activate the T cells to clonally expand and differentiate to effector cells or memory cells. B cells on the other hand do not need antigen presentation as they bind to the antigen directly. Their activation threshold is lowered if the antigen is opsonized by complement by engaging CD21 on B cells. Once activated the B cells expand clonally and secrete antibodies after differentiating to plasma cells. The effector phase consists of modulation of the immune response to tailor to the pathogen and promote the release of antimicrobial products. In this phase cells of the adaptive immune system cooperate with all the cells of the innate immune system to promote

immunity against the specific pathogen. The immune response can be crudely classified into two components: cell mediated immune response and humoral immune response. Cell mediated immunity is mainly mediated by T lymphocytes. This response is generated against intracellular microbes, which survive and proliferate inside host cells. Cell mediated immunity can lead to the destruction of the microbes or the lysis of the infected cells. Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. Antibodies specifically neutralize antigens, and target them for elimination by other effector mechanisms, such as complement.

1.4 Leishmaniasis

Leishmaniasis is a disease that is endemic in regions of the Middle East, South America, South Asia, and Africa. According to WHO estimates, Leishmaniasis affects 12 million people in 88 countries with two million new cases every year[50]. *Leishmania* spp is a protozoan parasite that can be transmitted by the bite of sand flies (Phlebotomus and Lutzomyia). There are four different forms of the disease: visceral, cutaneous, muscocutaneous and diffuse cutaneous. Almost 90% of visceral leishmaniasis cases in the world are found in India, Brazil and Bangladesh. Recently it has been found that patients infected with HIV have increased risk to contract leishmaniasis. In turn, infection with Leishmania spp. facilitates in establishing the clinical symptoms of AIDS through synergistic immuno-suppression. This compounds the problems associated with the treatment of patients that suffer from HIV/Leishmania co-infection[51].

The *Leishmania* parasite spends half of its life cycle as a motile promastigote that lives in the gut of the sandfly and the other as a non-motile amastigote that lives inside host macrophages. The procyclic promastigotes can divide in the gut of the fly, and then become metacyclic to be able to infect mammalian hosts. Once transmitted into the host, these parasites can quickly differentiate into amastigotes after being taken up by macrophages. The amastigotes can then divide in the phagolysosomes of macrophages and are eventually released when these phagocytes burst. These released parasites are taken up by neighboring macrophages, thus gaining access into surrounding tissue through the mononuclear phagocyte system. When an uninfected sandfly takes a blood meal from the infected host, the amastigotes taken up into the fly can differentiate into promastigotes in the sand fly and the cycle repeats itself.

Leishmania infection in mice has long been considered by immunologists as a model to study the immune response by a host. BALB/c mice are naturally more susceptible to *Leishmania major* infections due to the development of an ineffective T_h2 immune response against the parasites Other strains like C57BL/6 and C3H are able to control the infection due to their ability to raise an effective T_h1 response predominated by the cytokine IFNγ, which is able to activate macrophages to kill the parasite and eliminate the infection[52].

Leishmania spp. activate the alternative complement pathway[53]. Interactions between complement and Leishmania are important for two different (and possibly opposite) reasons. First, the opsonization of the parasite by complement can target these organisms to macrophages and promote parasite

phagocytosis. This can actually be an advantage to the parasite which replicates inside macrophages. Second, complement activation can also result in the deposition of the terminal components of complement to lyse the parasite. Complement is thought to be important in resistance against Leishmania in humans, as it has been shown that nearly 90% of all promastigotes can die within 3 minutes in medium containing 50% normal human serum (NHS) at 37°C[54]. Complement thus forms an important arm of the innate immune system to exert a strong selective pressure on the promastigotes. However, *Leishmania* employs several mechanisms to subvert and evade the complement system. Lipophosphoglycan (LPG) can activate the alternative pathway of complement and enables Leishmania to target macrophages as the host cell. At the same time LPG can also protect Leishmania from complement attack as the LPG coat on its surface becomes longer during differentiation into the infective metacyclic stage, thereby providing a physical barrier for the insertion of MAC. Another Leishmania surface molecule called gp63 cleaves the membrane bound C3b molecule into the inactive iC3b form, which then acts as a ligand for the macrophage CR3 receptor. Thus LPG and gp63, can protect the parasite from complement-mediated lysis and are considered to be important virulence factors[55].

1.5 Summary

The predominant function of the complement system is to "complement" the activity of antibodies and provide the first line of defense against pathogens. It also shapes the immune response by facilitating recruitment of leukocytes, opsonizing pathogens for phagocytosis, and regulating the adaptive immune

response by interacting with various receptors. In this study, I elucidate a new function of the complement system where it activates leukocytes during complement-mediated phagocytosis, resulting in the activation of the NLRP3 inflammasome though the formation of MAC on their cell surfaces. *Leishmania major* is considered to be a silent pathogen that does not induce any cytokines or antimicrobial factors from the host macrophages during infection. *Leishmania major*, by activating complement, is shown here to influence cytokine production in macrophages through the activation of NLRP3 inflammasome. The inflammasome, a previously uncharacterized player in Leishmania infection, is known to activate innate immunity by the processing and release of IL-1β and IL-18.

Chapter 2: Materials and Methods

2.1. Mice

C57BL/6, Balb/C, C5 deficient (B10.D2-Hc0 H2d H2-T18c/oSnJ), C5 sufficient (B10.D2-Hc1 H2d H2-T18c/nSnJ) and OT-II transgenic mice, which were 6-10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in high efficiency particle air-filtered Thoren units (Thoren Caging Systems, Hazleton, PA) at the University of Maryland. All animal studies were reviewed and approved by the University of Maryland Institutional Animal Care and Use Committee. The generation of $Nlrp3^{-/-}$, $Asc^{-/-}$, and $Casp1^{-/-}$ mice has been described previously [33]

2.2. Reagents

ATP, zymosan, and inulin were purchased from Sigma-Aldrich (St. Louis, MO). Glybenclamide and Compstatin were purchased from Tocris Bioscience (Bristol, UK). Normal human serum, serum deficient for complement proteins C3, C5, C6, C8 or C9, and purified C5b6, C7, C8, C9 proteins were purchased from Complement Tech (Tyler, TX). Particulate inulin was prepared by dissolving one gram of inulin in 40 ml water containing 0.1% ammonia and heated at 60°C. Inulin was frozen at -20°C overnight and subsequently precipitated at 37°C for 3 days in the presence of chloroform. The resulting precipitate was centrifuged at 2000xg for 10 min and re-suspended in water to a concentration of 10mg/ml and stored as 1 ml aliquots. The particles were sonicated before use.

2.3. Murine Macrophages

Bone marrow derived macrophages (BMMϕ) were prepared as previously described[56] Briefly, bone marrow cells was flushed from the femurs and tibias of mice with PBS+penicillin/streptomycin and cells were plated in petri dishes in DMEM/F12 supplemented with 10% FBS, 10 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20% conditioned medium from the supernatants of M-CSF secreting L929 (LC14) fibroblasts conditioned medium (LCCM).Cells were fed with the same media on day 3. On day 7, macrophages were removed from petri dishes and cultured in above mentioned media without LCCM.

RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% FBS with penicillin, streptomycin and glutamine.

2.4. Human Macrophages

Human peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers with their informed consent in accordance with the Declaration of Helsinki and institutional review board approval. The mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. .Monocytes were purified by attachment to culture dishes and differentiated into macrophages in the presence of DMEM medium supplemented with 10% heat inactivated human AB serum for 7 days.

2.5.Leishmania Parasites

Leishmania major Friedlin strain, clone V1 (MHOM/ IL/80/Friedlin), Ds-Red *L. major*, parasites were maintained as previously described[57] Stationary-phase promastigotes were propagated in 50:50 Schneider's insect medium (Sigma-Aldrich) and M-199 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine at 25˚C. Transgenic Ds-Red *L. major* parasites were grown in the presence of 50 µg/ml of Geneticin (G418; Sigma- Aldrich).

2.6. Stimulation of bone marrow–derived macrophages

BMMϕ were primed with 10 ng/ml Ultrapure LPS (Invivogen, San Diego, CA), for 4-6 hrs. LPS-primed BMMϕ were either challenged with 1:10 multiplicity of infection of *Leishmania major,* or were treated with ATP/zymosan/inulin at concentrations (1mM to 5mMin the presence or absence of 5% of normal human serum for 4 hrs. Supernatants were collected and assayed for IL-1β or IL-18. Antibody pairs for IL-1β ELISA were purchased from eBioscience (San Diego, CA) and for IL-18 ELISA from MBL International (Woburn, MA).

2.7. Cell Viability and FLICA Staining

Viability of BMMϕ was assessed by 7AAD and Annexin V staining (Molecular Probes). FAM-FLICA™ Caspase-1 assay was performed following manufacturer's protocol (Immunochemistry Technologies, Bloomington, MN). Cells positive for active caspase-1 were quantified by flow cytometric analyses.

2.8. Invitro Leishmania killing assay

$1x10^5$BMMϕ were plated on glass coverslips in a 24 well plate in 500µl DMEM media with 10% FBS and incubated overnight. The cells were stimulated with 20ng/ml IL-1β, 100U/ml IFNγ, 10µg/ml LMW-HA in appropriate wells. Ds-Red *Leishmania* promastigotes were added to each well at a ratio of 10 *Leishmania* parasites to 1 macrophage with 5% C5 deficient serum (serum from DBA2 mice). After 48 hours incubation, the cells were lysed with 1% triton-X solution in water. Leishmania viability was quantified by measuring the total amount of DsRed fluorescence using a fluorometer. The results were normalized with total protein concentration from the cell lysates as determined by absorbance at A280.

2.9. Lentiviral transduction

Lentivirus was produced in human embryonic kidney (HEK293T) cells transfected with the FUGW-based expression vector encoding CFP-ASC (a kind gift from Dr. Katherine Fitzgerald), lentiviral packaging plasmid psPAX2 and envelope plasmid pMD2.G using TransIT®-293 Transfection Reagent (Mirus Bio, Madison, WI). Supernatants containing lentivirus were collected after 48 hours and were used to infect RAW264.7 cells.

2.10. Immunofluorescence

For immunofluorescence imaging BMMϕ were cultured on cover slips, LPS-primed and activated with AlexaFluor®488-conjugated zymosan A (source *S. cerevisiae*; Life Technologies) or Ds-Red expressing *L. major*. To visualize complement MAC deposition on macrophages, C9-deficient (C9-D) serum was supplemented with Alexa647-labeled C9 protein at the time of image acquisition. Labelling of C9 protein with Alexa647 was performed following the

manufacturer's protocol (Alexa647 protein labelling kit, Life Technologies, Grand Island, NY). Cells were fixed with 4% paraformaldehyde, 3 min after C9 addition when the MAC deposition was found to occur at maximum on surface. Cover slips were mounted on clean glass slides using Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA).

To visualize inflammasome activation, RAW264.7 cells grown on cover slips were transduced with Lentivirus encoding CFP-ASC, LPS-primed and activated with Alexa488 conjugated zymosan A in the presence of 5% normal human serum for 45 min. The cells were then fixed and imaged under Leica SP5X Confocal Microscope (Leica, Exton, PA), in 100X oil immersion objective with numerical aperture of 1.32. The fluorochromes were excited using argon laser for Alexa-488 and white light laser for Ds-Red and Alexa-647 set at appropriate excitation wavelengths. The images acquired using Leica LAS software and processed with LASAF Lite and Adobe Photoshop CS4.

