Oxidized Low Density Lipoprotein Immune Complexes Prime the NLRP3 Inflammasome and Modulate T cell Responses in Atherosclerosis

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

August 11, 2017

Nashville, Tennessee

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To my parents, Greg and Teri,

who embody everything that I hope to be when I grow up,

and

to my fiancé, Brian,

who I want along with me every step of the way.

Acknowledgements

Most importantly, I must begin by thanking my thesis advisor, Dr. Amy S. Major. Under her guidance, I have grown from a hesitant, naive student to a confident, independent researcher. I am sure that there are many days that I made Amy regret her “door is always open” policy, but I am eternally grateful for her patience and willingness to teach. Amy is one of the only Principal Investigators I know that still works at the bench regularly. This is a quality that I not only respect and admire, but also hope to embody moving forward on my own career path. As a student, it is immensely encouraging to see that your mentor still loves science at its purest form. My thesis work has been completed during a particularly dark period for scientific research, and Amy has been a role model for resilience and perseverance. I truly cannot thank her enough for everything that she has taught me.

The next group of people that I would like to acknowledge are the members of my thesis committee: Dr. Daniel J. Moore (chair), Dr. Mark R. Boothby, Dr. David G. Harrison, and Dr. Holly M. S. Algood. Each member of my committee brought a unique perspective and expertise to the table. They provided a great deal of encouragement and feedback. My work has benefitted greatly from the input of these incredible scientists. Dan has always kept me on my toes, asked the hard questions, and pushed me to my extreme limits. I am very grateful for his mentorship, as well as his organization and timeliness in running my committee meetings and completing other “chairly” duties.

I owe a huge thank you to Dr. Ashley J. Wilhelm, a former post-doctoral researcher in the Major Lab. Ashley taught me every technique that I know and dedicated countless

hours helping me to plan and troubleshoot. We spent many long hours in the lab together carrying out experiments while scheming ways to be more efficient the next time. As a scientist and teacher, Ashley is thoughtful, meticulous, and patient. But more importantly, as a friend, she is irreplaceable. I cannot say thank you enough for her guidance and support both in and out of the lab.

It takes many people behind the scenes to keep everything running smoothly. I would like to thank Dr. Christopher R. Aiken, the Director of Graduate Studies for the Microbiology and Immunology Department, and Lorie Franklin of Microbiology and Immunology administration for everything that they have done to keep me moving forward in my studies. They go above and beyond the call of duty to make everything as painless as possible. I am also very grateful for Stacy Johnson and Amanda Renick-Beech in the Biomedical Research and Training (BRET) office who have been extremely helpful in helping me to manage my funding.

My life would not have been as scientifically enriched or as fun without the members of the aforementioned Moore Lab: Chris Wilson, Blair Stocks, and Andrew Marshall. Each of them provided invaluable advice on protocol optimization and experimental set-ups. It was also extremely helpful that they always seemed to have the reagents that I was missing and a variety of snacks to choose from when I came to borrow them! Chris and I have shared many afternoon coffee pep-talks (with him doing most of the pepping) that have encouraged me to keep pushing when it doesn’t seem like there is a light at the end of the tunnel. I’m grateful for all the support and laughs that these guys have given me.

I would not be here today without the support of my family. My mom, Teri and my dad, Greg are the most selfless people that I have or will ever meet. For as long as I can remember they have taught me that anything is achievable with kindness and hard work. These were not just words- I have watched them employ these tenets in their own life day in and day out. They treat everyone that they meet with compassion and work tirelessly to reach their goals. My mom is my best friend and role model. I am so grateful for her constant love and encouragement. My dad is by far the most intelligent person and best problem solver that I know. He is always interested in hearing about my scientific endeavors and is a great resource for advice and support. I am so fortunate to have had such a solid foundation on which to grown and learn.

Last but not least, I need to thank my fiancé, Brian. Throughout this process he has maintained an incredible balance of understanding and encouragement. Brian has spent countless weekends in the lab and listened to more than his fair share of scientific babble. I can never express enough gratitude for his support (and wine pouring skills). Many people become complacent in relationships, but Brian makes me want to wake up every morning and work harder and be better. I could not be more excited for our life together.

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List of Abbreviations

ADCC: Antibody dependent cellular cytotoxicity

ADCP: Antibody dependent cellular phagocytosis

AHA: American Heart Association

Aim2: Absent in melanoma 2

APC: Antigen presenting cell

ApoE: Apolipoprotein E

Bcl10: B cell lymphoma/leukemia 10

BMDC: Bone marrow-derived dendritic cell

BMDM: Bone marrow-derived macrophage

CARD9: Caspase recruitment domain family member 9

CBM: CARD9/Bcl10/MALT1 complex

CD: Cluster of differentiation

CHD: Coronary heart disease

CVD: Cardiovascular disease

CXCL: Chemokine

DC: Dendritic cell

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked Immunosorbent Assay

ERK: Extracellular signal-related kinase

Fab: Antigen binding region of antibody

Fc: Constant region of antibody

FcγR: Fc gamma receptor

IC: Immune complex

Ig: Immunoglobulin

IL: Interleukin

ITAM: Immunoreceptor tyrosine based activation motif

ITIM: Immunoreceptor tyrosine based inhibitory motif

LDLr: Low density lipoprotein receptor

LPS: Lipopolysaccharide

MALT1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1

MFI: Mean fluorescence intensity

MHC: Major histocompatibility complex

Nlrp3: NACHT, LRR and PYD domains-containing protein 3

Nlrc4: NLR family CARD domain-containing protein 4

NF-κB: Nuclear Factor kappa B

OxLDL: Oxidized low density lipoprotein

OxLDL-IC: OxLDL containing immune complex

OVA: Ovalbumin

OVA-IC: OVA containing immune complex

PAMP: Pathogen Associated Molecular Pattern

PBMC: Peripheral blood mononuclear cell

RA: Rheumatoid arthritis

RNA: Ribonucleic acid

SHIP: SH2-containing inositol 5′-phosphatase

SLE: Systemic Lupus Erythematosus

Syk: Spleen tyrosine kinase

TCR: T cell receptor

TGFβ: Transforming Growth Factor beta

TLR: Toll Like Receptor

TNFα: Tumor necrosis factor α

T_{reg}: Regulatory T cell

TREM2: Triggering Receptor Expressed on Myeloid Cells

WHO: World Health Organization

CHAPTER 1

Background and Research Goals

Introduction

Cardiovascular disease (CVD) represents a major public health burden both in the United States and across the globe. According to the American Heart Association (AHA) and the World Health Organization (WHO), approximately 801,000 Americans and 17.5 million people worldwide succumbed to CVD complications in 2013 (1, 2). These staggering statistics indicate that CVD is responsible for 31% of deaths both nationally and abroad. Of these CVD related mortalities, 1 in 7 American deaths and 2 out of 5 global deaths are a direct result of Coronary Heart Disease (CHD)(1, 2). CHD occurs when plaque begins to form in the arteries, narrowing the lumen and restricting the flow of oxygen rich blood to the heart (3). This formation of plaque in the arteries is also referred to as atherosclerosis. Complete occlusion of the vessel either by plaque build-up or rupture often results in a myocardial infarction or stroke (4).

Atherosclerosis has been plaguing humans for thousands of years. Detrimental changes have been observed in the arteries of ancient Egyptian mummies, and these observations are consistent with the pathology currently seen in both vascular surgery and post-mortem histology (5, 6). Pathologic changes in the vasculature garnered scientific interest as early as the 19th century. At this time the pathologists Carl von Rokitansky of Vienna, Austria and Rudolf Virchow of Berlin, Germany both made the observation that there were changes in cellular composition within the vessel walls of atherosclerotic plaques (7, 8). As a humoral pathologist, Rokitansky believed that the

cellular changes were secondary to the changes to the vessel; however, Virchow believed that these cells played a causal role (9).

At the turn of the 20th century, Adolf Windaus observed that atherosclerotic plaques were comprised of cholesterol and calcified connective tissue (10). Just three years later, Nikolai Anitschkow and Semen Chaltow showed that atherosclerosis could be induced by feeding rabbits a diet high in cholesterol (11). These studies not only identified cholesterol as an important risk factor for the development of atherosclerotic lesions, but also gave way to the field of thinking that lipids were key players in atherosclerotic plaque formation and cellular changes were secondary.

Despite the observations of Rokitansky and Virchow in the mid 1800's that atherosclerotic plaques were accompanied by cellular changes in the vessel, it was not until approximately 30 years ago that the cell biology of atherosclerosis became a topic of interest again. While lipid-engorged foam cells were seen in the plaques of both humans and experimental animals, the development of monoclonal antibody technology finally allowed investigators to determination that the majority of these cells were macrophages (12). This important finding resurrected the early work of Virchow, lending support to his theory that cellular inflammation may drive the progression of atherosclerosis. Further analysis using immunohistochemical staining in human atherosclerotic plaques showed the presence of many different immune cell subsets including monocytes, macrophages, dendritic cells, neutrophils, and $CD4^+$ and $CD8^+$ T cells (13, 14). Furthermore, high levels of MHC-II staining on antigen presenting cells in the lesions suggested that an active immune response was occurring (14). Interestingly, these subsets of immune cells were even found in the arteries of children and young

adults at predicted sites of later plaque formation such as the aortic intima and in fatty streaks, further implicating immune inflammation in atherosclerosis development (15). While these descriptive studies suggested that an immune reaction was occurring in atherogenesis, the antigen(s) driving these interactions was unknown. In 1989, Palinski *et al*. showed that oxidized low density lipoprotein (oxLDL), a modified cholesterol found in the atherosclerotic plaque, could induce systemic antibody formation (16). Later, Goran Hansson's group demonstrated that $CD4^+$ T cells, specifically recognizing oxLDL associated apoB-100 are found in humans and in animal models of atherosclerosis. Follow-up studies further suggested a role for oxLDL as an immunologic antigen by showing that T cell clones isolated from atherosclerotic plaques became activated by oxLDL (17). These studies were important not only for identifying an atherosclerosis-specific antigen but also for defining atherosclerosis as a systemic disease and not just localized inflammation. A model for the role of the immune response to oxLDL can be found in Figure 1.1.

These pioneering studies offer compelling evidence that atherosclerosis is at least in part mediated by cellular inflammation. However, despite the many advances and discoveries made since this initial work, many questions remain surrounding the immune response in atherosclerosis, as well as other diseases of sterile inflammation. Additionally, although there are treatment options available for atherosclerosis, it remains the number one cause of death both nationally and internationally, highlighting the importance for further understanding of this disease and identification of new and novel therapeutic targets.

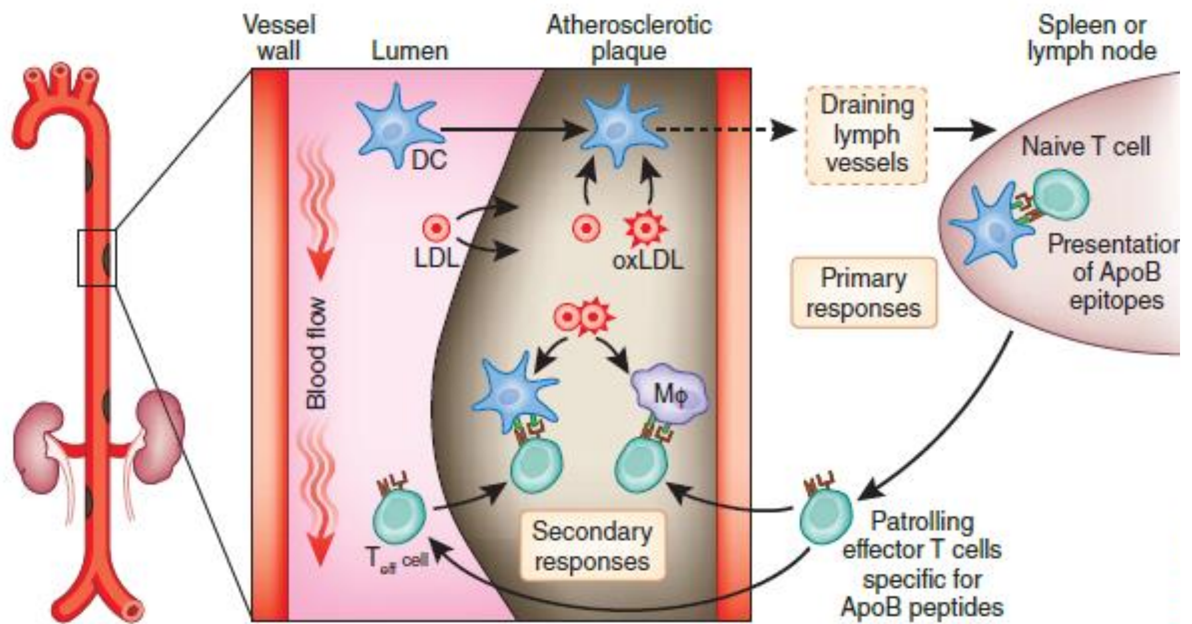

Figure 1.1. Immune Responses to oxLDL in the Atherosclerotic Plaque. Macrophages and DCs within the plaque take up oxLDL and prime specific effector T cell responses. From (186).
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B cell studies suggest systemic inflammation in atherosclerosis

B cells are an important component of the adaptive immune response given their unique ability to produce antibody; however, very few can be detected in atherosclerotic lesions or the surrounding adventitia(18, 19). In 2002, Major *et al*. showed that atherosclerosis-susceptible LDL receptor (LDLr) knock-out mice that received a bone marrow transplant from μMT (B cell deficient) mice had increased atherosclerotic lesion size in the proximal aorta compared to controls when placed on a high fat diet (20).This increase in atherosclerosis was accompanied by diminished titers of oxLDL antibodies and decreased production of pro-inflammatory cytokines, suggesting a pathogenic role for B cells (20). A follow-up study by Ait-Oufell *et al*. investigated the implications of removing mature B cells from circulation using anti-CD20 treatment in both $LDLr^{-/-}$ and $ApoE^{-/-}$ mice. In contrast to Major *et al*., treatment with anti-CD20 provided atheroprotective effects. The authors observed that depleting B cells with anti-CD20 dramatically decreased anti-oxLDL IgG, but only minimally reduced levels of anti-oxLDL IgM, effectively increasing the IgM to IgG ratio. This led the authors to hypothesize that IgM plays a protective role while IgG is inflammatory (21). Kyaw *et al*. confirmed this hypothesis in a series of elegant experiments in which he adoptively transferred either IgM-secreting B1a cells or conventional IgG-secreting B2 B cells into $ApoE^{-/-}Rag2^{-/-}$ or $ApoE^{-/-}$ mice. The results of these studies showed that B1a B cells abrogated atherosclerosis compared to controls, while B2 B cells increased lesion size over 300% (22). In a second complementary study, Kyaw confirmed these findings with genetic deletion of B2 B cells using $TNFRSF13B^{-/-}$ mice crossed to the $ApoE^{-/-}$ background. When these mice were placed on high fat diet they had significantly smaller

atherosclerotic lesions in the proximal aorta compared to $ApoE^{-/-}$ accompanied by decreased titers of IgG and decreased levels of inflammatory cytokines (23). Kyaw, Tay, and Krishnanmurthi *et al*. further demonstrated the atheroprotective role of IgM using a splenectomy model that specifically depleted B1a but not B2 B cells. They found that following splenectomy, protection against atherosclerosis was achieved with adoptive transfer of wild-type, but not $IgM^{-/-}$ B1a cells. Mice that received protective wild-type B1a cells also had increased titers of anti-oxLDL IgM (24). While the protective nature of IgM is well established at this point, the potentially pathogenic role of IgG is less understood.