2.11. In vivo experiments

For in vivo models of inflammasome activation, the complement-activating and complement-depleting functions of cobra venom factor (CVF) were exploited in this study. Age-matched male C5 deficient or sufficient mice were injected with 2U/mouse CVF (Quidel, San deigo, CA) and 20 mg/kg LPS intraperitoneally. After 3 hours, blood was collected by penetrating the retro-orbital sinus of mice to measure serum levels of IL-1β by ELISA. To examine if complement activation plays a role in leukocyte infiltration, C57Bl/6 mice were injected with saline or 1mg of inulin particles intraperitoneally and assessed for intraperitoneal infiltration

of GR1 (1A8) positive cells after 6 hrs in peritoneal lavage in mice previously complement-depleted with CVF. CVF was administered 24 hrs to prior to inulin administration at 2U/mouse concentration. The percentage of GR1 positive cells were assessed by flow cytometry.

2.12. T cell Activation

Spleen were removed from mice and placed in a petri-dish filled with PBS. Single cell suspension was obtained after the spleen or lymph nodes were meshed with a plunger of 10-ml syringe through cell strainer of 70μm nylon (BD Biosciences, San Jose, CA). $CD4^+$ T Cells Isolation Kit (Miltenyi Biotec Inc) was used to magnetically separate and purify T cells from these single cell suspension solutions following the manufacture's recommendation.

For antigen presentation, macrophages were plated at the concentration of 2×10^5/well and primed with 10ng/ml LPS for 4 hrs. Cells were then washed, and activated with 150 μg/ml OVA and inflammasome activators like inulin (100 μg/ml) in the presence of human serum (5%). Alternatively purified complement proteins C5b6 (2μg/ml), C7, C8, C9 (10μg/ml each) alone were added to macrophages to activate inflammasome. Macrophages were washed after 2 hours and co-cultured with 5×105 CD4+T cells for a week. Following primary stimulation, $CD4^+$ T cells were harvested, washed, and re-stimulated with immobilized CD3 antibodies (BD Biosciences, CA) for 48 hrs. Levels of IL-17 were measured in the supernatants following secondary stimulation (BD Biosciences, CA).

2.13. Statistical Analysis

Data analysis was performed using SigmaPlot (Systat Software, San Jose, CA) and analyzed using the Student's t-test. The statistical differences between groups, with the p-values are indicated in the related graphs as: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Chapter 3: The Role of Complement induced phagocytosis in activating NLRP3 inflammasome

3.1. IL-1β production following complement mediated phagocytosis

The ability of complement opsonized particles to activate macrophages were tested by incubating particles with macrophages along with normal serum (that serves as a source of complement). Different particles were chosen depending on their ability to activate complement. As shown in Figure 3A zymosan and inulin particles were potent activators of the alternative complement pathway whereas latex beads were not. These particles were added to LPS-primed murine macrophages, and the release of IL-1β was measured in supernatants following phagocytosis. Zymosan is a potent activator of the alternative complement pathway. Increasing doses of zymosan were added to macrophages in the presence or absence of normal human serum as a source of complement proteins. Zymosan bound avidly to macrophages in the presence or absence of serum (Figure 3B), but only serum-opsonized zymosan activated complement and induced robust release of IL-1β from macrophages (Figure 3B). As a control for these studies latex beads, which do not activate complement were added to macrophages (Figure 3A). These beads bound avidly to macrophages in the presence or absence of serum, and neither condition resulted in substantial IL-1β release by macrophages (Figure 3D, E).

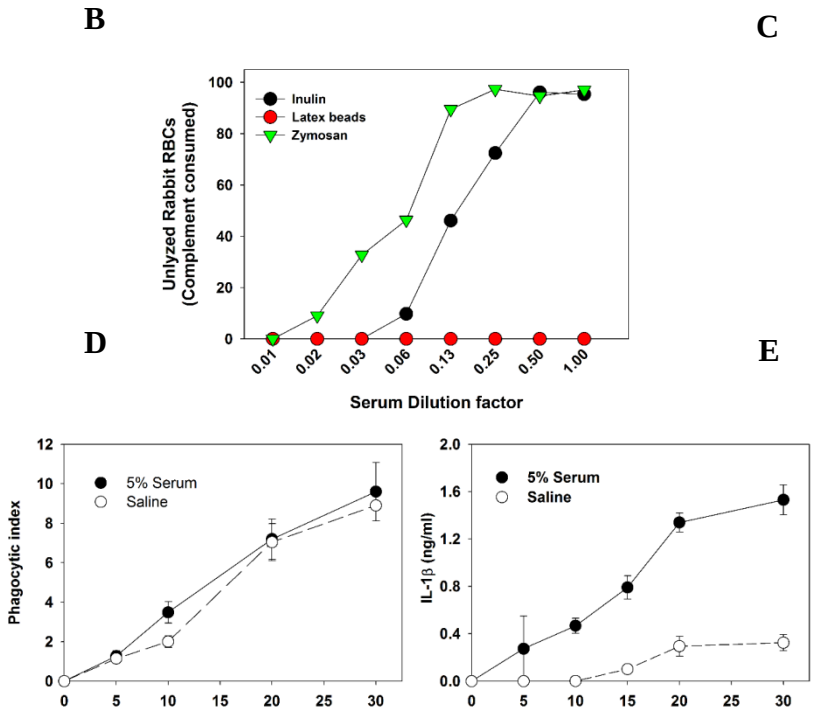

Figure 3: IL-1β production following complement mediated phagocytosis:

(A) Complement consumption to measure Alternative Complement Pathway activation by zymosan (black circles), inulin (green triangles), and latex beads (red circles) was assessed by analyzing the percent of unlyzed rabbit red blood cells. The percent lysis was measured at 550 nm to detect released hemoglobin after 30 minutes of incubation. (B) The phagocytosis of zymosan by mouse bone marrow derived macrophages (BMMϕ) was measured in the presence (closed circles) or absence (open circles) of 5 % normal human serum. The number of particles per 100 macrophages was counted under a 400X light microscope. Phagocytic index represents the average number of particles/macrophage (3 experiments). (C) IL-1β secretion by LPS primed macrophages was measured four hours following incubation with increasing amounts of zymosan in the presence (closed circles) or absence (open circles) of normal human serum (3 experiments). (D) The phagocytosis of latex beads by mouse bone marrow derived macrophages was measured in the presence (closed circles) or absence (open circles) of 5 % normal human serum. The average number of particles per 100 macrophages was counted and expressed as phagocytic index (2 experiments). (E) IL-1β secretion by LPS primed macrophages was measured four hours following incubation with increasing amounts of latex beads in the presence (closed circles) or absence (open circles) of 5% normal human serum (2 experiments).

3.2. Effect of opsonization on macrophage activation

Macrophages are important components in the immune system as they are often the first cells to come in contact with foreign pathogens. Serum opsonins like complement and antibodies help in detecting these pathogens and targeting them for destruction though phagocytosis in macrophages. To evaluate the specificity of this effect, we added another control particle, IgG coated sheep red blood cells (SRBCs), which bind to macrophage Fcγ receptors. We also added another complement activator, called inulin. Inulin is a polysaccharide that has been previously shown to be an efficient activator of complement. We were not able to detect any cytokine response by macrophages in response to IgG opsonization in complement free conditions (Figure 4A) but we could readily detect IL-1β secretion from complement opsonized inulin. IL-1β secretion from macrophages did not occur when serum complement was inactivated by heat (Figure 4B).

3.3 Secretion of inflammasome effectors following complement activation

The release of IL-18 from macrophages was also examined, and there was a similar dose-dependent release of IL-18 from macrophages following their interaction with zymosan (Figure 5A) in the presence but not the absence of complement. Thus complement-mediated phagocytosis by macrophages results in the release of IL-1β And IL-18. IL-1β is a protein made as an inactive zymogen, pro-IL-1β. The ELISA antibody pair does not discriminate between the cleaved/active form and the pro form of IL-1β. To analyze the specific form that is in the supernatant, fresh antibody pairs that only recognized proIL-1β was used.

The IL-1β that was released from macrophages was in the mature/cleaved form, because little pro-IL-1β was detected in the supernatants of these cells following the phagocytosis of complement-opsonized inulin as shown in Figure 5B. The role of complement was implied from the lack of activation seen when particles are opsonized with heat inactivated serum. To validate the importance of complement, Compstatin, a cyclic tridecapeptide that is a potent C3 inhibitor, was added to macrophages in the presence of normal serum with inulin. The induction of IL-1β by inulin occurred when inulin was added to macrophages in the presence of normal serum, but not in serum treated with Compstatin (Figure 5C).

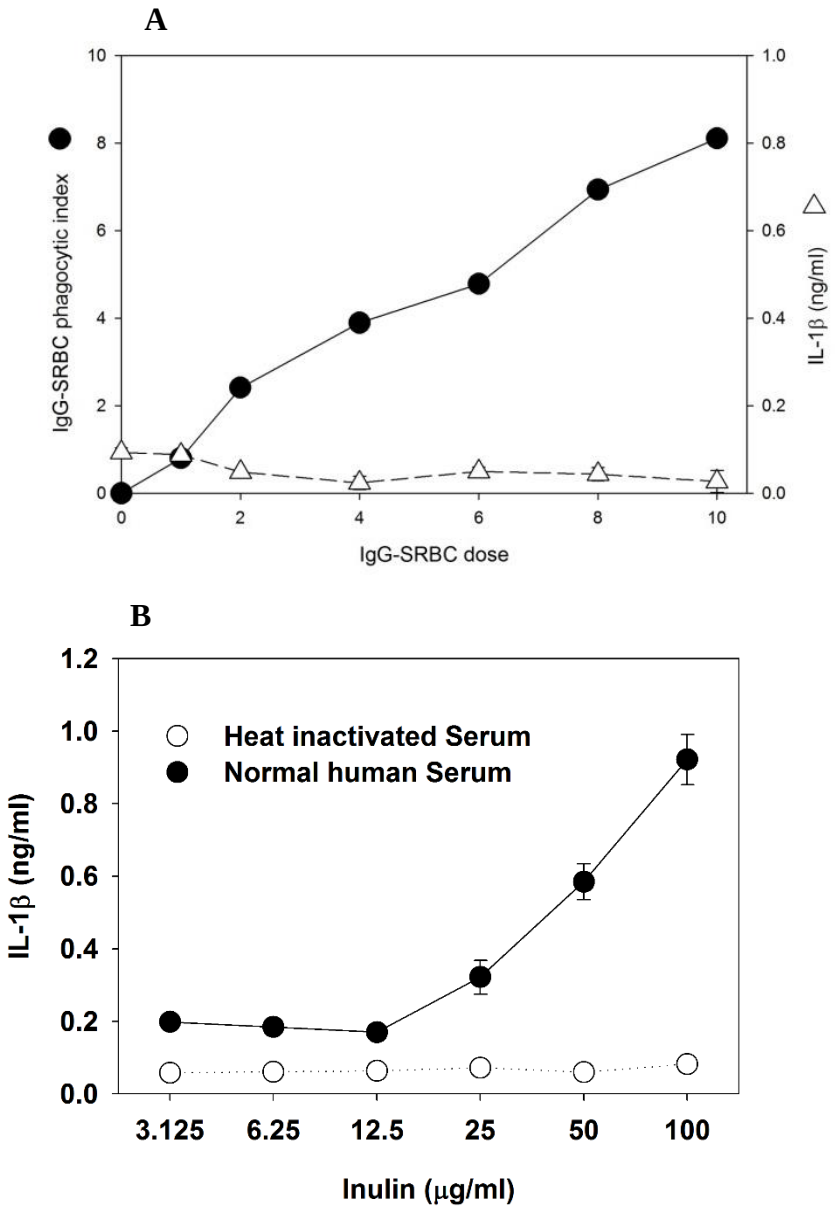

Figure 4: IL-1β production from particles opsonized with IgG or complement:

(A) IL-1β release (open triangles, right axis) following IgG-SRBC phagocytosis. The phagocytic index (left axis, closed circles) represents the average number of IgG-SRBC/macrophage (B) IL-1β secretion by LPS primed macrophages was measured four hours following incubation with increasing amounts of inulin in the presence (closed circles) of 5% normal human serum or 5% Heat inactivated serum (open circles) (3 experiments).