Immune Complexes in Sterile Inflammation

B cell studies identified an important role for antibodies in the development of atherosclerosis. Shortly after the observation that antibody responses were generated in response to oxLDL, it was discovered that titers of circulating anti-oxLDL antibodies could be used as a biomarker for atherosclerosis disease severity (16, 25). While the majority of studies on the immune response in atherosclerosis focus on unbound oxLDL as the driving antigen in atherosclerosis, likely due to the early observations of Windaus, Anitschkow, and Chaltow, it has been shown that up to 90% of the oxLDL in circulation is bound to specific antibody, forming immune complexes (ICs) (10, 11, 26). Interestingly, it has been observed in both humans and hyperlipidemic animals that the majority of antibodies contained in oxLDL-ICs are IgG in nature, suggesting a potentially pathogenic role for these ICs (27, 28).

Generally speaking, an IC is a solitary unit formed by the binding of antibody to its soluble antigen that, when formed, acts as an antigen of its own. Many sterile inflammatory disorders including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are associated with disease specific ICs that not only correlate with disease severity, but also play a mechanistic role in pathogenesis. RA is a common form of autoimmune arthritis characterized by joint pain and bone destruction especially of the hands and feet (29). Over two thirds of RA patients are considered "seropositive," meaning that they have ICs containing citrullinated proteins, as well as antibodies to the constant region (Fc) of self IgG antibodies (also known as rheumatoid factor). Citrullinated fibrinogen containing ICs precipitated from plasma have been shown to induce inflammatory Tumor Necrosis Factor alpha (TNFα) production from peripheral blood mononuclear cells (PBMCs) (30). Antibodies contained in RA associated ICs bind to Fc gamma receptors on the surface of maturing osteoclasts, increasing differentiation that ultimately leads to bone erosion (31, 32). Binding of these ICs to osteoclasts also results in the secretion of CXCL8 (IL-8) which binds to its cognate receptor on sensory neurons, resulting in joint pain and swelling (33–36).

Like RA, SLE is another sterile inflammatory disease driven by specific ICs. SLE has a wide variety of symptoms including a malar rash, alopecia, joint pain, and nephritis; however, the presence of anti-nuclear and anti-double stranded DNA ICs even in the absence of other symptoms is considered sufficient for an SLE diagnosis (37). These ICs can initiate experimental lupus nephritis by depositing in the kidneys and binding directly to the basement membrane of the glomerulus (38). In addition, nuclear antigen and double stranded DNA containing ICs initiate the complement

cascade. While this is a normal immune reaction for the clearance of immune complexes, chronic activation of complement has been observed to increase nephritis and proteinuria in mouse models of lupus (39, 40).

Work from the Lopes-Virella lab showed that oxLDL containing ICs (oxLDL-ICs) elicit increased cellular activation, inflammatory cytokine production, and foam cell formation from the human macrophage cell line THP-1 *in vitro* (41). However, it is currently unknown how oxLDL-ICs modulate the immune response and whether oxLDL-ICs play a mechanistic role in atherosclerosis progression or are simply biomarkers of disease. Interestingly, atherosclerosis patients are not the only individuals with increased levels of oxLDL-ICs. Increased titers of these ICs are also observed in other sterile inflammatory disorders including type1 and type 2 diabetes, SLE, and RA (26, 42–44). It is important to note that all of these diseases also have increased risk of cardiovascular complications as a comorbidity. Thus, it is important to understand the specific role of oxLDL-ICs in inflammation. The primary focus of my dissertation work has been to uncover the mechanism by which oxLDL-ICs influence inflammation and to determine whether oxLDL-ICs directly affect atherosclerosis outcomes.

Fcγ Receptors as Indicators of IC Pathogenesis

Fcγ receptors (FcγR) are the canonical receptors for IgG ICs by binding to the constant (Fc) region of the antibody. Generally speaking, these receptors can be either activating (FcγRI, FcγRIII, and FcγRIV in mice; FcγRI, FcγRIIa, FcγRIII, and FcγRIV in humans) or inhibitory (FcγRIIb in mice and FcgRIIba). FcγRs are expressed on a wide

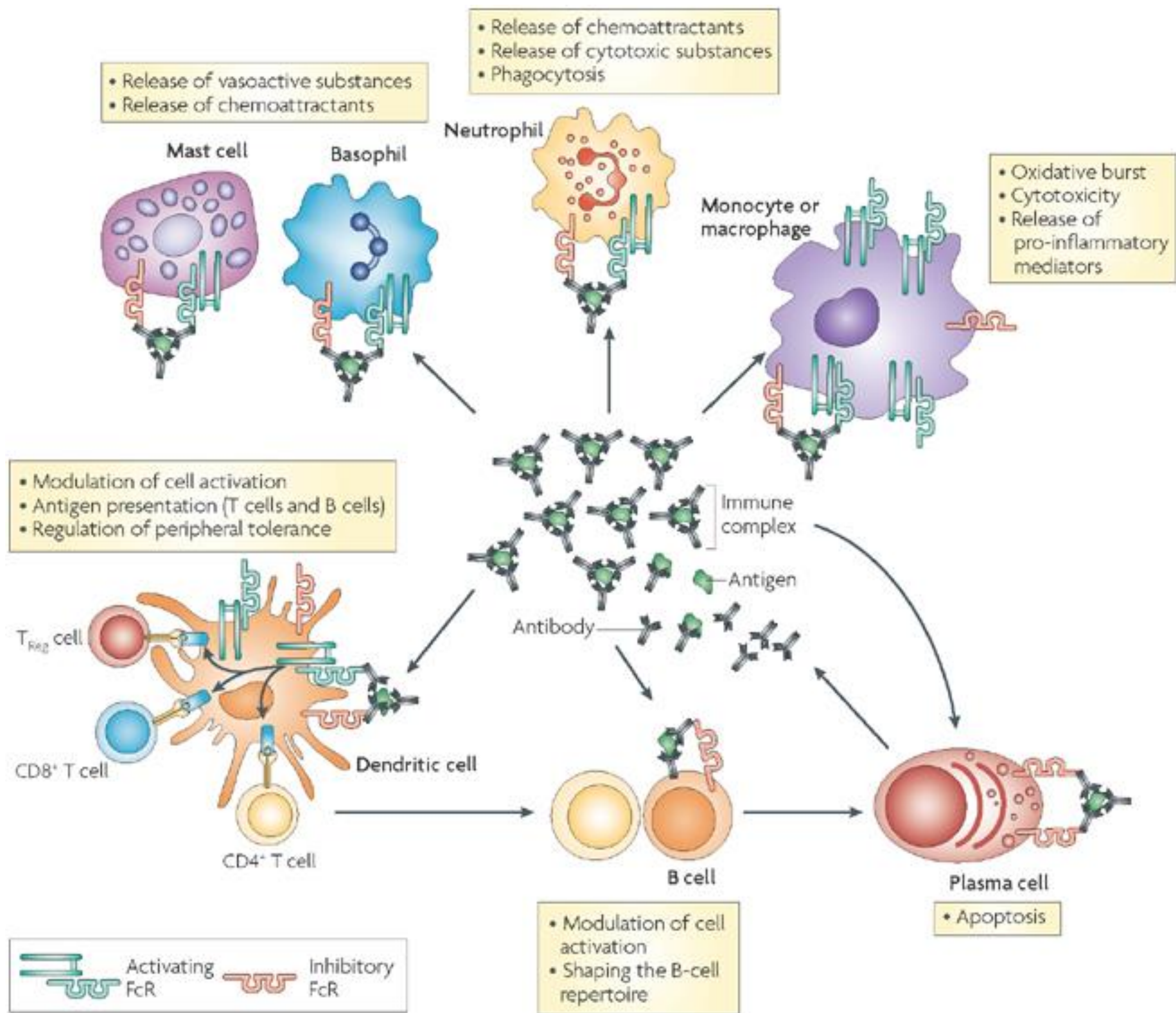

Figure 1.2. Various effector functions of FcγRs. From (46).
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variety of cell types including macrophages, dendritic cells (DCs), neutrophils, and eosinophils. Activating receptors signal through an immunoreceptor tyrosine based activation motif (ITAM) which results in recruitment and phosphorylation of spleen tyrosine kinase (Syk). Effector functions resulting from activating receptor ligation include antibody dependent cellular phagocytosis (ADCP), antibody dependent cell mediated cytotoxicity (ADCC), and release of pro-inflammatory cytokines and chemokines. The inhibitory receptor FcγRIIb signals through an immmunoreceptor tyrosine based inhibitory motif (ITIM) resulting in recruitment and phosphorylation of SH2-containing inositol 5′-phosphatase (SHIP). FcγRIIb ligation results in immunomodulatory responses (Summarized in Figure 1.2) (reviewed in 31–33).

Human studies suggest a regulatory role for FcγRs. It has been observed that FcγRs are expressed in atherosclerotic plaques, and polymorphisms in the activating receptor FcγRIIa are associated with exacerbated CHD (48–50). In $ApoE^{-/-}$ mice on high fat diet, treatment with whole human immunoglobulin (containing the Fc portion), but not treatment with human Fab fragments (lacking the Fc portion) was protective against atherosclerosis (51). *In vitro* studies indicate that FcγR (specifically the high affinity receptor FcγRI) mediated uptake of oxLDL-ICs facilitates inflammation and foam cell formation in human and mouse macrophages (52, 53).

Studies in mice globally deficient in FcγR subsets further support a potential role for IC pathogenesis in atherosclerosis. Mice lacking both of the activating receptors FcγRI and FcγRIII ($FcR\gamma^{-/-}$) on either the $ApoE^{-/-}$ or $LDLr^{-/-}$ background are protected from atherosclerosis compared to controls (54, 55). This protection is accompanied by decreased levels of pro-inflammatory cytokines in the aortas. Conversely, both $LDLr^{-/-}$

mice that received a bone marrow transplant from inhibitory receptor FcγRIIb$^{-/-}$ mice and FcγRIIb$^{-/-}$ mice crossed to the ApoE$^{-/-}$ background had increased atherosclerotic lesion size and increased inflammation compared to controls (56, 57). A more recent study of FcγRIIb/ApoE double knock-out mice on a congenic, rather than mixed, background showed opposite results. These mice had decreased atherosclerotic burden and inflammation compared to their control counterparts. The authors attributed this opposing finding to upregulation of lupus-associated Slam genes (involved in expression of many receptors on hematopoetic cells) in the mixed but not the congenic mice (58). However, despite this discrepancy, these studies indicated a potentially important role for oxLDL-ICs in atherosclerosis associated inflammation.

ICs Can Bind to Multiple Receptors to Modulate Immune Reponses

While FcγR are the canonical IC receptors, it has been widely observed that the antigenic portion of the IC can bind other cell surface receptors, as well. In many cases, ICs have been found to also bind various Toll Like Receptors (TLRs). TLRs recognize distinct molecular patterns and are key players in innate immune responses(reviewed in 60). In the context of SLE, single stranded RNA containing ICs are able to bind to TLR7, enhancing inflammation and glomerular nephritis (60). Similarly, double stranded DNA containing-ICs bind to TLR9 following internalization to enhance dendritic cell and B cell mediated inflammation in SLE (61). Sokolove *et al.* demonstrated that RA associated citrullinated fibrinogen ICs concomitantly bind to FcγRs and TLR4, enhancing the production of inflammatory TNFα from macrophages (30). Yet another study from Duffy

et al. showed that IgG opsonized *Francisella tularensis* enhanced IL-6 and IL-1β production from macrophages by binding to both FcγRs and TLR2 (60).