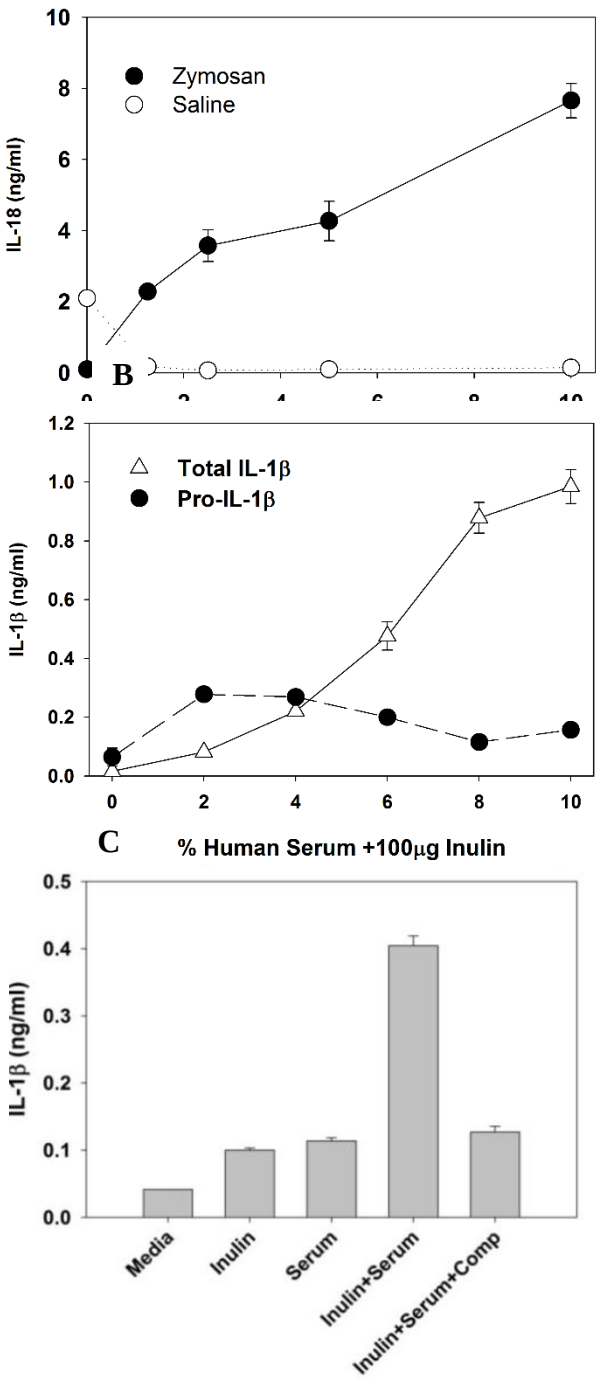

Figure 5: Macrophage activation following phagocytosis of opsonized particles:

(A) IL-18 secretion by LPS primed macrophages was measured four hours following incubation with zymosan (10 μg/ml) (closed circles) or vehicle control (open circles) and increasing concentrations of normal human serum (2 experiments). (B) Total IL-1β (open triangles) and pro-IL-1β (closed circles) were measured in supernatants by ELISA four hours after incubating macrophages with 100 μg/ml inulin and increasing concentrations of normal human serum. (C) The inhibition of inulin activated-complement induced secretion of IL-1β by 100μM compstatin

3.4. Membrane Attack Complex induced secretion of IL-1β

To identify the complement components necessary for macrophage inflammasome activation, serum deficient in specific complement components was examined. Macrophages failed to secrete IL-1β in response to complement activation by inulin when any individual complement proteins were depleted from serum (Figure 6A). This included serum deficient in the terminal components, C8 and C9, indicating a role for the membrane attack complex (MAC) in IL-1β release. To directly show that the MAC complex was required for inflammasome activation, the terminal complement components C5b, C6, C7 C8 and C9 were directly added sequentially to LPS-primed macrophages to assemble the MAC on the macrophage plasma membrane. IL-1β was only detected in cells exposed to the complete C5b-9 membrane attack complex (Figure 6B).

3.5. NLRP3 inflammasome activation during complement-mediated phagocytosis.

Macrophages have a number of pattern recognition receptors (PRRs) that can sense danger and promote host inflammatory responses. One of the receptors, NLRP3, is present in the cytosol and is believed to sense cytosolic danger through ionic imbalance and increased reactive oxygen species generation. We hypothesized that complement-mediated phagocytosis could cause the activation of NLRP3 inflammasome and subsequently, Caspase-1 to release IL-1 and IL-18.

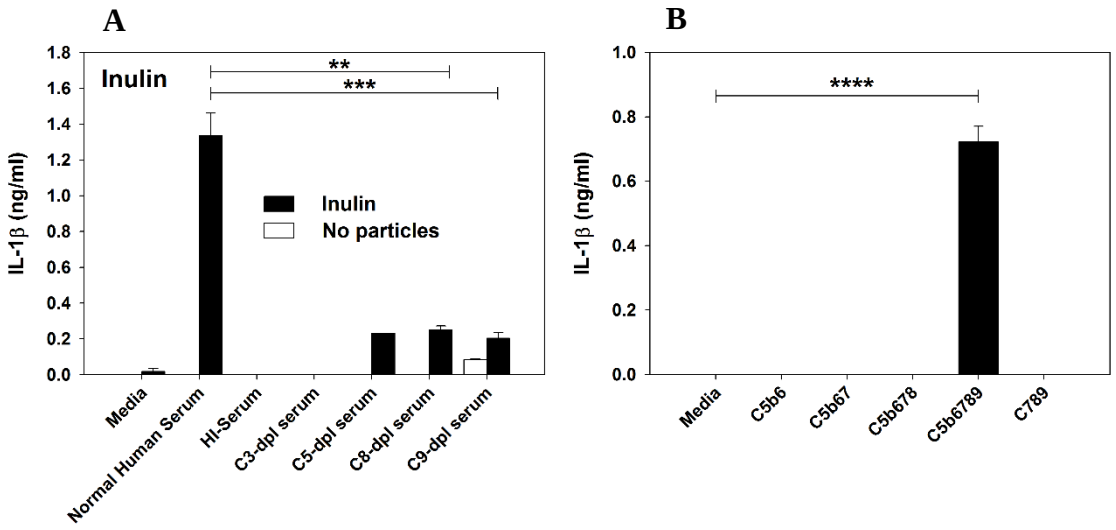

Figure 6: Macrophage activation by complement membrane attack complex:

(A) IL-1β secretion was measured in supernatants following the incubation of macrophages with inulin (100µg/ml) plus normal serum or serum deficient in individual complement components. (B) IL-1β secretion was measured in supernatants of macrophages after the addition of purified terminal complement components to macrophages (2 experiments).

To investigate the role of inflammasome activation following phagocytosis, BMMϕ from mice deficient in NLRP3, NLRC4, ASC, or caspase-1 were examined and compared to macrophages from WT mice. LPS-primed macrophages from WT or NLRC4$^{-/-}$ mice secreted IL-1β in response to complement activation by inulin (Figure 7A), zymosan (Figure 7B) or *L. major* (Figure 7C). However, macrophages from mice deficient in NLRP3, ASC, or caspase-1 failed to secrete detectable IL-1β. Caspase-1 activation was directly measured by FLICA staining of macrophages. Macrophages responding to complement activation by zymosan inulin *or L. major* exhibited caspase-1 cleavage. Incubation of these particles in serum deficient in the sixth component of complement failed to induce caspase-1 activation (Figure 8).

To visualize inflammasome formation, a macrophage-like cell line RAW264.7, which does not express endogenous ASC, was transduced with CFP-ASC and exposed to complement activators. These macrophages were primed with LPS for four hours and then exposed to fluorescent Zymosan particles in the presence of normal serum. ASC-specks were observed in macrophages that actively took up zymosan (Figure 9A, B). The ASC specks were not observed in the cells that received zymosan without normal serum (Figure 9C). The specks observed were very similar in size but not as plentiful as those in macrophages exposed to the well-known NLRP3 agonist ATP (Figure 9D).

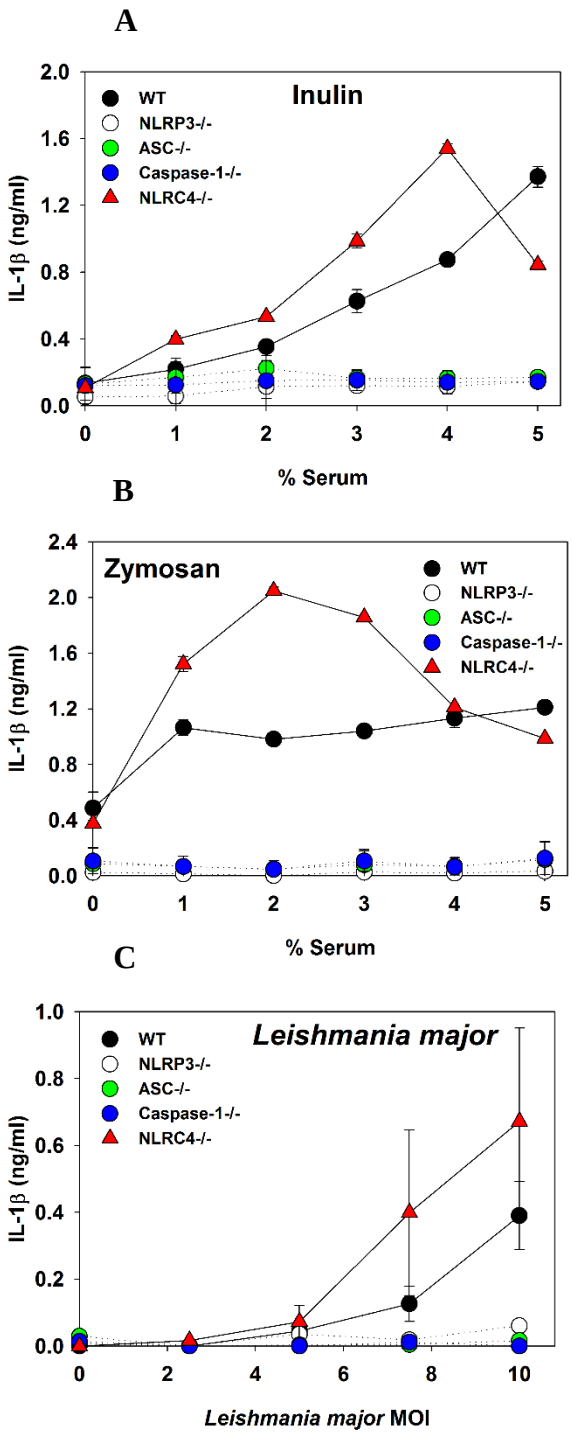

Figure 7: NLRP3 inflammasome activation during complement-mediated phagocytosis. :

IL-1β production from LPS primed bone marrow derived macrophages (BMMϕs) from wild type (WT) mice (closed circles) or mice deficient in NLRP3 (open circles), ASC (grey squares), caspase-1 (closed inverted triangle), or NLRC4 (open triangle) was measured by ELISA following their incubation with inulin (A), zymosan (B), or *L. major* (C) in the presence of 5% normal human serum.