Much like the antigens contained in the ICs discussed above, oxLDL has been shown to bind TLRs through molecular mimicry (63, 64). Binding of oxLDL to TLR4 enhances production of pro-inflammatory cytokines and facilitates foam cell formation in human and mouse macrophages (65–67). As a modified cholesterol, oxLDL also binds the scavenger receptor CD36 (68). While the scavenger receptor CD36 is one of the main receptors responsible for lipid loading and foam cell formation in macrophages, it has also been shown to facilitate sterile inflammation by forming a heterotrimer with TLR4 and TLR6(69, 70). Formation of the TLR4/TLR6/CD36 heterotrimer results in increased production of the pro-inflammatory cytokine IL-1β (70, 71). Given that oxLDL-ICs may be able to bind multiple receptors on the cell surface, the studies discussed in this section highlight a potential mechanism by which oxLDL-ICs may modulate the immune response.

Dendritic Cells are the Potential Drivers of IC Induced Immune Responses

To date**,** many studies of the immune response in atherosclerosis focus on macrophages. This is likely due to their prevalence in atherosclerotic lesions, as well as their propensity to become lipid-laden foam cells (reviewed in 60). However, there are many other immune cell types that are involved in inflammation and atherosclerosis. Dendritic cells (DCs) are specialized antigen presenting cells (APCs) that provide an important link between the innate and adaptive immune response. Although they derive from a common progenitor cell in the bone marrow, DCs are unique from macrophages

in both morphology and in their ability to potentiate an adaptive immune response (73, 74). Early studies of DCs found them to be especially potent at activating T cells in mixed leukocyte cultures, and uncovered that DCs are two times more proficient at capturing and presenting antigen to T cells than any other APC (75, 76). DCs survey the periphery where they capture antigens and bring them to the draining lymph node to initiate immune responses by presenting the antigen in the context of the MHC.

DCs are a heterogeneous population of cells with a wide variety of specialized functions. However, classical DCs are thought to be $CD11c^{+}MHCII^{+}F4/80^{-}$. These markers differentiate DCs from macrophages which are CD11c- and $F4/80^{+}$. $CD11c^{+}$ DCs are found in areas of the aorta prone to development of atherosclerosis in both humans and mice, and DC numbers increase as lesions grow (19, 77, 78). The DCs observed in atherosclerotic lesions cluster with T cells and are thought to be activated based on the expression of co-stimulatory molecules such as CD86 (78, 79).

There have been many studies implicating DC/T cell interactions in the pathogenesis of atherosclerosis. $LDLr^{-/-}$ mice globally deficient in MHCII are protected from atherosclerosis, and this protection is accompanied by a reduction in T cell activation (80). Lievens *et al*. discovered that disrupting signaling of the immunomodulatory cytokine Transforming Growth Factor beta (TGFβ) in $CD11c^{+}$ cells of $ApoE^{-/-}$ mice caused expansion of effector T cells and increases in atherosclerotic lesion size(81). Yet another study by Subramanian *et al*. showed that MyD88 (a critical protein downstream of TLR4) signaling for oxLDL in $CD11c^{+}$ cells is required for regulatory T cell (T_{reg}) mediated protection from atherosclerosis in $LDLr^{-/-}$ mice (82).In addition to using *in vivo* manipulations to study DC/T cell interactions in atherosclerosis,

researchers have also investigated the effects of treating DCs *in vitro* prior to adoptive transfer into $LDLr^{-/-}$ or $ApoE^{-/-}$ mice. It has been shown that bone marrow-derived dendritic cells (BMDCs) treated with oxLDL and adoptively transferred into $LDLr^{-/-}$ mice generate specific $CD4^{+}$ T cell responses and confer protection against atherosclerosis (83). On the other hand, a second study found that injecting $ApoE^{-/-}$ mice with BMDCs pulsed with malondialdehyde-modified LDL increases atherosclerosis by inhibiting the proliferation of T_{regs} (84).

Interestingly, classical DCs express moderate to high levels of all of the FcγRs, and DCs in atherosclerotic lesions express robust levels of the high affinity activating receptor FcγRI(85, 86). FcγRs provide an important link between the humoral and cellular immune response as internalization of ICs by FcγRs allows for the antibody bound antigen to be shuttled to the endosome for subsequent presentation to T cells on Major histocompatibility complex (MHC) I and II (87–89). Thus, it stands to reason that oxLDL-IC binding to DCs may be important to both the innate and adaptive immune response in atherosclerosis.

Research Goals and Summary of Data

Atherosclerosis is a disease of sterile inflammation that represents a major public health burden both in the United States and worldwide. The majority of studies to date focus on the role of free oxLDL in the immune response in atherosclerosis; however up to 90% of circulating oxLDL is bound to specific antibody in ICs. Many sterile inflammatory disorders such as RA and SLE are characterized by the prevalence of disease specific ICs that are known to play a mechanistic role in pathogenesis. While it

is known that oxLDL-IC titers correlate with disease severity, it is currently unknown how oxLDL-ICs modulate the immune response. The goal of this study is to determine the contribution of oxLDL-ICs to inflammation and atherosclerosis.

In Chapter II, I show that oxLDL-ICs act as a priming signal for the NLRP3 inflammasome by binding to the receptors FcγR, TLR4, and CD36. Signaling through these receptors converges on the adaptor protein CARD9 and results in formation of the CARD9-Bcl10-MALT1 complex, exploiting a pathway that is commonly associated with fungal pathogenesis. This chapter will also provide evidence that oxLDL-ICs directly influence atherosclerosis outcomes. Chapter III investigates how oxLDL-ICs modulate the adaptive immune response. In this chapter, I demonstrate that oxLDL-IC mediated IL-1α and IL-1β from DCs promotes Th17 responses, while IL-23 inhibits IFNγ production. The findings from these two chapters are summarized in Chapter IV and future directions for each are provided. Ultimately these studies move from bench to bedside, identifying a pathological role for a long standing biomarker of atherosclerosis disease severity.

CHAPTER 2

OxLDL Immune Complexes Prime the NLRP3 Inflammasome via CARD9 and Exacerbate Atherosclerosis

The majority of this work was published in The Journal of Immunology Volume 198 in the year 2017 under the same name. Figures and text from the original manuscript have been modified and data have been added.

Abstract

OxLDL has been shown to initiate inflammatory responses in many different cell types including macrophages and DCs. While many studies focus on the effects of free oxLDL on the immune response, the majority of oxLDL in circulation is complexed to specific antibody forming ICs. Elevated titers of oxLDL-ICs can be found in a number of sterile inflammatory disorders including atherosclerosis, Type 1 and 2 diabetes, RA, and SLE. Levels of oxLDL-ICs often correlate with atherosclerosis disease severity; however, little is known about how oxLDL-ICs modulate the immune response and effect atherosclerotic disease outcomes. In this chapter, I demonstrate that bone marrow- BMDCs incubated with oxLDL-ICs for 24 hours are more activated and secrete significantly more IL-1β compared to BMDCs treated with free oxLDL, but there was no difference in levels of TNFα or IL-6. OxLDL-IC treatment increased expression of inflammasome-related genes *Il1a*, *Il1b*, and *Nlrp3*. Pre-treatment of BMDCs with a caspase 1 inhibitor decreased IL-1β secretion in response to oxLDL-ICs. To prime the inflammasome, oxLDL-ICs signaled through multiple receptors including FcγR, TLR4, and CD36. OxLDL-IC signaling in BMDCs converged on the adaptor protein CARD9, resulting in formation of the CARD9-Bcl10-MALT1 signalosome complex and NF-κB translocation to the nucleus. Finally,

oxLDL-IC injection significantly increased atherosclerotic lesion burden in $LDLr^{-/-}$ mice compared to saline and oxLDL injected controls.

Introduction

Immune complexes (ICs) are formed by the binding of a specific antibody to its soluble antigen creating a solitary unit. Many sterile inflammatory disorders are characterized by increased serum titers of disease-specific ICs, which can have mechanistic roles in pathogenesis including RA, SLE and atherosclerosis (90, 91). In atherosclerosis, antibodies are directed at oxLDL forming oxLDL-ICs(25). Although it has been shown that oxLDL-ICs can elicit increased inflammatory cytokine production from a human macrophage cell line, little is known about how oxLDL-ICs modulate the immune response or effect atherosclerotic outcomes(41).

Interestingly, many of the sterile inflammatory disorders characterized by high serum titers of ICs are also associated with chronic inflammasome hyperactivation (92–94). The inflammasome is a multi-protein oligomer that requires both a priming and activating signal for initiation. Activation of the inflammasome results in robust secretion of the inflammatory cytokine IL-1β (95). The inflammasome was originally identified as an innate immune mechanism necessary for the clearance of many bacterial and fungal pathogens (96, 97). Unfortunately, hyperactivation of the inflammasome has been found to exacerbate many inflammatory diseases (98). To combat the negative effects of chronic inflammasome activation, IL-1β blockade is used clinically to treat IC-related diseases including RA and juvenile SLE. (99, 100). Inhibition of inflammasome mediated IL-1β is also protective in atherosclerosis as it has been shown that knocking out the inflammasome- related gene *Nlrp3* in mice completely abolishes atherosclerosis (101).

However, despite the observations that sterile inflammatory disease have both increased serum IC levels and inflammasome activation, a direct connection has not been made between these two factors.

This study demonstrates that oxLDL-ICs act as a priming signal for the Nlrp3 inflammasome by concomitant signaling through FcγR, TLR4, and CD36. OxLDL-IC mediated inflammasome priming occurs in a receptor dependent fashion and does not require previously established mechanisms such as cholesterol crystal formation (101). Signaling through these receptors converges on the adaptor protein CARD9, resulting in formation of the CARD9-Bcl10-Malt1 complex and nuclear translocation of NF-κB. Finally, this study demonstrates that oxLDL-ICs increase atherosclerotic lesion size and are not simply a biomarker for disease severity.

Materials and Methods

Mice. C57BL/6J (B6), B6N.129-Nlrp3$^{tm1Hhf/J}$ (*Nlrp3*$^{-/-}$), B6.129P2 (SJL)-Myd88tm1Defr/J (*Myd88*$^{-/-}$), and B6.Cg-Tg (TcraTcrb) 425Cbn/J (OT-II) mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained and housed at Vanderbilt University. All mice used in these studies were on the B6 background. Procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

oxLDL and oxLDL-ICs. Human native LDL was purchased from Intracel Resources (Frederick, MD) or Sigma-Aldrich (St. Louis, MO). OxLDL was made by dialyzing human LDL for 24 hrs against 0.9 M NaCl at 4°C with two buffer changes, followed by dialysis against 0.9 M NaCl containing 20 μM $CuSO_4$ for 4 hrs at room temperature. Oxidation was terminated by dialysis against 1 mM EDTA in 1X PBS for 16 hrs with two buffer changes. Extent of oxidation was determined by TBARS assay (Cell Biolabs, Inc., San

Diego, CA). OxLDL-ICs were generated by incubating polyclonal rabbit anti-human apoB-100 (Alfa Aesar, Ward Hill, MA) with oxLDL at a ratio of 10:1 (500 μg of antibody, 50 μg of oxLDL) overnight at 37°C. Unbound antibody and antigen were removed by size exclusion filtration. For all experiments, immune complex concentrations were normalized based on oxLDL concentration to ensure that equal amounts of oxLDL were used in both the oxLDL and oxLDL-IC conditions. Fab_2 fragments were made using the Pierce Fab Fragmentation Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocol. OxLDL enriched immune complexes were obtained from the serum of *Apoe*$^{-/-}$ mice fed Western diet (21% saturated fat, 0.15% cholesterol) for 12 weeks. Whole blood was obtained by retro-orbital bleeding. Serum was incubated with protein G beads for 1 hr at room temperature. Immune complexes were eluted from protein G beads and protein concentration was calculated by BCA assay according to manufacturer's instructions (Thermo Fisher Scientific).

Cell Culture. BMDCs were generated as previously described (102). Briefly, bone marrow from hind legs was flushed with RPMI-1640 (Corning, Corning, MA) supplemented with 10% FBS (Gibco, Grand Island, NY), 10 mM HEPES (Corning), and 1× Penicillin/Streptomycin/L-glutamine (Sigma-Aldrich) (hereafter referred to as TCM). Cells were plated in 100 mm^2 petri dishes at $2×10^5$ cells/mL in TCM containing 20 ng/mL recombinant GM-CSF (R&D Systems, Minneapolis, MN). Media was replaced on days 3 and 6 and cells were harvested on day 9. To make BMDCs from various transgenic strains femurs were shipped overnight. Femurs from *Cd36*$^{-/-}$ mice were obtained from Dr. Kathryn Moore (New York University, New York, NY). *Cd11c*^{cre}/*Syk*$^{flox/flox}$ femurs were obtained from Dr. John Lukens (University of Virginia, Charlottesville, VA). Femurs from

Card9$^{-/-}$ mice were received from Dr. Thirumala Kanneganti (St. Jude Children's Research Hospital, Memphis, TN) (103).

ELISA and Western Blotting. IL-1β, IL-6, and TNFα (BD Biosciences, San Jose, CA) ELISAs were performed according to manufacturer's instructions. For Western blotting experiments, 1×10^6 BMDCs were treated with indicated stimuli for 24 hrs. Cells were lysed with 1× RIPA buffer and lysates were separated by 4%-20% reducing SDS-PAGE. Blots were incubated with anti-mouse caspase-1 monoclonal antibody (Adipogen, San Diego, CA) or anti-mouse NFκB p65 antibody (Cell Signaling Technology, Danvers, MA), overnight at 4°C followed by IRDye 680RD goat anti-mouse or goat anti-rabbit (LI-COR, Lincoln, NE) for 30 min at room temperature. Bands were visualized using the LI-COR Odyssey System.

Immunoprecipitation. CBM complex formation was assessed in whole cell lysates from BMDCs stimulated for 2 hrs with oxLDL or oxLDL-ICs. Cells were lysed in 1× RIPA buffer followed by immunoprecipitation with antibody to MALT1, CARD9, or Bcl10 (Santa Cruz Biotechnology, Dallas, TX). Western blot analysis was performed as described above with anti-CARD9, anti-Bcl-10, and anti-MALT1 (Cell Signaling Technologies).

Real-Time Quantitative PCR. BMDCs were treated with indicated stimuli for two hrs. Total RNA was isolated from cells using Norgen Total RNA Isolation Kits (Norgen Biotek Corporation, Thorold, Ontario, Canada). RNA concentrations were normalized and RNA was reversed transcribed with a high capacity RNA to cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). The reverse transcription product was used for detecting mRNA expression by quantitative real time PCR using the QuantStudio 6-flex

System (Life Technologies, Grand Island, NY). The cycling-threshold (C_T) value for each gene was normalized to that of the house keeping gene *Ppia*, and relative expression calculated by the change in cycling threshold method ($\Delta\Delta C_T$).

Flow Cytometry. To measure FcγR expression, BMDCs were then stained on ice for 30 min with CD16.2-APC, CD16/32-FITC, CD32-Alexa Fluor 488, or CD64-APC in the absence of Fc-block. The CD16.2, CD16/32, and CD64 antibodies were purchased from BD Bioscience and diluted 1:200. Antibodies were diluted 1:200 in FACS buffer containing HBSS, 1% BSA, 4.17mM sodium bicarbonate, and 3.08mM sodium azide. The CD32 antibody, a gift from Dr. Jeffrey Ravetch (The Rockefeller University, New York, NY), was labeled using an Alexa Fluor 488 Antibody Labeling Kit (Thermo Fisher Scientific). Cells were washed and re-suspended in 2% PFA for analysis on a MACSQuant seven color flow cytometer (Miltenyi Biotech) and data were analyzed using FlowJo Single Cell Analysis Version 7.6.5. To measure pSyk and pErk, cells were stimulated with LPS, oxLDL, oxLDL-Fab_2 or oxLDL-IC for 5 or 15min. Cells were then fixed for 10minf in 1× lyse/fix buffer (BD Bioscience) and permeabilized for 30min using Perm Buffer III (BD Bioscience). After permeabilization, cells were Fc blocked for 15min followed by staining with either CD11b-V450 (BD Bioscience), CD11c-FITC (BD Bioscience) and pSyk Y525/526- PE (Cell Signaling Technology); or, CD11b-V450 (BD Biosciences), CD11c-PeCy7 (BD Biosciences) and pERK1/2-FITC (BD Biosciences).

In vivo studies. For atherosclerosis studies 8-10 week old male $LDLr^{-/-}$ mice were retro-orbitally injected every two weeks with saline, 10μg oxLDL, or 25μg oxLDL-IC (equivalent concentrations of oxLDL). Mice were placed on Western diet (21% fat, 0.15% cholesterol)

one week after the first injection for the duration of the study. After 8 weeks on diet, mice were sacrificed and aortic root lesion area was evaluated using Oil Red O staining.

Statistical Analyses. Where appropriate statistical significance was determined using a Student's t test. If more than two groups were compared, a one way Analysis of Variance (ANOVA) with Bonferroni correction was used. In all cases $p<0.05$ was considered statistically significant.

Results

OxLDL-ICs increase DC activation. It has been demonstrated that ICs containing TLR ligands can enhance inflammatory responses in DCs and macrophages (30, 61). To determine if oxLDL-ICs increase DC activation compared to free oxLDL, BMDCs were incubated with oxLDL or oxLDL-ICs for 16 hours followed by staining for the activation markers CD40, MHCII, and CD86 (Figure 2.1). Treatment of BMDCs with oxLDL-ICs increased expression of CD40 and MHCII compared to oxLDL treatment, indicating that oxLDL-ICs enhance DC activation.

OxLDL-ICs elicit robust IL-1β production. In order to test whether increased activation was accompanied by differential cytokine responses, BMDCs were incubated with either oxLDL or oxLDL-ICs for 24hrs. No differences were observed in TNFα or IL-6 production between the treatment groups; however, oxLDL-ICs induced almost 10-fold more IL-1β production compared to free oxLDL (Figure 2.2A). To control for anomalies that may be associated with lab-generated oxLDL-ICs, BMDCs were also treated with oxLDL-enriched ICs isolated from hyperlipidemic *ApoE* deficient mice (ApoE-IC). Given that results elicited by ApoE-ICs were similar to those obtained with oxLDL-ICs, the

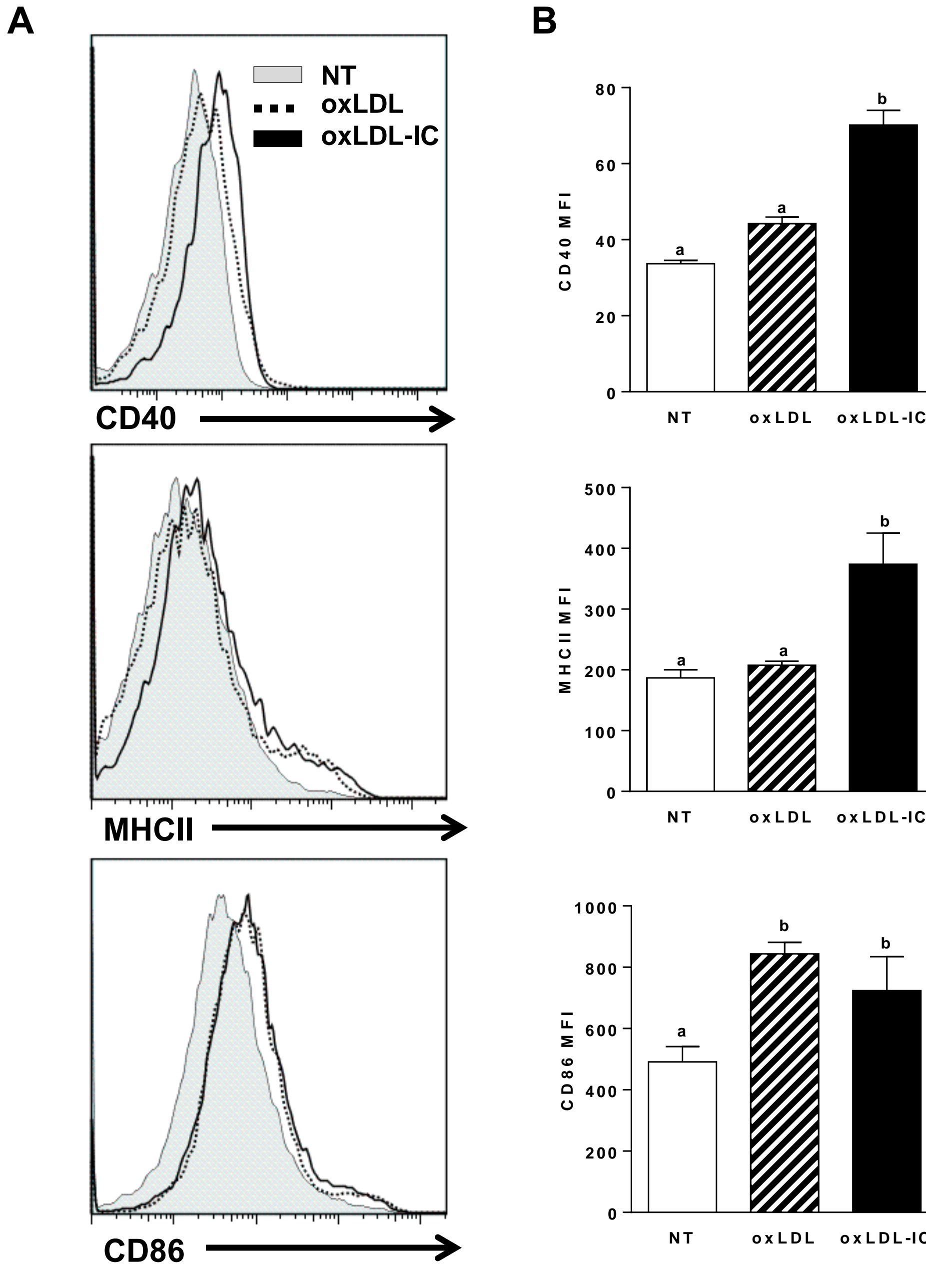

Figure 2.1. OxLDL-ICs increase activation of BMDCs. BMDCs were incubated with oxLDL or oxLDL-ICs for 24 hours. CD40, MHCII, and CD86 expression were measured by flow cytometry. Representative histograms of the respective activation markers are shown in A (Gated on $CD11b^{+}CD11c^{+}$ cells) for oxLDL (dashed) and oxLDL-IC (solid) treated BMDCs. Activation marker expression is quantitated based on mean fluorescence intensity (B) where n=3 mice/experiment and at least 3 experimental repeats Solid gray histograms are isotype controls. Unlike letters denote significance ($p<0.05$) by Student's *t* test and error bars represent SEM.

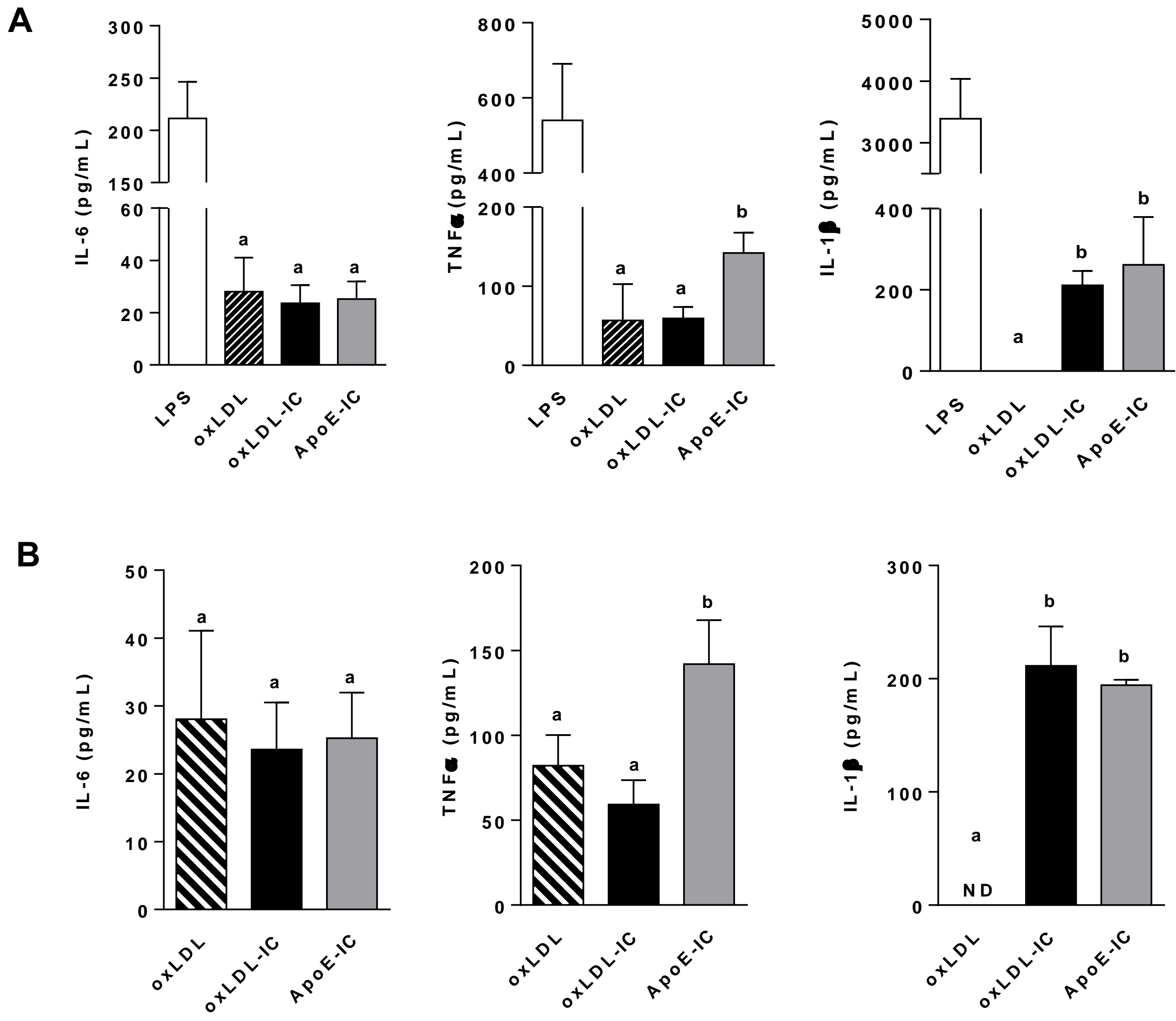

Figure 2.2. OxLDL-ICs induce potent IL-1β secretion from BMDCs and BMDMs. (A) BMDCs and (B) BMDMs were treated for 24 hours with oxLDL, oxLDL-ICs, or ICs isolated from the serum of ApoE -/- mice. Cytokine levels in culture supernatants was measured by ELISA. Shown are representative experiments where n= at least three biological and technical replicates. Error bars indicate SEM. Unlike letters denote significance ($p<0.01$) by Student's *t* test.

enhanced IL-1β secretion elicited by oxLDL-ICs is likely to be physiologic and not simply an artifact. Bone marrow-derived macrophages (BMDMs) were also treated with oxLDL or oxLDL-ICs for 24hrs. The cytokine profiles obtained mirrored those of the BMDCs, suggesting that this observation was not a DC-specific phenomenon and represented a fundamental difference in signaling between oxLDL and oxLDL-ICs (Figure 2.2B).