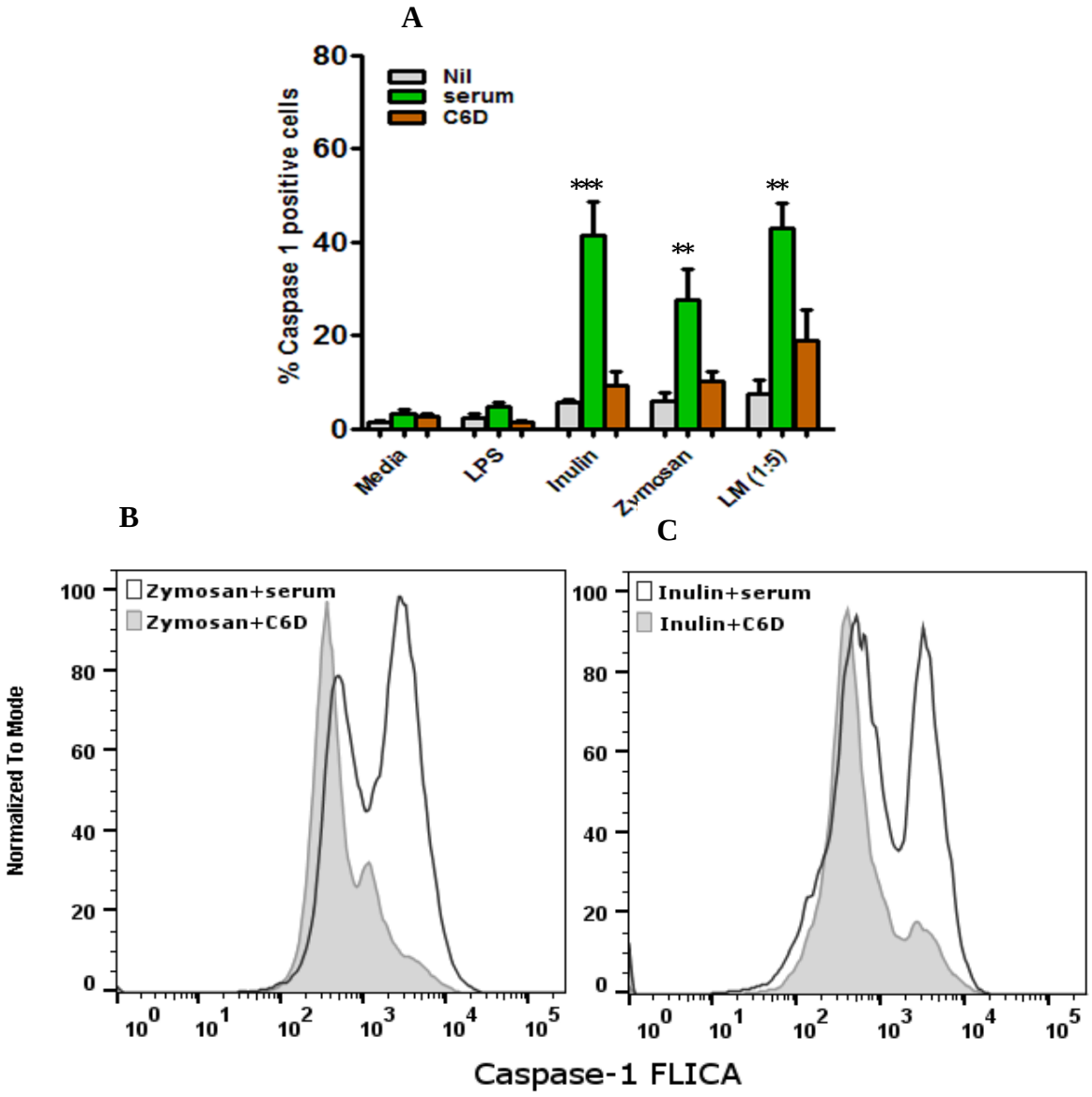

Figure 8: NLRP3 inflammasome activation during complement-mediated phagocytosis:

(A) Caspase-1 positive cells were assessed by flow cytometry using a FAM-FLICA dye based assay. Cells were primed with LPS for 6 hours followed by antigen stimulation in the presence of 5% complete or C6D human serum. Representative FACS plots of FLICA staining following activation of LPS primed macrophages by (B) zymosan or (C) inulin with normal serum (blue) or with C6 deficient serum (red).

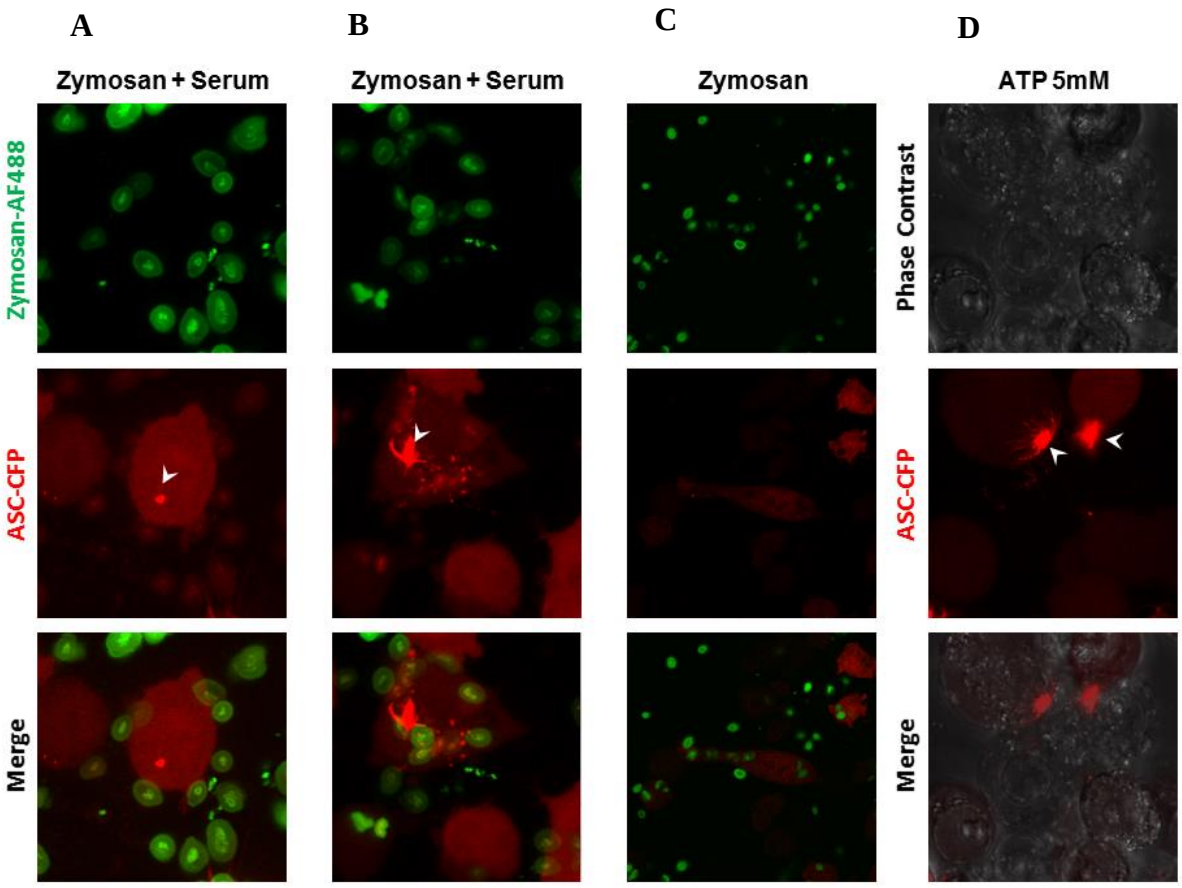

Figure 9: Visualization of Inflammasome activation following complement activators.

ASC-CFP transduced macrophages (red) were LPS primed for four hours. There were then activated by Alexa488 conjugated zymosan (green) and serum (A and B) or zymosan alone for 45mins (C) or by ATP (D) for 20mins. Cells were visualized using Leica SP5 X Confocal Microscope.

3.6 Chapter summary:

The activation of complement pathway is known to induce and acute inflammatory response. These responses not only induce recruitment of cells to the site of infection but also induces the activation of cells to produce cytokines required to promote an immune response. The induction of inflammation by complement was previously thought to be solely due to the production of anaphylatoxins C3a and C5a. In this study, we show that the MAC can induce the activation of macrophages by activating the assembly of the NLRP3 inflammasome. This activation has been shown to induce the maturation and release of IL-1β and IL-18, both of which have been implicated in the promotion of acute phase responses and inflammation[58]. The MAC proteins can be now seen as attractive targets to modulate inflammatory conditions as it has been shown to directly induce the activation of macrophages and promote cytokine secretion. The activation though MAC regardless of the type of particle being used also raises the possibility that NLRP3 inflammasome plays an important role in the acute responses against any pathogen that happens to activate complement.

Chapter 4: Activation of Membrane Attack Complex and its role in promoting inflammation through NLRP3

4.1 Membrane Attack Complex Bystander Effect

Complement activation contributes to host defense though opsonization of particles and lysis of target cells though the membrane attack complex (MAC). Complement activation also leads to inflammation, and people with genetic deficiencies in complement regulatory proteins frequently suffer from inflammatory immunopathology. Although complement–mediated inflammation has been largely attributed to the generation of the complement anaphylotoxins, C5a and C3a, it is currently unclear if the MAC also plays a role in promoting an acute inflammatory response. We showed the importance of MAC in inflammasome activation, but it became important to determine how the MAC interacted with the plasma membrane of the macrophage.

The “reactive lysis” of unsensitized erythrocytes by the complement MAC was first described by Lachmann and colleagues in 1970. These early studies demonstrated that the terminal complement components were not irreversibly bound to target particles, but rather could “jump” to neighboring un-sensitized “bystander” erythrocytes. This could result in the lysis of RBCs that were themselves not activators of complement. Although the bystander mechanism

proved to be helpful for the study of the structure and function of the MAC in later years, the physiological relevance of this phenomenon remained unclear. In light of our previous observations, this provided a mechanistic hypothesis as to how the MAC could form on the macrophages leading to NLRP3 activation and result in inflammation (Figure 10).

To demonstrate bystander activation in this work, the original description of "reactive lysis", was adapted to detect macrophage inflammasome activation. Complement pathway can be crudely divided into two phases: the activation step and the execution step. The activation step (regardless of way complement pathway is initiated through the classical, lectin or the alternative pathway), is primarily a series of proteolytic steps that requires divalent ions like Mg^{2+} and Ca^{2+} as cofactors. The products of this pathway form functional convertases that interact with the surface of the target particle by forming a covalent bond. The execution step which, involves the assembly of MAC involves protein interactions between C5b, C6, C7, C8 and C9 and inserts into the target particle through hydrophobic interactions (Figure 11). In the reactive lysis experiments, the complement C5 convertase was formed on the surface of a complement-activating particle, such as inulin, by incubating inulin with complete serum in 5mM Mg^{2+}/EGTA at 17°C. This enables effective formation of convertases on the surface of inulin by allowing the activation step to proceed. The inulin was then washed and added to unsensitized RBCs in the presence of serum and 3mM EDTA, conditions which block complement activation but allow the non-enzymatic formation of the MAC complex (execution step).