OxLDL-ICs prime the Nlrp3 inflammasome. High IL-1β production is a hallmark of inflammasome activation, and previous studies have shown that oxLDL activates the inflammasome through the formation of cholesterol crystals (101). I hypothesized that oxLDL-ICs may activate the inflammasome through a similar mechanism. Inflammasome activation is a two-step process. The first signal “primes” the inflammasome, resulting in the production of pro-IL-1β. This signal is typically conferred by a pathogen associated molecular pattern (PAMP) such as LPS (95). The second signal, or the activating signal causes cleavage of pro-caspase 1 to caspase 1 which subsequently cleaves pro-IL-1β into its mature form (Figure 2.3). Cellular damage, extracellular ATP, cholesterol crystals, and uric acid crystals have all been identified as activating signals for the inflammasome (104). To test if oxLDL-ICs can serve as an activating signal for the inflammasome like cholesterol crystals, BMDCs were primed with LPS for 3hrs followed by treatement with oxLDL (25μg/ml) or increasing concentrations of oxLDL-ICs (containing 10, 25 or 50μg/mL total oxLDL) for 3 additional hrs. OxLDL-ICs were able to act as an activating signal, however even the highest concentration of oxLDL-ICs elicited IL-1β levels similar to that of oxLDL (Figure 2.4A, left). To test whether oxLDL-ICs act as a priming signal for the inflammasome, BMDCs were incubated with oxLDL or oxLDL-ICs in increasing concentration for 3hrs followed by ATP for one additional hour. As a priming signal,

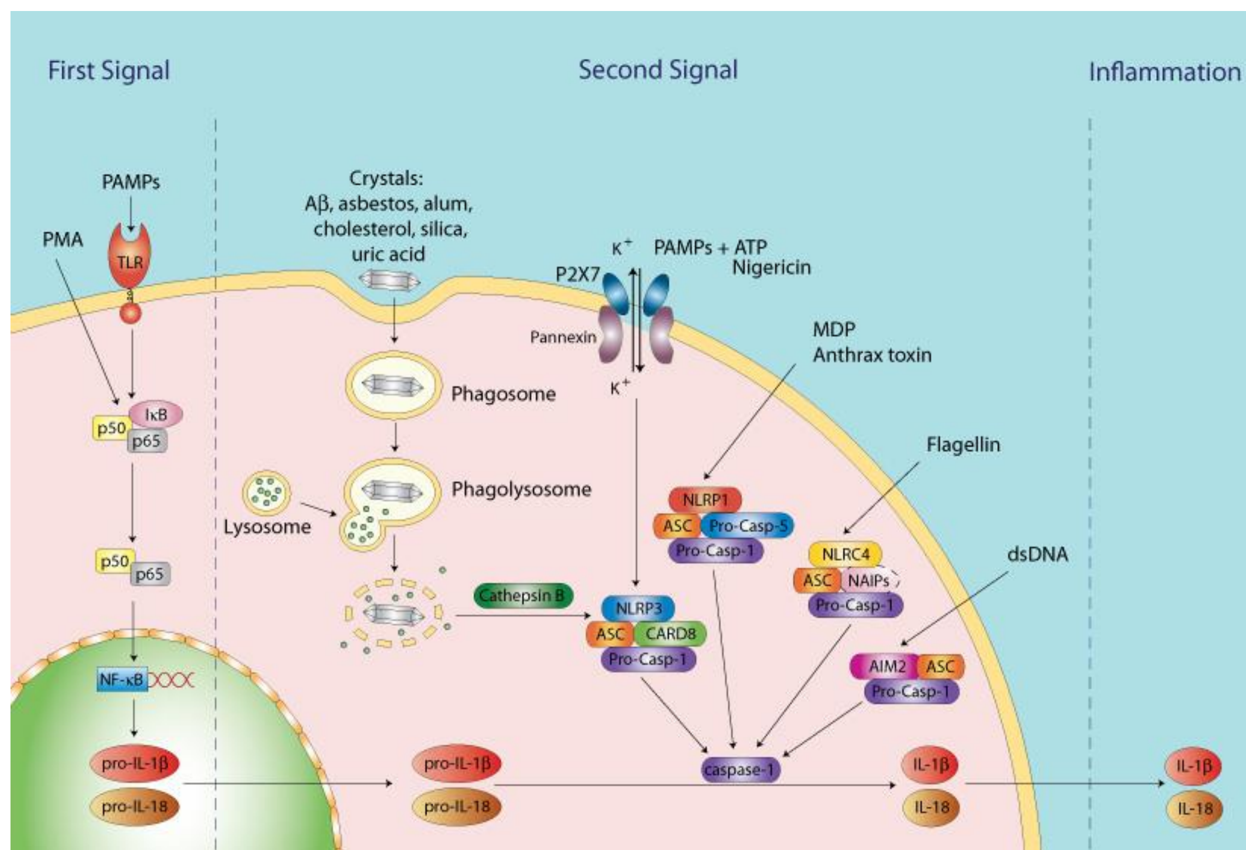

Figure 2.3. Inflammasome priming and activation. Inflammasome priming (first signal) typically occurs via PAMP signaling and results in the transcription of inflammasome related genes. Inflammasome activation (second signal) can happen through a variety of mechanisms including ATP or crystal formation and results in the cleavage of pro-caspse-1 and the subsequent cleavage of pro-IL1β. From http://www.invivogen.com/review-inflammasome.
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oxLDL-ICs elicited significantly more IL-1β than free oxLDL (Figure 2.4A, right panel). To rule out the possibility that OxLDL-ICs were inducing IL-1β production through the formation of crystals, OxLDL-ICs did not promote IL-1β through formation of cholesterol crystals, BMDCs were incubated with oxLDL or oxLDL-ICs for three hours and analyzed by polarizing light microscopy. Twenty-four hour incubation with oxLDL and the reverse cholesterol transport inhibitor CLI-067 was used as a positive control for crystal formation. Three hour incubation of BMDCs with oxLDL or oxLDL-ICs was not sufficient for crystal formation (Figure 2.4B). Endotoxin contamination of IC preparations is another scenario that could give false positive results for IL-1β production. Pretreatment of BMDCs with the LPS inhibitor polymyxin B prior to exposure to oxLDL or oxLDL-ICs had no effect on elicited IL-1β production, ruling out this possibility (Figure 2.4C).

OxLDL-IC priming of the inflammasome is dependent on both Nlrp3 and caspase-1. To confirm that oxLDL-ICs were acting as a priming signal for the inflammasome, qPCR analysis was performed on RNA from BMDCs treated with oxLDL or oxLDL-ICs for 2hrs. Increased transcription of inflammasome-related genes *Il1a*, *Il1b*, and *Nlrp3* was observed with no change in inflammasome-related genes *Aim2*, *Nlrc4*, or *Il18* (Figure 2.5A). These data indicate that oxLDL-ICs induce *Nlrp3* mRNA levels, suggesting that oxLDL-ICs specifically prime the Nlrp3 inflammasome. In order to confirm this finding, wild-type and *Nlrp3*$^{-/-}$ BMDCs were treated with oxLDL-ICs for 3 hrs followed by ATP for an additional hr. IL-1β was measured in culture supernatants by ELISA. As expected, absence of *Nlrp3* completely abolished mature IL-1β production (Figure 2.5B). To confirm that oxLDL-IC mediated inflammasome activation was caspase-1 dependent, Western blot analysis was performed on whole cell lysates and supernatant from oxLDL

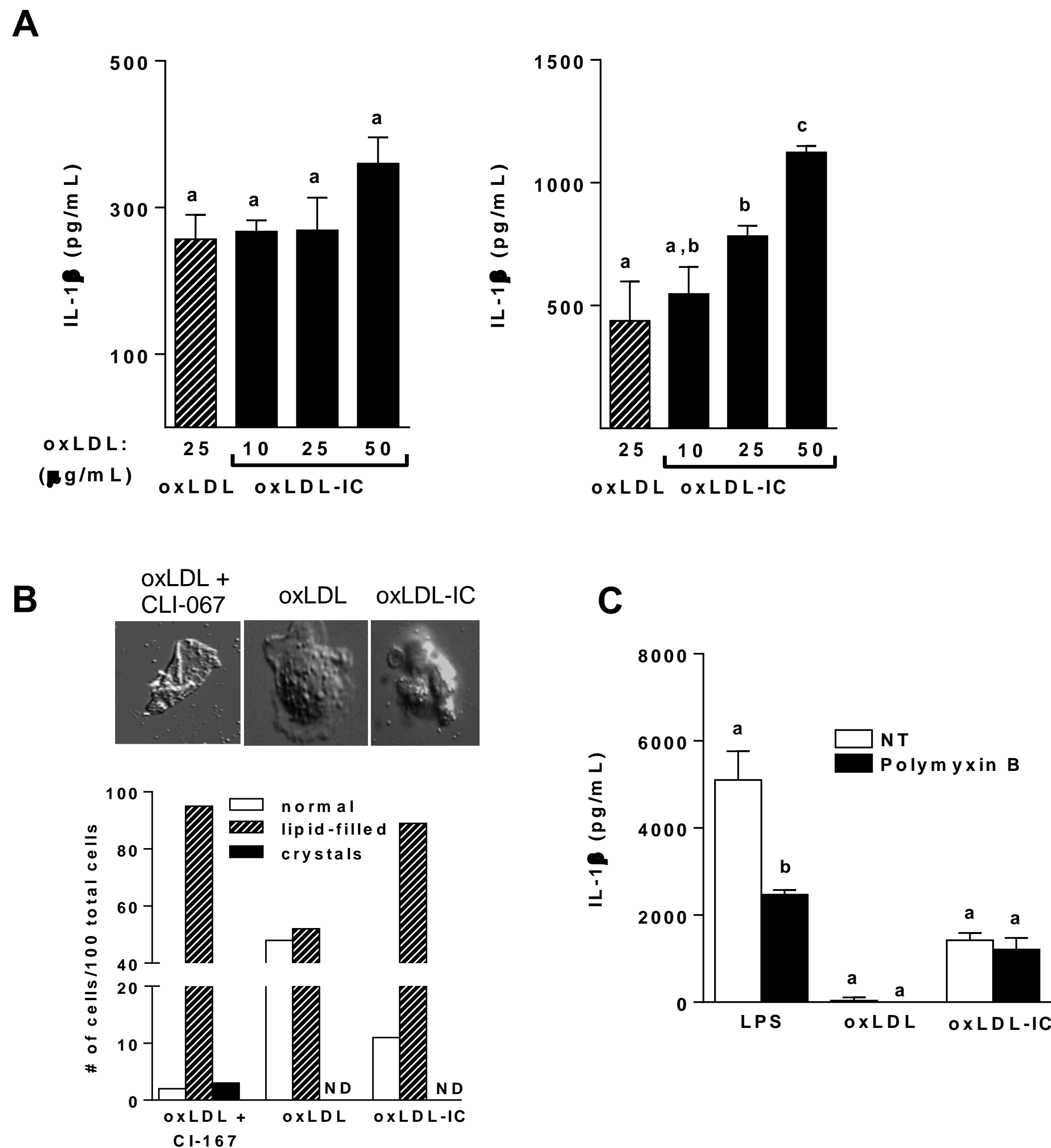

Figure 2.4. OxLDL ICs prime the inflammasome. (A) oxLDL ICs were tested for their ability to act as an activating (left panel) or priming (right panel) signal for the inflammasome. Culture supernatants were tested for IL-1β by ELISA. Shown is one representative of three experiments with three mice per experiment. Unlike letters denote significance ($p < 0.05$) by Student t test, and error bars represent SEM. (B) BMDCs were treated with oxLDL or oxLDL ICs for 3h or with oxLDL and the ACAT inhibitor CLI-067 (positive control) for 24 h, crystal formation was analyzed by polarizing light microscopy. Lipid-filled cells and crystal formation were quantified; representative images are depicted. Shown is one representative of two experiments. Original magnification ×1000. (C) BMDCs were treated with oxLDL ICs in the presence of polymyxin B. Shown is one representative of two experiments. IL-1β in culture supernatants was measured by ELISA. Unlike letters denote significance ($p < 0.01$) by one-way ANOVA with a Bonferroni posttest, and error bars represent SD.

and oxLDL-IC treated BMDCs. Treatment of BMDCs with oxLDL-ICs increased levels of cleaved caspase-1 that was not seen in lysates from cells pre-treated with caspase-1 and pan-caspase inhibitors (Figure 2.5C). Measurement of IL-1β production from BMDCs pre-treated with caspase-1 and pan-caspase inhibitors confirmed that oxLDL-IC mediated inflammasome activation was capsase-1 dependent (Figure 2.5D). Taken together, these data show that oxLDL-ICs elicit robust IL-1β production from BMDCs by inducing production of pro-IL-1β and Nlrp3.