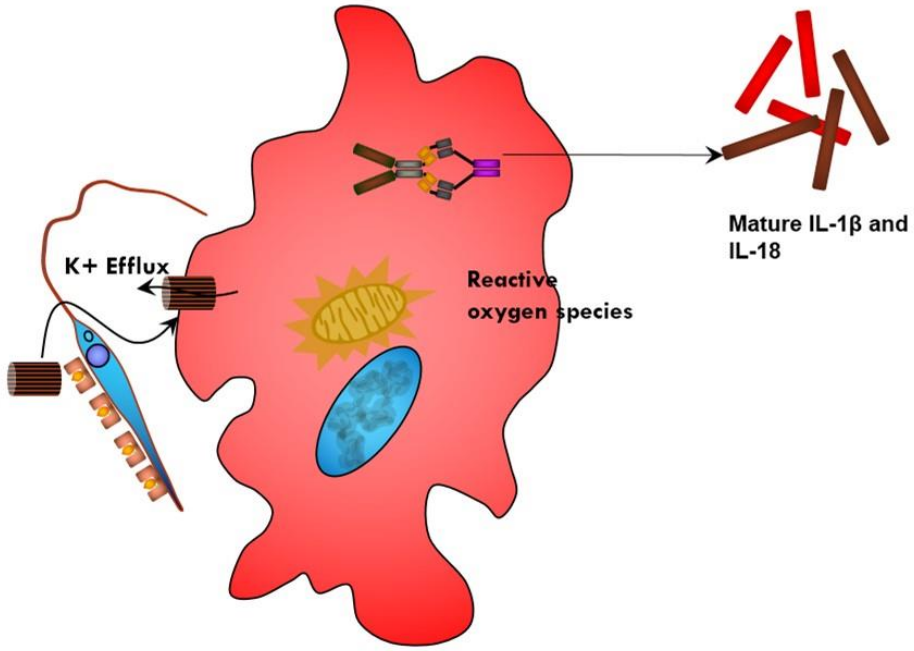

Figure 10: Bystander activation induced inflammasome activation.

The process of phagocytosis brings into close contact any complement activating pathogen and macrophages. The assembly of the membrane attack complex (MAC) takes place on the complement convertases present on pathogens. The MAC is then either formed on pathogens to effect lysis or on host macrophages to induce inflammasome activation through the K+ efflux induced cell stress leading to the secretion of mature IL-1β and IL-18.

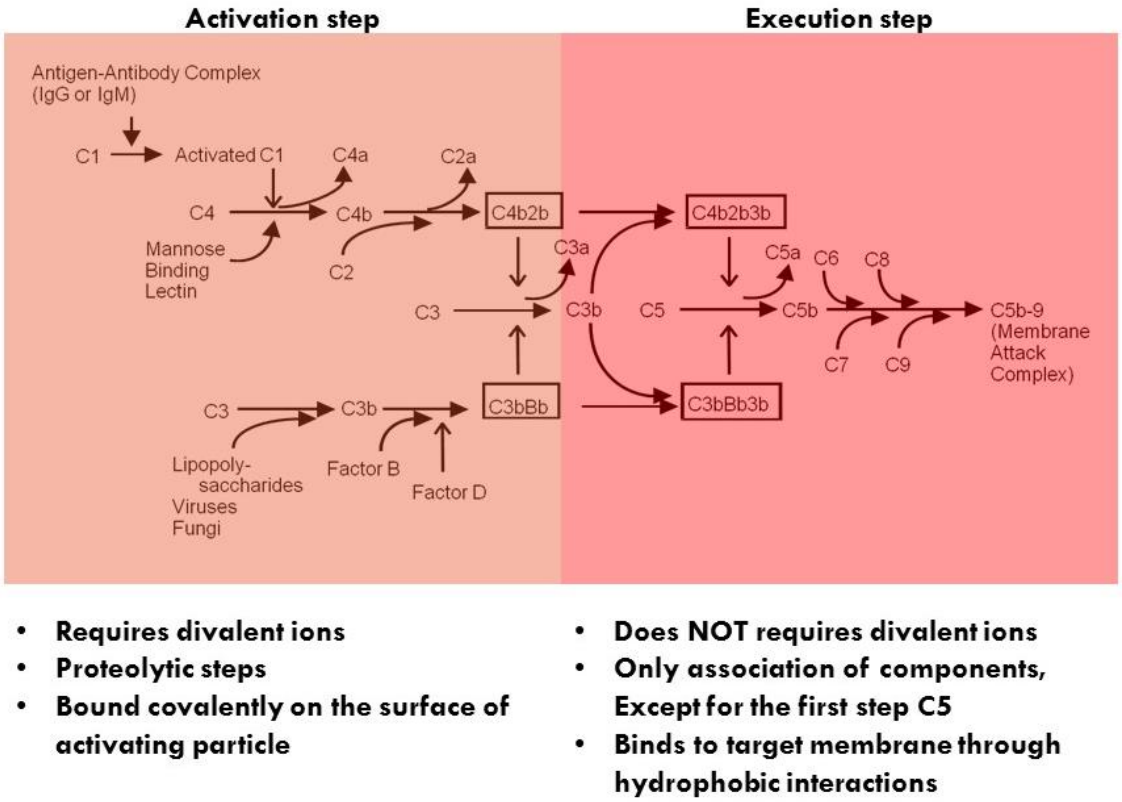

Figure 12: Salient features of complement activation.

The complement pathway can be divided into two main parts: the activation step and the execution step. Although complement can be activated through any of the three activation mechanisms, they share common features like requirement of divalent ions as cofactors, and being bound covalently to target particles. The execution step primarily involves the assembly of the membrane attack complex that involves assembly of multiple proteins (C5b, C6, C7, C8 and C9. This step does not require divalent ions and products of this step interact with the particle though hydrophobic interactions to establish a MAC pore.

The lysis of "bystander" RBCs under these conditions indicates that the MAC had been transferred onto RBCs after being activated by the C3-convertase previously formed on inulin (Figure 12A)[39].A similar approach to the reactive lysis system was taken, but adding macrophages as the bystander acceptor cell. Rather than measuring lysis, IL-1β secretion from macrophages was measured. A serum dose-dependent increase in IL-1β secretion by macrophages was observed under these conditions indicating that complement activation can induce IL-1β release from "bystander" macrophages (Figure 12B).

To demonstrate that this phenomenon occurs across mammalian species, homologous human and mouse systems were compared. Human macrophages release IL-1β in a human serum dose-dependent manner (Figure 13A), and murine macrophages release IL-1β in a murine serum dose-dependent manner in response to activation by inulin (Figure 13B). Mouse serum has previously been shown to be particularly labile, exhibiting reduced serum lytic activity in vitro, therefore it is not surprising that higher concentrations of mouse serum were required to elicit detectable inflammasome activation in this assay[59].

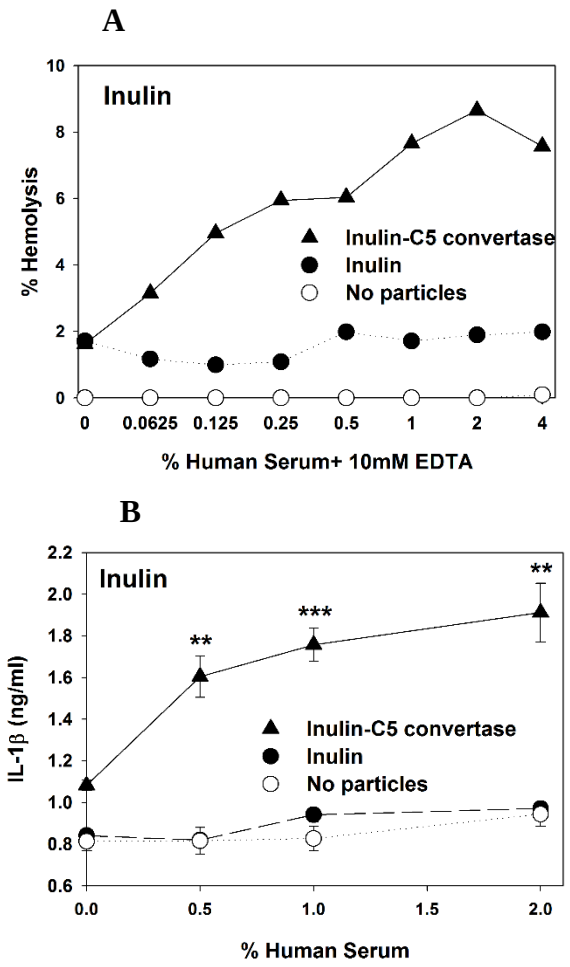

Figure 12: Bystander activation following complement mediated phagocytosis.

(A) The "reactive lysis" of bystander erythrocytes (see Methods) was measured by quantitating hemoglobin release from lysed SRBC after incubating C5b-inulin in increasing serum concentrations in 3mM EDTA. (B) Inflammasome activation by "bystander" complement activation was assessed by measuring the release of IL-1β following co-incubation of macrophages with C5b-inulin in 3mM EDTA and increasing amounts of human serum (2 experiments).

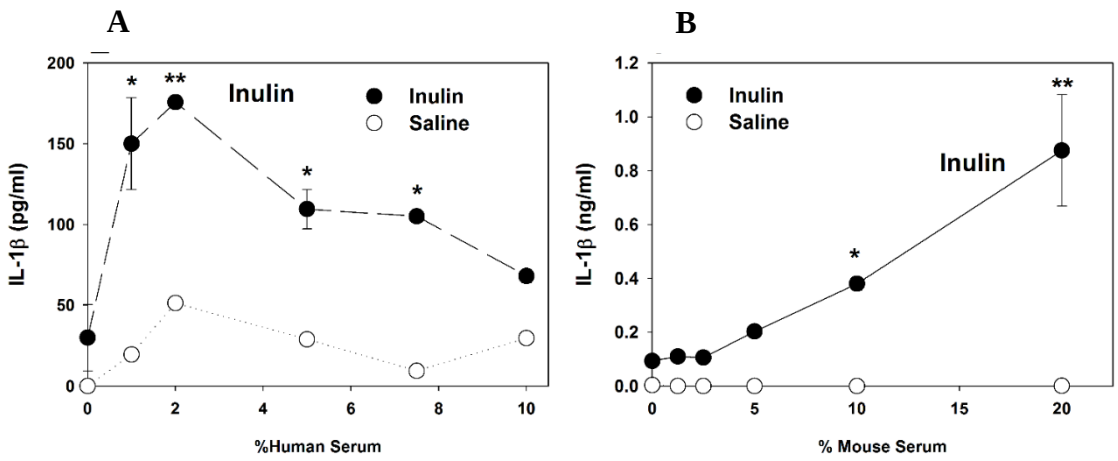

Figure 13: Complement mediated phagocytosis induced inflammasome activation across species.

IL-1β secretion from human (A) or mouse (B) macrophages after incubation of inulin plus increasing concentration of human (A) or mouse (B) serum.

4.2 Deposition of MAC during phagocytosis

To visualize MAC formation on the macrophage plasma membrane during the uptake of complement-activating particles, fluorescence microscopy was used to detect AF-649-tagged C9 deposition. As expected, C9 was easily visualized on the surface of complement-opsonized particles, but MAC formation was also detected on the surface of bystander macrophages when Ds-Red expressing *L. major* or zymosan was added to macrophages (Figure 14A). The bystander MAC deposition occurred primarily on the surfaces of macrophages actively involved in the phagocytosis of complement-opsonized particles, and it also could be visualized on the surface of cells directly adjacent to these cells, but not on cells distant from those undergoing phagocytosis (Figure 14B). Not all of the macrophages undergoing complement-mediated phagocytosis were decorated with C9 (Figure 14C). As controls, macrophages not receiving complement activators had little to no C9 deposition on their surface. C9 could also be detected inside macrophages, but we could not determine whether this was due to the endocytosis of MAC by macrophages or a bystander transfer occurring within macrophage phagolysosomes.