OxLDL-ICs elicit IL-1β production via FcγR, TLR4, and CD36. FcγRs are the canonical receptors for IgG containing-ICs, however it has been previously shown that oxLDL activates the inflammasome through formation of a heterotrimer containing TLRs and the scavenger receptor CD36 (45, 105). To tease out the potential contribution of each of these receptors to inflammasome activation, I first determined the baseline expression of FcγRs on BMDCs. Results indicated that BMDCs mainly express the activating receptors FcγRI and FcγRIV (Figure 2.6A). BMDCs were then treated with oxLDL-ICs or oxLDL-Fab_2 (lacking the Fc portion of the antibody to prevent binding to FcγRs) in the presence or absence of the TLR-4 inhibitor CLI-095 for 3 hrs followed by ATP for an additional hr. Treatment of BMDCs with the Fab_2 complex or the TLR4 inhibitor decreased IL-1β production by approximately 50% (Figure 2.6B). Interestingly, treatment of BMDCs with both the TLR4 inhibitor and oxLDL-Fab_2 further decreased IL-1β suggesting an additive role for these receptors (Figure 2.6B). The importance of TLR signaling to oxLDL-IC mediated inflammasome activation was confirmed using *Myd88*$^{-/-}$ BMDCs, and *Cd36*$^{-/-}$ BMDCs implicated a role for the scavenger receptor, as well (Figure 2.6C and D). These results show that oxLDL-IC priming of the inflammasome occurs in a

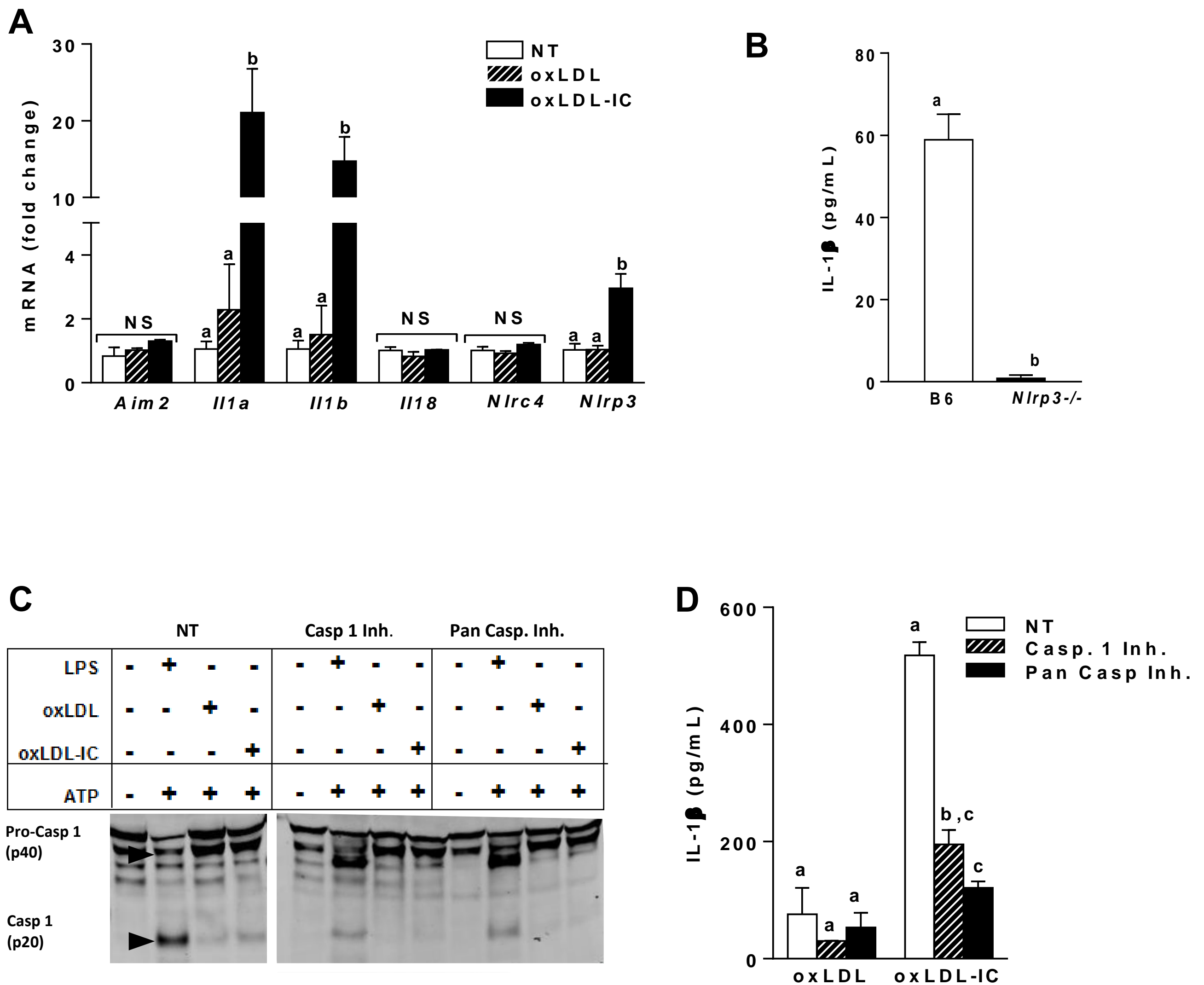

Figure 2.5. Inflammasome priming is Nlrp3 and caspase-1 dependent. (A) BMDCs were stimulated for 2 h with oxLDL or oxLDL ICs. Expression of inflammasome-related genes was measured using real-time RT-PCR and expressed as the 2−ΔΔCT method compared with the no-treatment group (n = 6 mice). Unlike letters denote significance ($p < 0.01$) by one-way ANOVA with a Bonferroni posttest. (B) B6 and Nlrp3−/− BMDCs were treated for 3 h with oxLDL or oxLDL ICs, followed by ATP for 1 h. IL-1β production in culture supernatants was measured by ELISA. Shown is one of three experiments with three mice per experiment. Unlike letters indicate significance ($p < 0.01$) by the Student t test, and error bars represent SEM.(C) Cells were stimulated for 3 hours with oxLDL or oxLDL-IC followed by lysis in RIPA buffer and Western blot for pro-caspase 1 and cleaved caspase-1. (D) BMDCs were treated as in (B) in the presence or absence of a caspase-1 inhibitor (Z-VAD-FMK) or a pan-caspase inhibitor (Z-YVAD-FMK). IL-1β production in culture supernatants was measured by ELISA. Shown is one of three experiments with three mice per experiment. Unlike letters denote significance ($p < 0.01$) by one-way ANOVA with a Bonferroni posttest, and error bars represent SD.

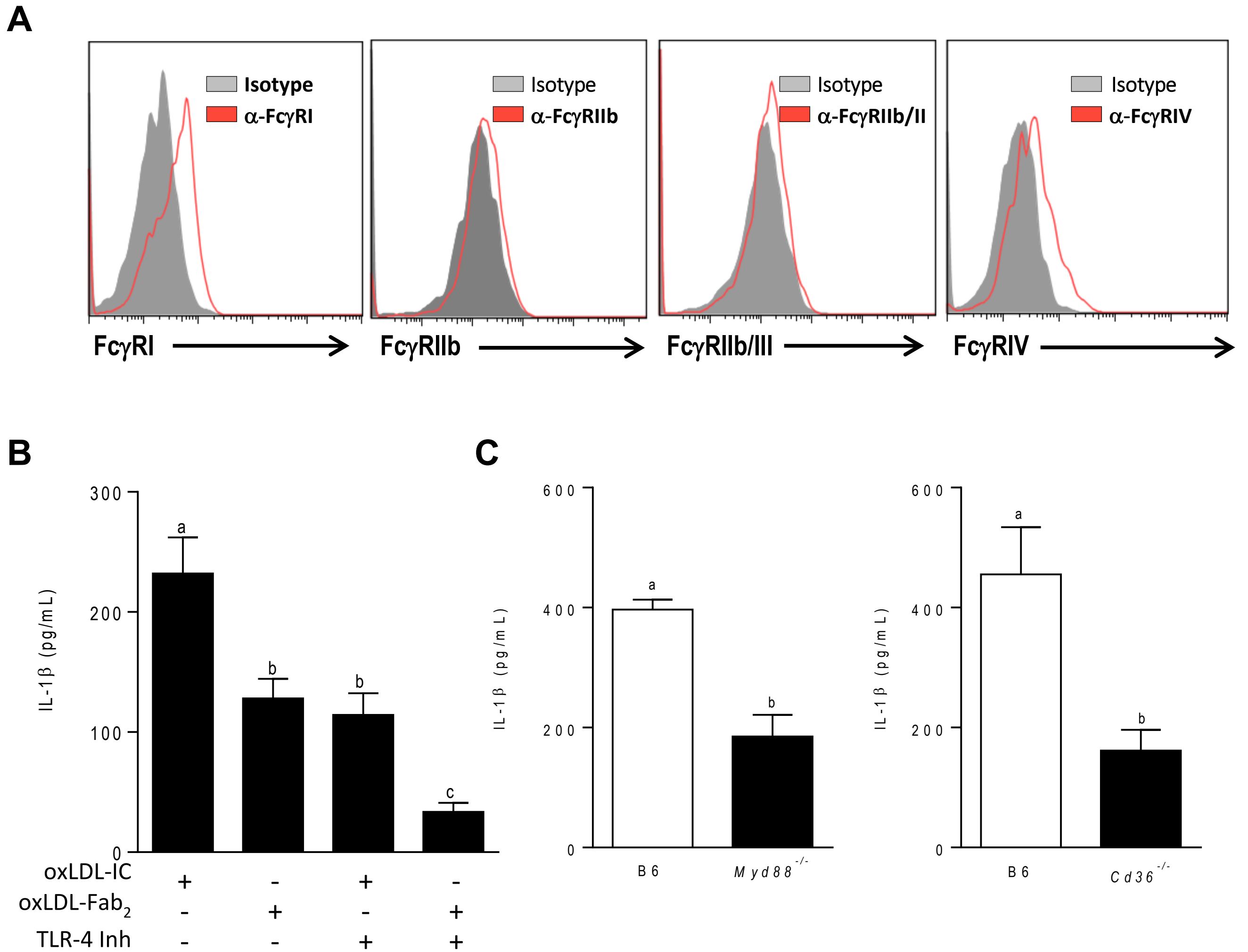

Figure 2.6. OxLDL-ICs elicit IL-1β production via FcγR, TLR4, and CD36. (A) Surface expression of FcγRs on BMDCs was measured by flow cytometry. Shown is one representative of three experiments. (B) BMDCs were treated with the TLR4 inhibitor CLI-095 prior to treatment with oxLDL IC or oxLDL Fab2 for 3h and ATP for an additional hour. Culture supernatants were tested for IL-1β by ELISA. Shown is one of three experiments with similar results. Unlike letters denote significance ($p < 0.01$) by one-way ANOVA with a Bonferroni posttest. (C) BMDCs from Myd88 -/- (left panel) and CD36-/- (right panel) mice (n = 3 per group) were treated with oxLDL or oxLDL ICs for 3 h, followed by ATP for an additional hour. IL-1b in culture supernatants was measured by ELISA. Unlike letters denote significance ($p < 0.01$) by Student t test, and error bars represent SEM.

receptor dependent fashion and suggests collaboration between FcγRs, TLR4, and CD36.

OxLDL-IC induce Syk phosphorylation downstream of FcγRs. After observing that BMDCs express high levels of activating FcγRs, and that BMDCs treated with oxLDL-Fab_2 produced lower levels of IL-1β similar to those elicited by free oxLDL (Figure 2.6B), I hypothesized that oxLDL-ICs may enhance IL-1β production by induce phosphorylation of Syk downstream of activating FcγRs. To answer this question, BMDCs were treated with oxLDL or oxLDL-ICs for 15 minutes and Syk phosphorylation was measured by phosphoroflow cytometry. Treatment of BMDCs with oxLDL-ICs increased levels of pSyk, however treatement with oxLDL did not result in Syk phosphorylation (Figure 2.7A left). To confirm that oxLDL-IC mediated Syk phosphorylation a direct result of FcγRs ligation, BMDCs were treated with oxLDL-Fab_2 or oxLDL-ICs. Like free oxLDL, oxLDL-Fab_2 also did not cause phosphorylation of Syk (Figure 2.7A middle). BMDCs were also treated with non-specific OVA-containing ICs (ova-ICs) as an additional control. While ova-ICs did caused Syk phosphorylation, it was slightly less than the levels elicited by oxLDL-IC treatment (Figure 2.7A right). It is important to note that although ova-ICs increased levels of pSyk, they did not induce increased IL-1β production (Figure 2.7B). This observation suggests that enhanced IL-1β production requires concomitant ligation of multiple receptors. Further confirming the need for engagement of multiple receptors, unbound anti-oxLDL also did not elicit IL-1β production from BMDCs (Figure 2.7B). Given that ligation of FcγRs can result in signaling through ERK to promote production of anti-inflammatory cytokines such as IL-10, I tested the possibility that oxLDL-ICs generally