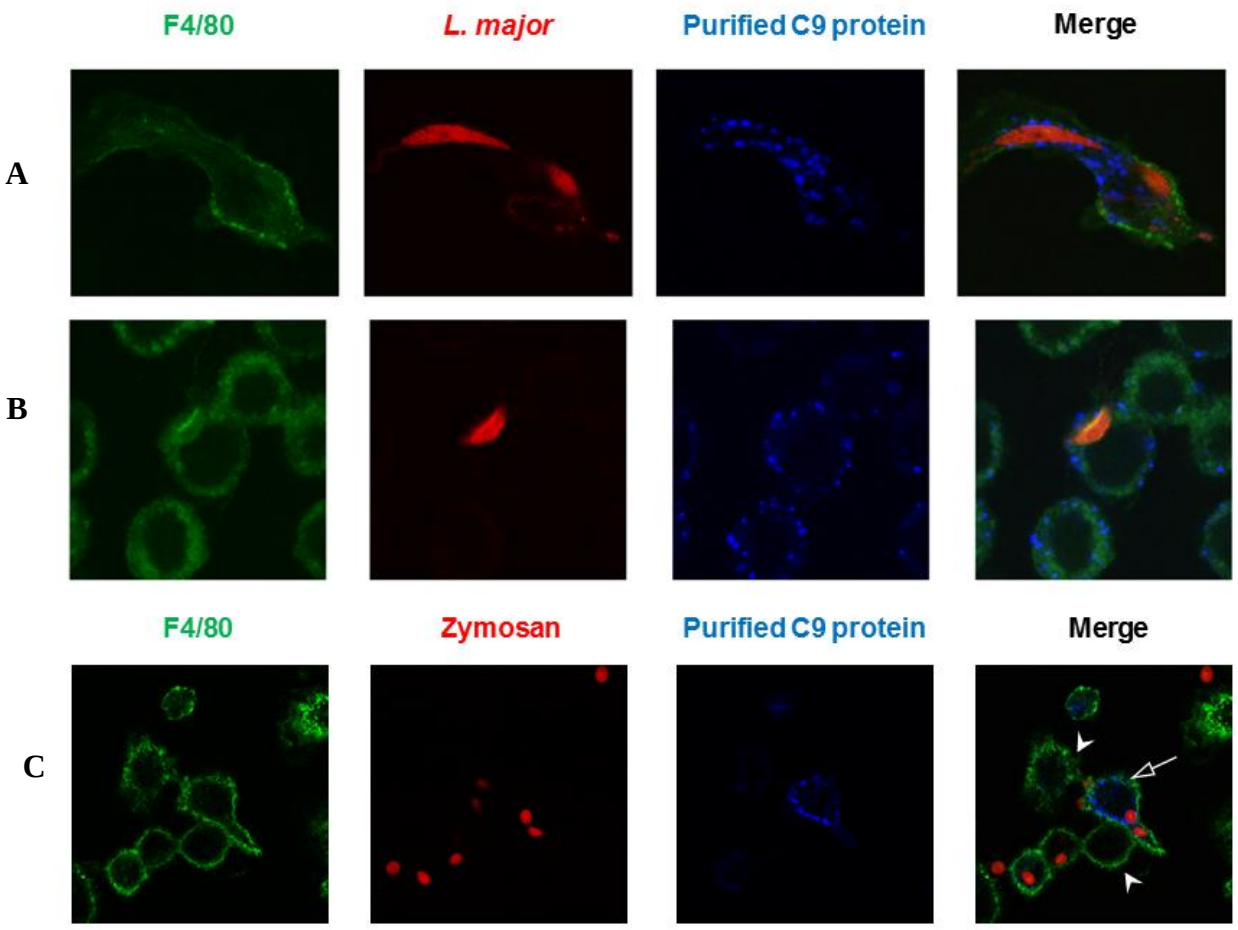

Figure 14: Bystander MAC deposition on macrophages.

LPS primed BMMϕs were incubated with Ds-Red Leishmania (shown in red, A, B) or zymosan AF488 (C) for 3mins with C9 deficient human serum supplemented with AF-647 labelled C9 (blue). Complement activation was stopped by placing the cells on ice and macrophages were stained with (A, B) FITC-F4/80 (green), or (C) PE-F4/80 (green). (A) C9 (blue) was found to be deposited on L. major (red) and on the surface of macrophages (green). (B) C9 (blue) was deposited on the surface of L. major (red) infected macrophages as well as macrophages immediately adjacent to the infected macrophage. (C) C9 (blue) was found on the surface of some zymosan (red) containing macrophages (open arrow) but not on the surface of others (arrow heads)..

4.3 Imbalance in Intracellular ions leads to the activation of Inflammasome

The MAC has been implicated in a variety non-lytic effects that differ according to the nature of the target cell. On phagocytes (neutrophils and macrophages), the MAC induces K+, ATP, and Ca2+ transport across membranes. Each of these molecules has been shown to influence the activation of the NLRP3 inflammasome. To determine the importance of secreted ATP in complement mediated secretion of IL-1β, we used macrophages from $P2X7R^{-/-}$ mice that lacked the ability to activate the inflammasome in response to high concentration of extracellular ATP. $P2X7R^{-/-}$ macrophages retained their ability to secrete IL-1β in response to complement activation by inulin, indicating that MAC-mediated pore formation does not require the P2X7R for NLRP3 inflammasome activation (Figure 15).

The presence of the MAC pore on the cell surface of host cells can also induce reactive oxygen species (ROS) due to mitochondrial stress. As ROS have been implicated in NLRP3 activation, we looked into the effect of ROS inhibition on IL-1β secretion. The ROS chelator N-acetyl cysteine (NAC) induced a dose dependent reduction in IL-1β secretion by LPS-primed macrophages in response to inulin and complement (Figure 16A). Pore formation usually induces K+ efflux from cells, and this efflux is required for the activation of the inflammasome. To examine the role of K+ efflux in MAC pore induced bystander inflammasome activation, K+ efflux was blocked by adding the drug glybenclamide, or inhibited by increasing the extracellular concentrations of K+. Both conditions resulted in decreased IL-1β secretion in response to the phagocytosis of complement

opsonized inulin, indicating that K+ efflux is required for MAC-mediated inflammasome activation (Figure 16B, C).

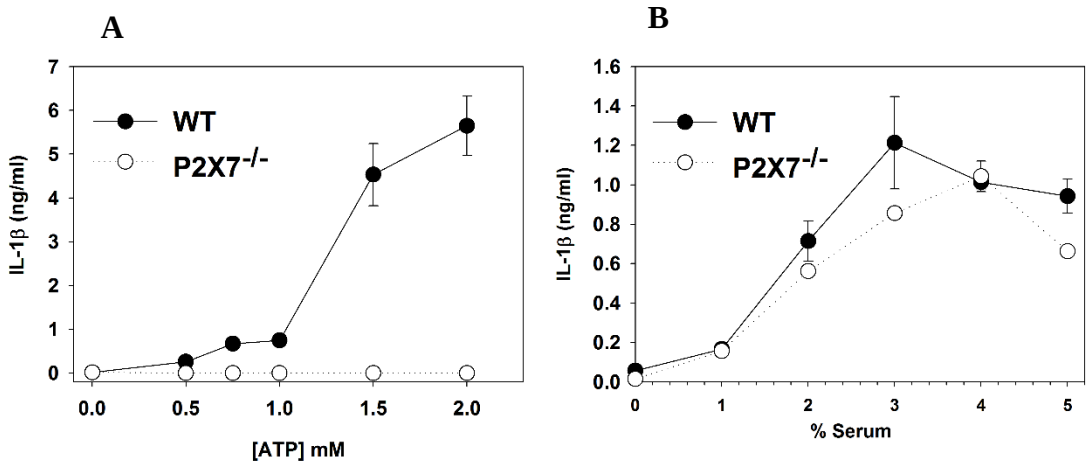

Figure 15: Complement mediated Inflammasome activation is P2X7 independent.

IL-1β production from macrophages deficient in P2X7 (open circles) was compared to WT macrophages (closed circles) after stimulation with increasing concentrations of ATP (A) or inulin plus increasing concentrations of normal human serum (B)

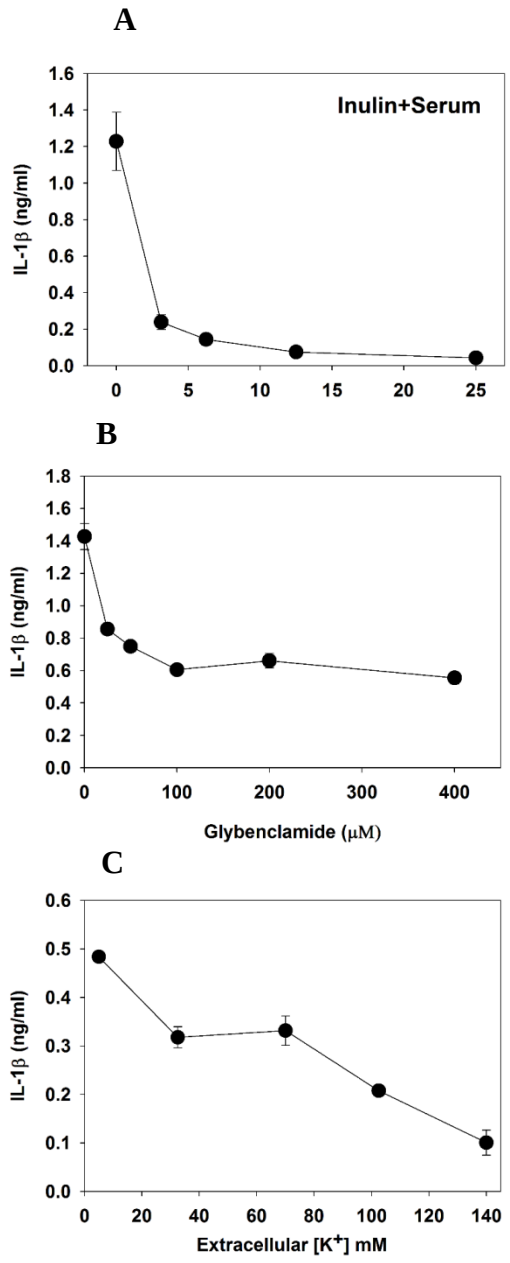

Figure 16: MAC mediated K+ efflux and ROS production drives inflammasome activation.

IL-1β production from macrophages was measured by ELISA after inhibiting reactive oxygen species by adding increasing concentrations of N-acetyl cysteine (NAC) (A) , or after inhibiting potassium efflux by incubating macrophages in increasing concentrations of Glybenclamide (B) or extracellular potassium (C) during Inflammasome activation by Inulin and 5% Serum.

4.4 MAC mediated Cell repair and Cell death

The MAC pore also allows the influx of Ca2+ and increased intracellular Ca2+ mediates many of the consequences of MAC insertion. One major consequence of Ca2+ influx is the promotion of membrane repair mechanisms in macrophages. In the absence of calcium signaling pores on host cells persist to induce greater cellular damage leading to apoptosis. Persistent pore accumulation on the macrophage plasma membrane could lead to increased inflammasome activation due to the persistence of danger signals. To analyze the importance of extracellular Ca2+ influx in inflammasome activation we incubated macrophages with MAC proteins C5b, C6, C7, C8, and C9 for 4 mins in the presence or absence of Ca2+ in the media. To check for the persistence of MAC pore on the plasma membrane, propidium iodide (PI) was added for 1 min and the cells were analyzed for PI incorporation by flow cytometry. PI is a vital dye and thus is excluded from intact viable cells, however if the membrane is compromised due to pores then PI can enter the cells and bind to nucleic acids making it fluorescent. We found that macrophages incubated with MAC in the absence of Ca2+ had persistent pores even after 4 mins, unlike macrophages incubated in normal media (Figure 17A). This persistence of MAC on the plasma membrane leads to increased IL-1β when it is deposited through bystander activation during phagocytosis of inulin (Figure 17B). Thus, membrane repair mechanisms not only prevents excessive inflammasome activation but also prevents macrophage cell death.

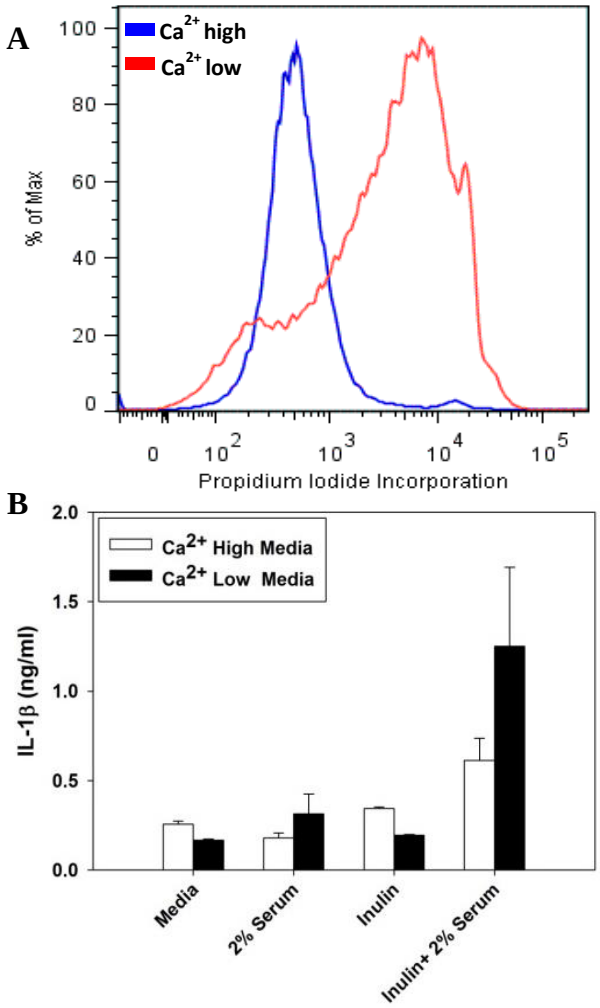

Figure 17: Ca2+ Mediated Membrane repair and modulation of Inflammasome activation.

(A) BMMϕ was incubated with Mac proteins in the Ca2+ low or Ca2+ high media for 4 mins. The integrity of the membrane after MAC deposition was analyzed by looking into PI incorporation through flow cytometry. (B) IL-1β was measured in supernatants of macrophages after activation with inulin and 2% serum for 4 hours in Ca2+ low or Ca2+ high media.

Complement was first recognized because of its capacity, in conjunction with antibody to lyse cells. Erythrocytes were among the first cells to be investigated with regard to the lytic mechanism of complement action. It has been shown that a single functional MAC in the membrane of a metabolically inert, aged erythrocyte is sufficient to lyse the cell by colloid osmosis. Unlike erythrocytes that can be lysed by a single C5b-9 channel, several channels are required to lyse a metabolically active nucleated cell, which appear to require C5b-9 containing multimeric C9. The multichannel requirement for nucleated cell lysis has been attributed to a defense response of the cell though ion pumps to stabilize membrane polarization and to promote elimination of TCC from the PM by endocytosis or by endocytosis and vesiculation. Nucleated cells are thus more difficult to kill with antibody and complement than are aged erythrocytes or liposomes. To directly monitor macrophage viability following bystander complement activation, macrophages were stained with 7AAD and Annexin V to measure cell viability. As shown in Figure 18, there was little evidence for cell death via apoptosis in response to complement activation by inulin or zymosan. Thus, macrophages undergoing complement-mediated phagocytosis remain viable while they cleave caspase-1 to release biologically active IL-1β and IL-18.

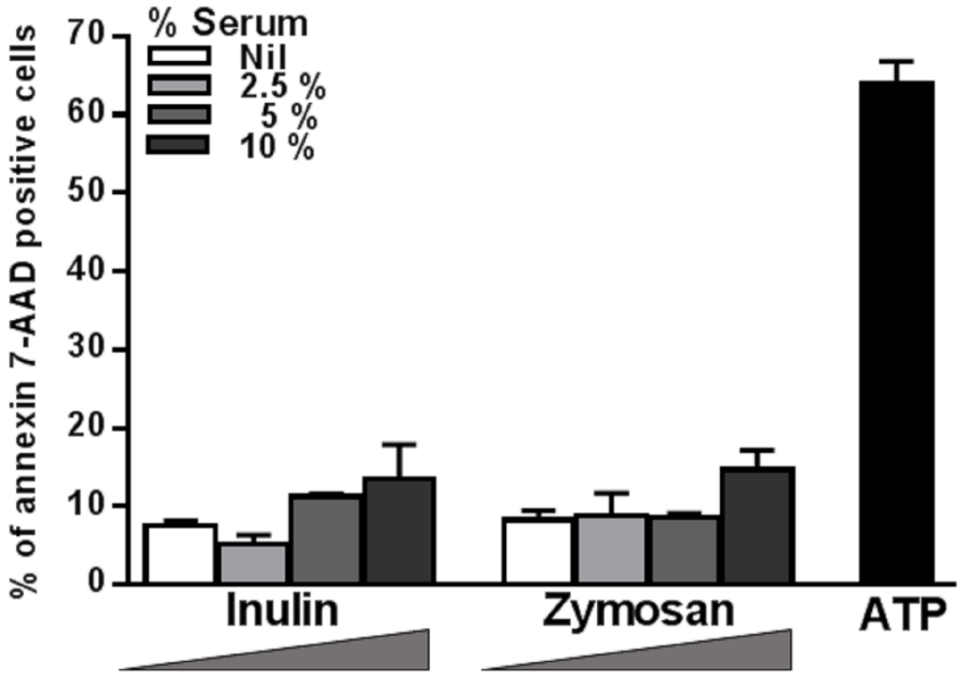

Figure 18: Macrophage cell death following inflammasome activation with MAC.

Macrophage viability was measured by the uptake of 7AAD and Annexin V staining after incubating LPS-primed macrophages with various complement activators.

4.5 Role of MAC bystander activation in modulating adaptive immunity

Inflammasome activation in many studies previously has been linked to a skewed T_h17 adaptive immune response. Macrophages can act as antigen presenting cells to promote T cell activation and adaptive immunity. In addition to its role in promoting inflammation in patients with autoimmune diseases, T_h17 and IL-17 play important roles in the clearance of extracellular bacterial and fungal infections. This enables the transition from inflammatory response to an immune response against a particular pathogen. To investigate a role for complement activation in the modulation of T cell responses, we examined the ability of bystander-activated macrophages to serve as antigen presenting cells. LPS primed macrophages were pulsed with Ova under conditions where the inflammasome was activated, either by the addition of purified MAC components (Figure 19A) or by bystander inulin activation (Figure 19B). Ova-specific CD4+ DO.11.10 T cells were added to macrophages and incubated for 7 days. T cells were re-stimulated at day 7 using immobilized antibody to CD3. Cytokine production from T cells was measured three days later. Bystander complement activation on macrophages induced T cells to make significantly higher levels of IL-17A. To verify the importance of inflammasome activation in T cell polarization, LPS-primed WT or NLRP3-/- macrophages on a BALB/c background were cultured with inulin and human serum along with 150µg/ml OVA and added to CD4+OT-II T cells. In primary stimulations, CD4+OT-II T cells co-cultured with WT macrophages showed an increase in IL-17A production that was not observed when NLRP3-/- BMMϕ, were added (Figure 20A). The re-stimulation of T cells showed a similar

NLRP3-dependent increase in IL-17A (Figure 20B) production. These data indicate that IL-1β, produced as a result of bystander complement activation on macrophages can influence T cell polarization.

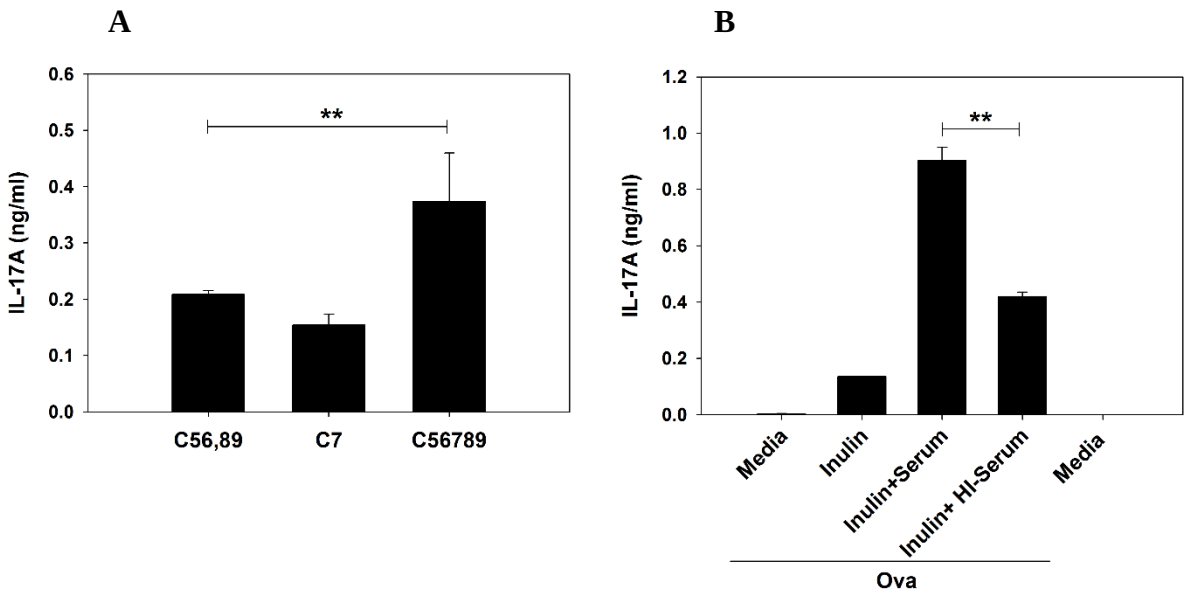

Figure 19: Bystander complement activation induced modulation of Th cell activation.

IL-17A production from OVA-specific D011.10 T cells was measured in vitro after stimulation with LPS primed Ova-pulsed macrophages in which the inflammasome was activated by purified terminal complement components (A) or by inulin and serum (B)

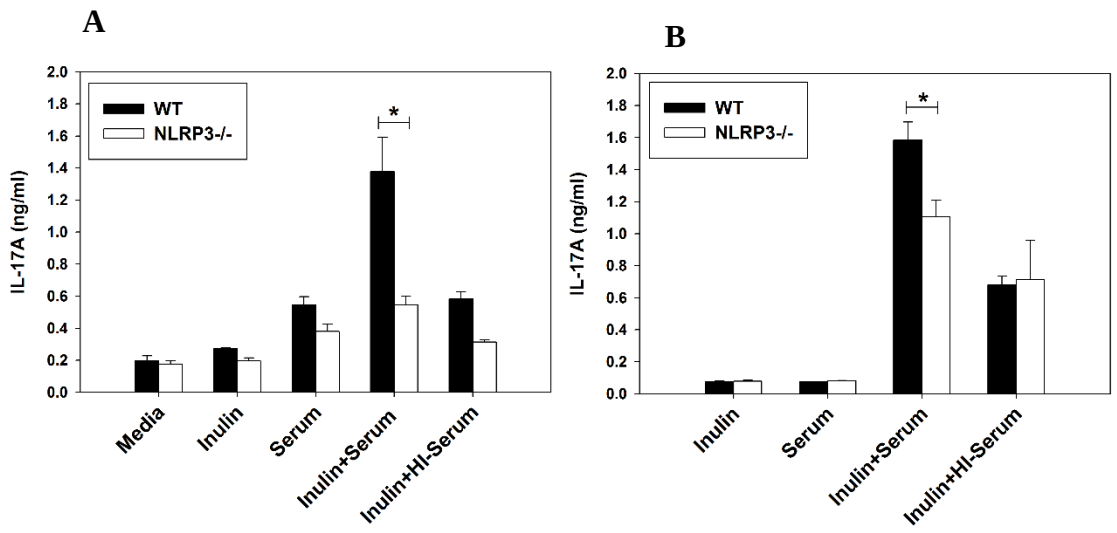

Figure 20: Role of NLRP3 in complement induced modulation of adaptive immunity.