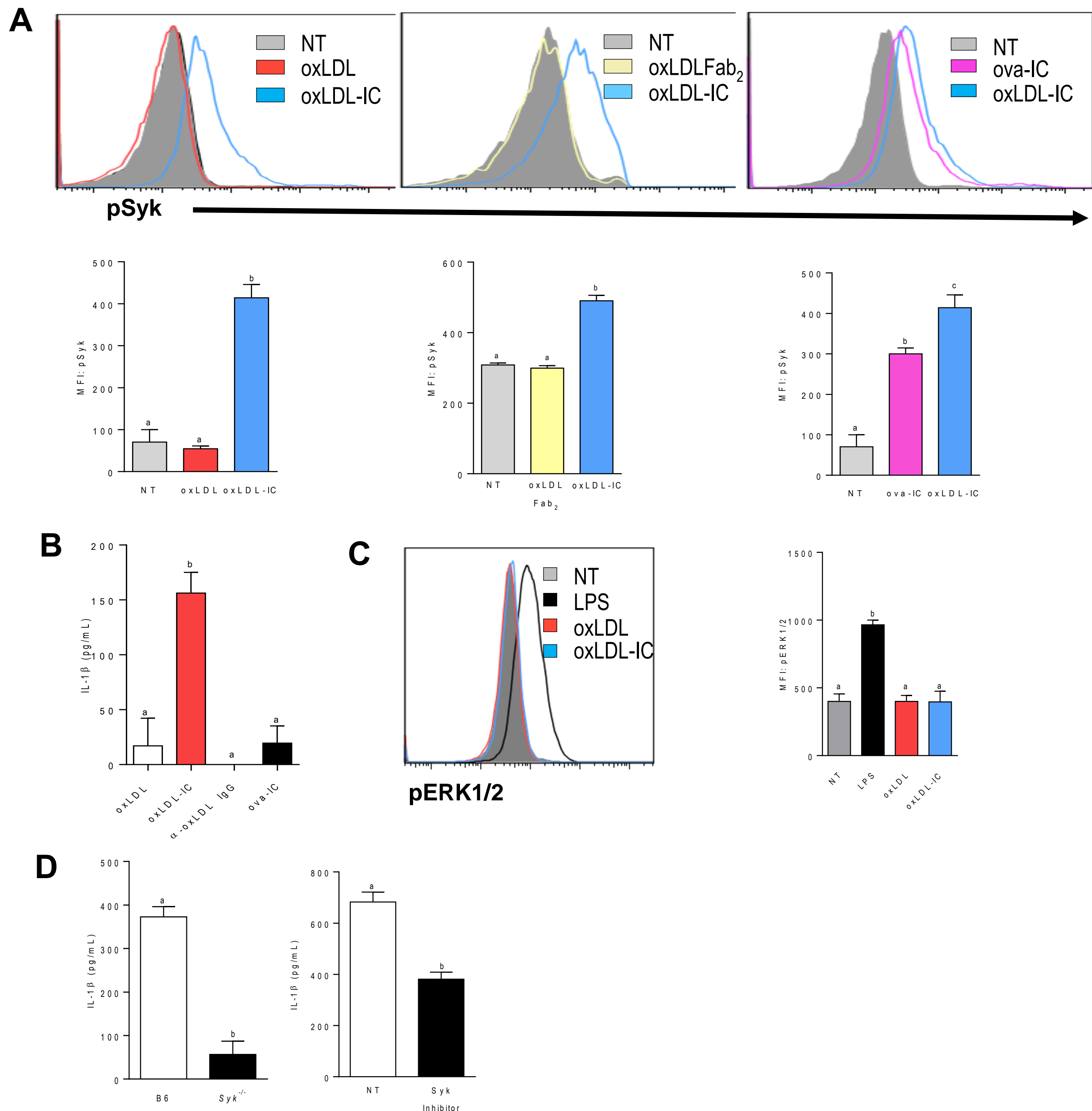

Figure 2.7. OxLDL ICs enhance IL-1β production via p-Syk. (A) BMDCs were incubated with oxLDL or oxLDL ICs, oxLDL-Fab2, oxLDL ICs, or ova-ICs for 15 min. Syk phosphorylation was measured by phospho-flow cytometry. Representative line graphs are shown (upper panels). n = 3 mice per group. Unlike letters indicate significance ($p<0.001$) by one-way ANOVA with Bonferroni posttest, and error bars represent SEM. (B) Cells were treated with oxLDL, anti-oxLDL, oxLDL ICs, or ova-ICs for 3 h, followed by ATP for an additional hour. IL-1β in culture supernatants was measured by ELISA. Shown is one representative of three separate experiments. Unlike letters denote significance by oneway ANOVA with a Bonferroni posttest, and error bars indicate the SD. (C) BMDCs were incubated with LPS oxLDL or oxLDL ICs for 15 min. Erk phosphorylation was measured by phosphoflow cytometry. A representative line graph is shown (left panel) and results are quantified by mean fluorescence intensity (MFI; right panel). n = 3 separate experiments. Unlike letters indicate significance ($p <0.001$) by one-way ANOVA with Bonferroni posttest, and error bars represent SEM. (D) B6 or Syk-/- BMDCs (left panel) or B6 BMDCs plus or minus the Syk inhibitor Bay61-3606 (right panel) were incubated with oxLDL or oxLDL ICs for 3 h, followed by ATP for 1h. n = 3 separate experiments. Unlike letters indicate significance ($p<0.05$) by the Student t test, and error bars represent SEM.

induce phosphorylation of all FcγR-associated kinases. However, treatment of BMDCs with oxLDL-ICs for 15 minutes did not produce detectable ERK phosphorylation (Figure 2.7C). The contribution of FcγR mediated Syk phosphorylation to oxLDL-IC mediate inflammasome activation was supported by the significant decrease in IL-1β production from oxLDL-IC treated BMDCs from *Syk*$^{-/-}$ mice and in the presence of a Syk inhibitor (Figure 2.7D). These data indicate that while Syk phosphorylation plays an important role in oxLDL-IC mediated IL-1β production, it is not sufficient to elicit enhanced levels of this pro-inflammatory cytokine.

OxLDL-IC-mediated inflammasome priming requires CARD9. Previous studies have shown that ITAM-coupled receptor and TLR signaling pathways converge on the adaptor protein CARD9, and CARD9-dependent inflammasome activation and resulting IL-1β production are critical for mounting an immune response to fungal pathogens(106–108). CARD9 is a key component of the CARD9-Bcl10-MALT1 (CBM) signalosome, a protein complex that facilitates translocation of NF-κB to the nucleus and production of pro-inflammatory cytokines (109). Although studies on fungal pathogens typically focus on the ITAM coupled receptors dectin 1 and 2, it is possible that oxLDL-ICs may also utilize the CARD9 pathway given that they bind to both TLRs and ITAM coupled FcγRs (104,105,107). To test if CARD9 is involved in oxLDL-IC-mediated inflammasome responses, wild-type and *Card9*$^{-/-}$ BMDCs were treated with oxLDL or oxLDL-ICs for 3 hrs followed by ATP for an additional hr in the presence or absence of either a TLR4 or Syk inhibitor. CARD9 deficiency had no effect on IL-1β responses to LPS priming (Figure 2.8A, left panel) or oxLDL-mediated IL-1β production (Figure 2.8A, right panel). Absence of CARD9 did, however, result in significantly decreased levels of IL-1β secretion from

oxLDL-IC treated cells (Figure 2.8A, right panel). As previously shown in figure 2.5A, pre-treatment of wild type BMDCs with a TLR-4 or Syk inhibitor reduced IL-1β levels b approximately 50% but did not completely abolish them, suggesting that that both TLR-4 and FcγRs are required for robust IL-1β production. Further confirming that oxLDL-IC signaling through these receptors converges on CARD9, pre-treatment of *Card9*$^{-/-}$ BMDCs with either TLR4 or Syk inhibitors completely ablated IL-1β production. This was accompanied by decreased expression of inflammasome genes *Il1a*, *Il1b* or *Nlrp3* in CARD9 knock-out cells treated with oxLDL-ICs, but not oxLDL (Figure 2.8B). Surprisingly, CARD9 deficiency did not affect production of IL-6 or TNFα (Figure 2.8C). These data confirm that oxLDL-ICs prime the IL-1β response by signaling through multiple receptors and converging on the adaptor protein CARD9.

OxLDL-ICs induce CBM complex formation and NF-kB translocation. To determine whether oxLDL-ICs signaling through CARD9 promoted formation of the CBM signalosome complex, BMDCs were treated for 2 hrs with oxLDL or oxLDL-ICs. Immunoprecipitation of whole cell lysates using antibodies to MALT1 and CARD9 showed that the entire CBM complex was formed when cells were treated with oxLDL-ICs, and not oxLDL alone (Figure 2.9A, left and middle). Interestingly, immunoprecipitation experiments with an antibody directed at Bcl10 resulted in detection of the entire CBM complex in both treatment groups. Although the entire CBM complex was pulled down in both treatment groups under this condition, the levels of MALT1 and CARD9 associated with Bcl10 were much higher in the BMDCs treated with oxLDL-ICs, suggesting that perhaps Bcl10 is rate limiting (Figure 2.9A, right). Given that CBM complex formation is associated with nuclear translocation of the transcription factor NF-κB, I next analyzed

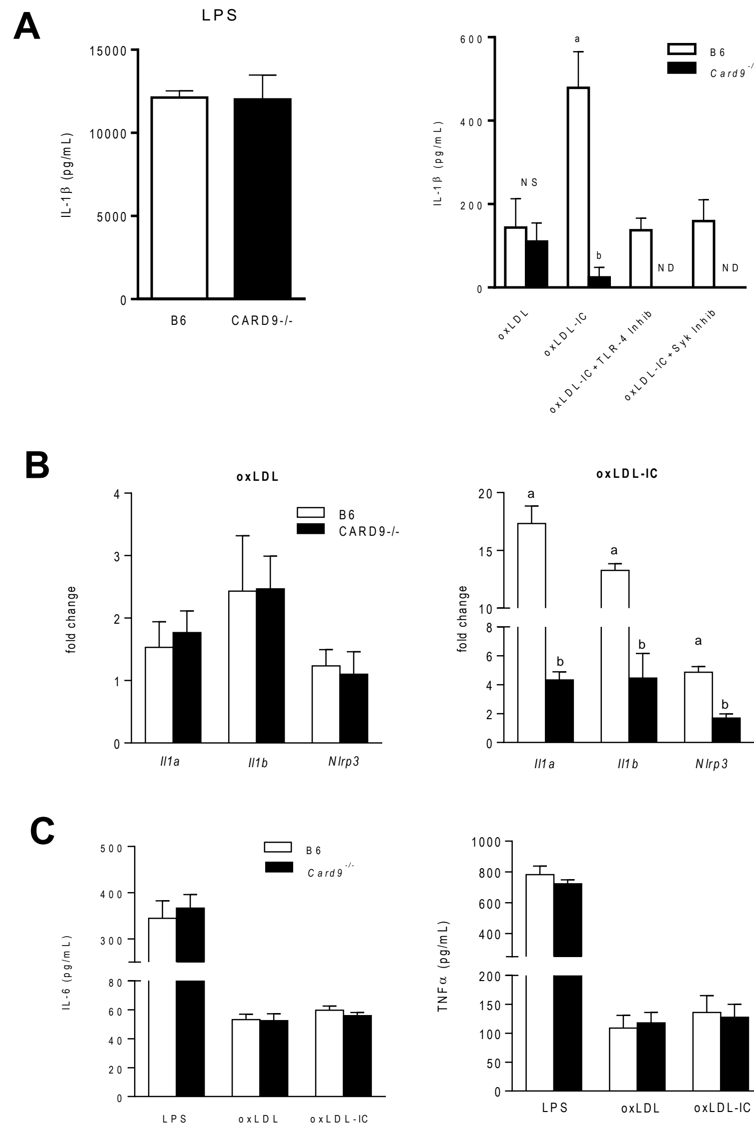

Figure 2.8. OxLDL-IC inflammasome priming is CARD9 dependent. (A) B6 and Card9-/- BMDCs were incubated with LPS, oxLDL, or oxLDL ICs in the presence or absence of a TLR4 or Syk inhibitor for 3 h, followed by ATP for an additional hour. IL-1β in culture supernatants was measured by ELISA (n = 3 mice per group). Unlike letters denote significance ($p < 0.001$) by the Student t test, and error bars indicate SEM. (B) B6 and Card9-/- BMDCs were incubated with oxLDL (left panel) or oxLDL ICs (right panel) for 2 h. Expression of inflammasome-related genes was measured using real-time RTPCR and expressed as $2^{-\Delta\Delta CT}$ compared with the no-treatment group (n = 3 mice per group). Unlike letters denote significance ($p < 0.01$) by one-way ANOVA with a Bonferroni posttest. (C) BMDCs from B6 and Card9-/- mice were incubated with LPS, oxLDL, or oxLDL ICs for 24 h. TNFα and IL-6 in culture supernatants were measured by ELISA. n = 3 mice per group; error bars represent SEM.

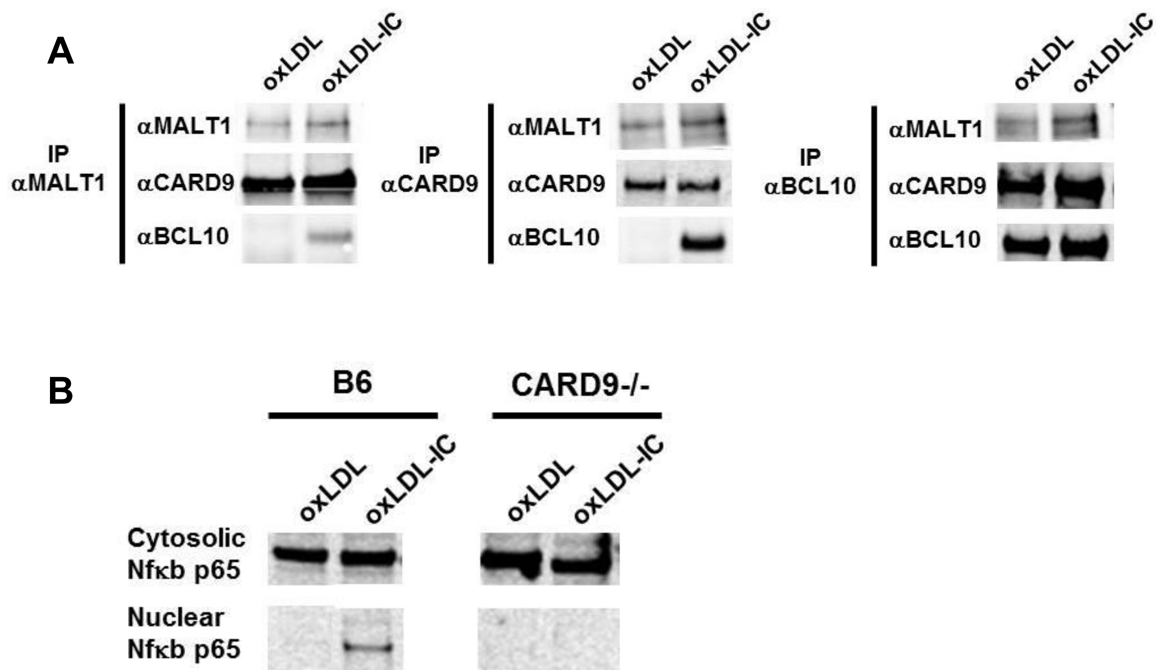

Figure 2.9. OxLDL-ICs cause CBM formation. (A) BMDCs were treated with oxLDL or oxLDL ICs for 2 h. Immunoprecipitation using Abs to MALT1, CARD9, and BCL10 was performed on whole-cell lysates, followed by Western blot analysis. Shown is one representative of three similar experiments. (B) BMDCs were treated with oxLDL or oxLDL ICs for 2h. Lysates were separated into nuclear and cytosolic fractions, followed by Western blotting for NF-κB p65. Shown is one of three representative experiments

levels of NF-κB p65 in the cytosolic and nuclear fractions of oxLDL and oxLDL-IC treated BMDCs. Unsurprisingly, OxLDL-ICs but not oxLDL induced nuclear translocation of NF-κB. However, nuclear translocation of NF-kB did not occur when *Card9*$^{-/-}$ BMDCs were treated with oxLDL-ICs (Figure 2.9B). Taken together, the results from figures 2.7 and 2.8