IL-17A production by OT-II T cells was measured following co-culture with WT (black bars) or NLRP3-/- macrophages that were pulsed with OVA under conditions that induced inflammasome activation (inulin + serum) or in which the inflammasome was not activated (inulin + HI-serum). (A) Supernatants of primary co cultures of CD4T cell and macrophages (B) Supernatant of T cells restimulated with immobilized CD3 antibody.

4.6 IL-1β induced activation of macrophages and the killing of Leishmania

Leishmania have developed intricate pathways to disrupt host signaling pathways that enable them to survive in macrophages. Thus it has been proposed that *Leishmania* is silent in its entry into macrophages. Leishmania do not induce the production of IL-12 from infected macrophages, nor do they induce TNF or NO production (data not shown). This is consistent with a quiescent mechanism of entry. To ascertain the importance of inflammasome activation in limiting *Leishmania major* replication in macrophages, we activated macrophages using culture supernatants of inflammasome activated macrophages. In line with our previous observations, *Leishmania* was not able to provide signals that synergized with IFN-γ to enable macrophage activation as measured by nitric oxide production. However, inflammasome activated supernatants together with IFN-γ activated macrophages to produce significantly higher amount of reactive nitrogen species that decreased parasite replication in macrophages *in vitro* (Figure 21A). The results were strikingly similar to TLR agonist and another endogenous danger signal, LMW- hyaluronic acid. There also was evidence of classical activation of the macrophage as both inflammasome activated supernatants and LMW-HA were able to synergize with IFNγ and induce the production of reactive nitrogen species NO as shown in Figure 21B. This indicates that products of inflammasome activation are capable of contributing to the killing of the parasites, like TLR agonists. Leishmania themselves do not induce any effector molecules as can be seen by the lack of killing of these

parasites in the presence of IFN-γ alone. Thus, inflammasome activation produces cytikines that can participate in the killing of *Leishmania* spp.

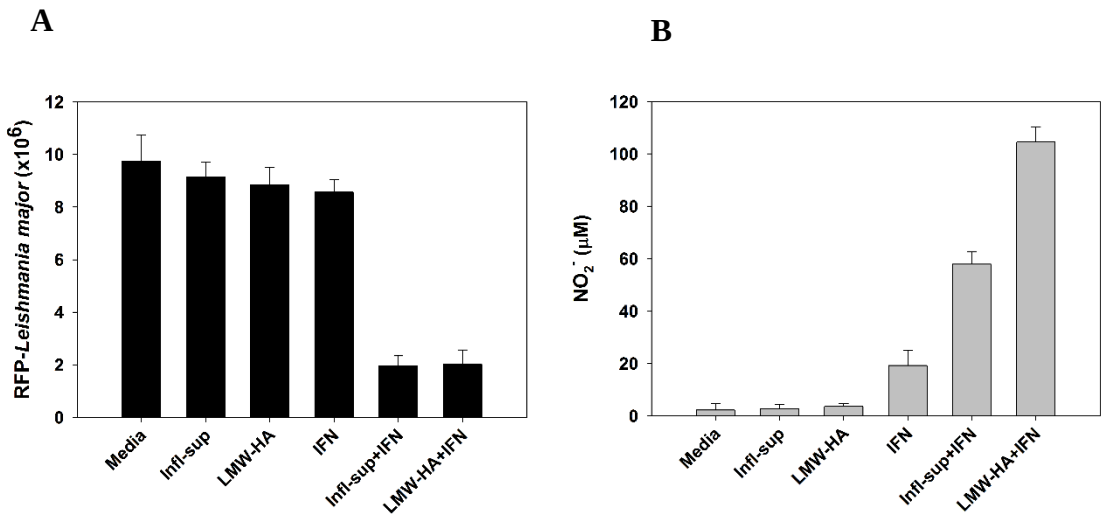

Figure 21: Products of Inflammasome activation synergizes with IFNγ to effect the killing of *Leishmania major*.

LPS-primed macrophages from WT were pulsed with ATP for 10 mins, and then washed and replaced with fresh medium for further incubation. Medium was collected after 1 hrs later. Secondary cultures were treated with collected medium (labeled IL-1β), and then infected with *L. major-RFP* for 48 hrs. Cells were lysed using TritonX-100 and lysates were quantified for RFP using a fluorimeter (A). Supernatants were analyzed for NO by Griess assay (B).

Chapter 5: Conclusions

Complement has long been recognized to be an integral component of host defense. The multiple complement activation pathways share the common terminal complement components C5b-C9n, which form membrane attack complexes (MAC) that create pores on membranes to cause the osmotic lysis of target particles. In this study, we demonstrate that the deposition of sublethal membrane attack complexes on macrophages during the process of complement-mediated phagocytosis can activate host inflammasomes, and thereby affect innate and adaptive immunity. It is shown here that IL-1β and IL-18 release from macrophages depends on a previously described[60] but poorly understood "bystander" deposition of MAC on the plasma membrane of phagocytic macrophages. The secretion of IL-1β and IL-18 did not occur with macrophages deficient in NLRP3, ASC or Caspase-1, indicating that the NLRP3 inflammasome is activated during complement-mediated phagocytosis. In this way, complement activation during phagocytosis of particles, generates key inflammatory signals through inflammasome activation and subsequent IL-1β and IL-18 cytokine release.

The so-called "reactive lysis" of unsensitized erythrocytes by the complement MAC was first described by Lachmann and colleagues in 1970[39]. These early studies demonstrated that the terminal complement components were not irreversibly bound to target particles, but rather could "jump" to neighboring un-sensitized "bystander" erythrocytes. This could result in the lysis of RBCs that were themselves not activators of complement. Although this bystander

mechanism proved to be helpful for the study of the structure and function of the MAC, the physiological relevance of this phenomenon remained unclear. The present study suggests that this bystander deposition of MAC can occur on macrophages during the process of complement-mediated phagocytosis. Instead of resulting in reactive lysis it resulted in inflammasome activation and the secretion of IL-1β and IL-18 by phagocytic macrophages. Thus, this work establishes a new physiological relevance for bystander MAC deposition.

Bacterial pore forming toxins have been previously associated with the activation of host cell inflammasomes[61]. The poly-C9 MAC also forms pores on membranes to cause host cell lysis. However, most eukaryotic cells have efficient membrane repair mechanisms to prevent host cell lysis. In particular, the expression of CD59 on mammalian cells can inhibit increased C9 deposition to prevent cell lysis. Despite these regulatory mechanisms, recent studies have shown that directing complement deposition on to host cells , with antibodies to cell surface proteins, can result in the activation of host inflammasomes[45]. This suggests that in autoimmune diseases, self-reactive antibodies could contribute to inflammation by activating host inflammasomes. In the present work, we demonstrate that auto-antibodies are not an absolute requirement to induce inflammasome activation in host cells. Rather, the phagocytosis of complement-opsonized particles alone is sufficient to activate inflammasomes and initiate similar inflammatory cascades. These observations may help explain why complement-mediated phagocytosis is associated with more inflammatory responses than non-opsonic phagocytosis.

The complement MAC has been shown to be involved in exacerbating the clinical pathology in a variety of autoimmune and autoinflammatory diseases ranging from gout[62], to rheumatoid arthritis[63] and coronary artery disease[64]. In many cases, antibodies to terminal complement components have been used to mitigate inflammation. Eculizimab, a humanized antibody targeting complement component C5, is being prescribed for patients with Paroxysmal Nocturnal Hemoglobinuria (PNH)[65], atypical hemolytic uremic syndrome (aHUS)[66], glomerulonephritis[67], SLE[68], and even asthma[69]. Interestingly, the administration of anti-complement antibodies not only prevents host cell lysis, but also reduces inflammatory markers in these patients[70]. It is tempting to speculate that part of this may be due to the inhibition of inflammasome induction by bystander deposition of MAC on host cells.

Several groups have proposed that complement can be a natural adjuvant to promote immune responses, and that complement activation by adjuvants[71] can enhance vaccine efficacy[72–74]. Complement activation is thought to play an important role in the adjuvant effects of alum[75], and novel adjuvants based on inulin have been shown to be potent inducers of T_h1 and T_h2 immune response[76]. Complement-opsonized antigens, like HIV[72,74] and anthrax[77], are better at activating T cells than their unopsonized counterparts. In the present studies we demonstrate that complement activation can be causally linked to IL-1β and IL-18 secretion by macrophages. This can have myriad effects on innate and adaptive immune responses. IL-1β is a potent proinflammatory cytokine that is known to induce fever, upregulate acute phase proteins, and induce adhesion molecule

expression on endothelial cells[27]. IL-1β is important for the development of T_h17 responses which can confer immunity to fungi, such as C. albicans[78]. IL-1β production has been associated with increased resistance to M. tuberculosis[79], and in some scenarios IL-1R1 signaling can also promote T_h2 responses in mouse models of asthma[80]. IL-18 is a potent inducer of IFNγ from NK cells and T cells, and in the presence of IL-12 or IL-15 can potentiate cell mediated immunity. IL-18 can also combine with IL-23 to promote T_h17 responses[81], and with IL-4 to promote T_h2 responses[82]. Therefore the mechanism of complement-mediated inflammasome activation provides a novel therapeutic target pathway that is relevant in multiple contexts from enhancement of vaccine responses to suppression of undesirable immunogenicity and inflammation. Our study also suggests that complement induced activation of the inflammasome in macrophages may lead to protective immune response against a variety of pathogens not only due to the direct lytic or opsonic effects of complement, but also by fundamentally changing the immune response to that organism.

Leishmania spp. is efficient at evading the immune response. In fact, it may actively inhibit macrophage responses to IFNγ [83]. Previous studies report that MyD88 is absolutely essential in promoting protective immunity to *Leishmania*. Since *Leishmania* does not elicit a proinflammatory response from macrophages, it becomes important to study IL-1β and IL-18 signaling because both of these cytokines signal through MyD88[58]. The study of the role of endogenous danger signals in regulating immune response during *Leishmania* spp. infections therefore becomes important. The NLRP3 inflammasome complex has been

shown to be activated by a number of different danger signals. One of the most important substrate for inflammasomes is IL-1β. IL-1β is released early during an infection and is capable of modulating many different aspects of innate immune response to an infection. Like TLRs, it also signals through a receptor that utilizes the adaptor MyD88[84]. These studies may uncover an unexpected way in which complement activation may skew adaptive immune responses. Inflammasomes regulate both the maturation of cytokines and also various other cellular activities like regulation of apoptosis of an infected cell by sensing danger signals like ATP and pore forming proteins. The ability of Inflammasome derived IL-1β to synergize with macrophages to initiate killing of parasites, can help us understand the mechanism of early control by innate immune system during *Leishmania* infection. It remains to be determined if the modulation of the adaptive immune response by inflammasome activation has any effect of *Leishmania* spp. pathogenesis.

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