OxLDL-ICs exacerbate atherosclerosis and cause aortic dissection in vivo. In order to determine whether oxLDL-ICs played a role in atherosclerosis disease severity or were simply a biomarker, I injected LDLr$^{-/-}$ mice with oxLDL or oxLDL-IC and measured atherosclerotic lesions in the aortic root after 8 weeks on Western diet. Representative images of lesions in saline, oxLDL, and oxLDL-IC treatment groups are shown in Figure 2.10A. Mice that received oxLDL-IC (equivalent concentration of oxLDL) injections had significantly larger atherosclerotic lesions than mice that received injections of saline or oxLDL (Figure 2.10B). Interestingly, although oxLDL-IC injected mice had increased total lesion are, they did not have significantly more ORO staining, indicating that the lesions were cellular in nature (Figure 2.10C). Changes in lesion area were not a result of changes in total body weight, serum cholesterol, or serum triglycerides (Figure 2.10D). Analysis of Oil Red O staining revealed that some of the atherosclerotic lesions seemed to be growing into the adventitia of the vessel causing vascular remodeling. Trichrome blue staining of the collagen fibers confirmed breaks in the intraelastic lamina of the vessel, indicating that these lesions were causing aortic dissection. A model figure of a blood vessel can be found in Figure 2.11A. Aortic dissection causes plaque instability, rendering the plaque more likely to rupture and cause a cardiovascular even such as heart attack and stroke. Interestingly, 4 out of 7 mice treated with oxLDL-ICs showed remodeling of the vessel (57%), whereas only 1 out of 6 in both the saline and oxLDL

Figure 2.10. OxLDL-ICs increase Atherosclerosis. LDLr-/- mice were injected with saline, 10µg oxLDL, or 25µg oxLDL-IC every 2 weeks for 10 weeks. Mice were placed on Western diet one week after the first injection. Atherosclerosis in the proximal aorta was determined by Oil Red O Staining. Representative images are depicted (A) and quantification of staining is graphed in (B). Data are representative of 6-7 mice per group. (C) Post study levels of serums cholesterol and triglycerides and body weight. Error bars represent SEM and unlike letters denote significance ($p<0.05$) by one-way ANOVA with a Bonferroni posttest.

treatment groups displayed aortic dissection (17%) (data not shown). Representative images of Oil Red O and trichrome blue staining in the proximal aorta from a mouse treated with oxLDL-ICs are shown (Figure 2.11B, C). Both lesion cellularity and aortic dissection have been associated with plaque instability, rendering the plaque more likely to rupture and cause a cardiovascular even such as heart attack and stroke(111–113). Taken together these data conclude that oxLDL-ICs increased atherosclerotic lesion size and promote aortic dissection and plaque instability.

Discussion

A number of past studies have provided indirect evidence that oxLDL-ICs have pathogenic potential. Experiments performed in hyperlipidemic *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice deficient in activating FcγRI/III exhibited decreased atherosclerosis, while atherosclerosis-susceptible mice lacking the inhibitory FcγRIIb show strain-dependent increases or decreases atherosclerosis (54, 57, 58). Furthermore, Kyaw *et al.* demonstrated that IgG is pathogenic whereas IgM is protective(22–24). However, despite strong indications that oxLDL-ICs may play an important role in inflammation in atherosclerosis, the majority of studies understanding this immune response in focus on free oxLDL (31–33). Work from the Lopes-Virella lab has shown that oxLDL-ICs elicit production of inflammatory cytokines from human macrophages *in vitro*, however very little is understood about how oxLDL-ICs contribute to inflammation and if they have direct effects on atherosclerotic outcomes *in vivo*(41). I have provided evidence that oxLDL-ICs act directly on DCs (and macrophages) to cause increased activation marker expression

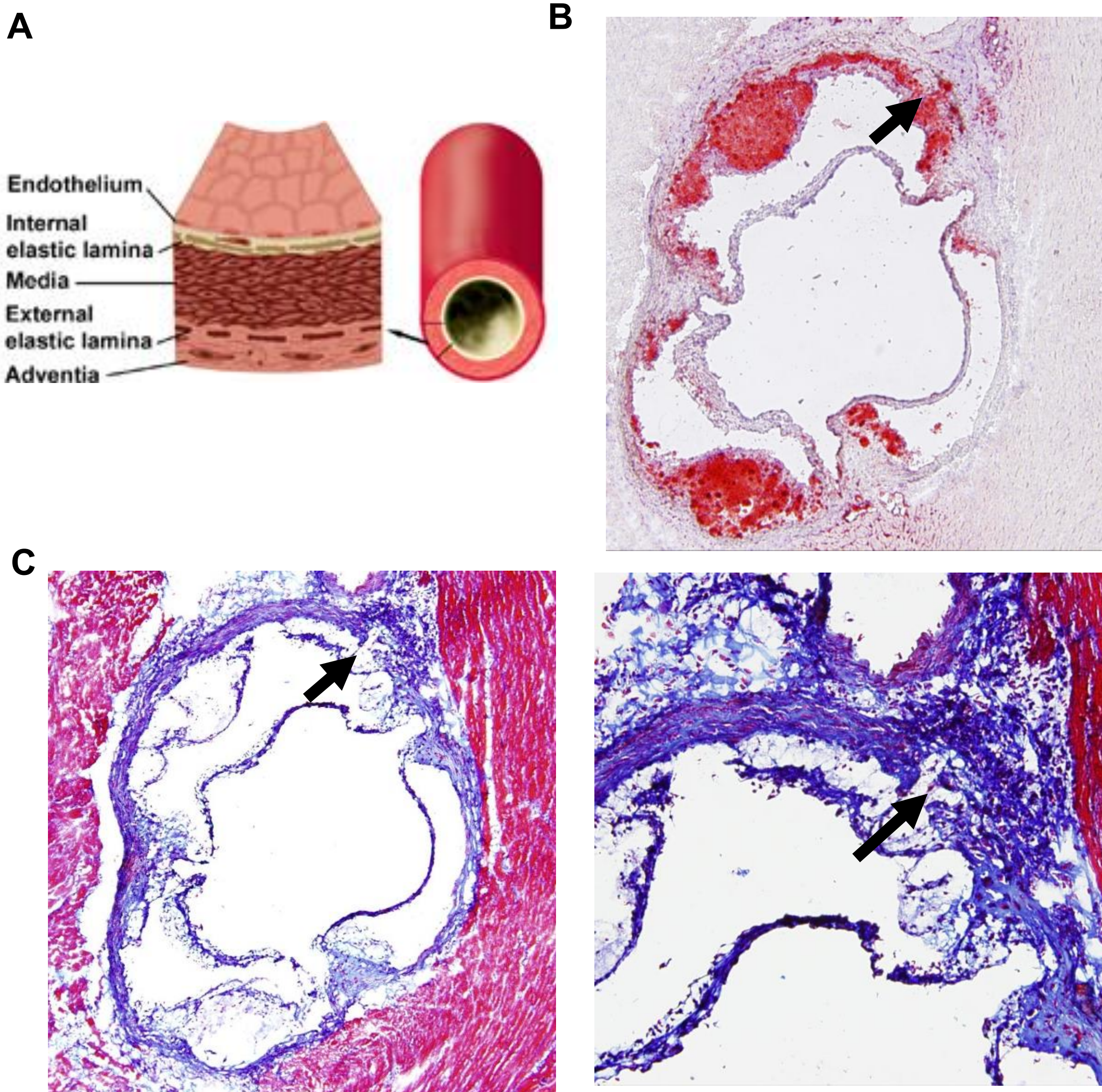

Figure 2.11. Aortic dissection in oxLDL-IC treated mice. (A) A reference diagram for the anatomy of an artery. Copyright © J. Norah. *Arterial Surgery of the Leg* (B) Oil Red O stained showing vascular remodeling (indicated with a black arrow) (C) Trichrome blue staining of the same aorta depicts a break in the intraelastic lamina denoted with an arrow at low (left) and high (right) magnification.

and differential cytokine production compared to oxLDL alone. This finding is novel and important as dendritic cells provide a link between the innate and adaptive immune response.

Furthermore, I have shown that oxLDL-ICs act as priming signals for IL-1β production and Nlrp3 inflammasome activation via FcγR, CD36 and TLR4. Previous work by Sheedy *et al.* elicited a role for oxLDL as an activating signal for the inflammasome (71). Entry of oxLDL into the cell was facilitated by a heterotrimer of TLR4/TLR6 and CD36. Following entry into the cell, oxLDL was able to act as an activating signal through the formation of crystals which resulted in lysosomal disruption. The authors of this study concluded that oxLDL was able to act as both a priming and activating signal for the inflammasome via TLR ligation and cholesterol crystal formation, respectively. Given that high levels of IL-1β were observed following 24 hrs of treatment with oxLDL-ICs (Figure 2.2A), it is likely that oxLDL-ICs are also able to act as both the priming and activating signal for the inflammasome even more efficiently than free oxLDL. Like oxLDL mediated inflammasome activation, oxLDL-IC priming of the inflammasome occurs in a receptor-dependent fashion. However unlike oxLDL, the primary mechanism of inflammasome activation is not cholesterol crystal formation. These conclusions are supported by data showing that 1) oxLDL-ICs enhance IL-1β production above oxLDL when used as a priming signal for the inflammasome; 2) IL-1β production is partially decreased by removing CD36, TLR4, or FcγR; and 3) oxLDL-ICs increase transcription of the inflammasome-related genes *Il1a*, *Il1b*, and *Nlrp3*. It is possible that oxLDL-ICs also act as an activating signal *in vivo* both by inducing cell death via pyroptosis resulting in the

release of cellular contents including ATP and by cholesterol crystal formation and lysosomal disruption following uptake via FcγRs.

The aforementioned study and others have shown that oxLDL induces inflammasome mediated IL-1β production from BMDMs; however, we were not able to detect IL-1β in BMDM supernatants under our treatment conditions (Figure 2.2B)(71, 117, 118). These different observations are likely related to both time and dose. Studies by Jiang *et al.* detected IL-1β production from BMDMs treated with increasing concentrations of oxLDL (25-200 μg/mL) for 12 hrs, choosing to do the majority of the experiments with 200 μg/mL of oxLDL (117). Similar work by Liu *et al*. used high concentrations of oxLDL (50-200 μg) for 24 hrs to look at production of IL-1β (118). The concentrations of oxLDL used in these studies elicit potent responses, however they are at the extreme upper limit of being physiologically relevant. To complete the studies presented in this chapter, I chose to use 10 μg/mL oxLDL to more closely mimic levels of circulating oxLDL *in vivo*. In addition to using higher concentrations of oxLDL, the studies by Jiang, Liu, and others stimulated cells for a minimum of 12 hours (117, 118). Long incubation periods allow time for the formation of cholesterol crystals which is the primary mechanism by which oxLDL activates the inflammasome. The studies presented herein were performed using a much shorter 3 hr incubation with the antigens in an attempt to tease apart the different mechanisms by which oxLDL and oxLDL-ICs induced IL-1β production.

Recently, Duffy *et al.* demonstrated that inactivated *Franciscella tularensis* (*F. tularensis*) opsonized with IgG activated the inflammasome in an FcγR/TLR dependent fashion (62). While this study did not directly demonstrate cross talk between these two

receptors, it is likely occurring given that TLRs and FcγRs are tightly clustered in glycoprotein microdomains (119). Although they do not bind to FcγRs, fungal antigens, such as those from *Candida albicans (C. albicans)*, have been shown to activate the inflammasome by binding to several other ITAM associated receptors including the C-type lectins dectin-1, dectin-2, and mincle (106, 108, 110). Binding of these antigens leads to recruitment and phosphorylation of Syk and further signal propagation resulting in the formation of a CARD9/Bcl10/MALT1 (CBM) complex and nuclear translocation of NF-κB (106). The study presented in this chapter shows that, like fungal pathogens, oxLDL-ICs utilize the CBM signaling pathway during sterile inflammation to enhance IL-1β responses in BMDCs. Surprisingly, increased CARD9-mediated NF-κB translocation did not result in increased production of TNFα or IL6, both of which are known transcriptional targets of NF-κB. There have been a handful of studies implicating CARD9 in increased TNFα production in models of fungal pathogenesis, and no reports connecting CARD9 signaling and IL-6 production to date (120–122). The studies examining CARD9 mediated TNFα production all required dectin-1 ligation. It is reasonable to hypothesize that the FcγR-CARD9 pathway is distinct from the dectin-1-CARD9 pathway and involves a phosphorylation or ubiquitination event that gives NF-κB greater affinity for the IL-1 promoter. In addition, it is also possible that TNFα levels are increased at an earlier or later time point given that they were only measured at 24 hrs. Greater understanding of the FcγR-CARD9 pathway in sterile inflammation is an area of continued interested and warrants further study.

In 2014, Janczy *et al.* published data that opposed the current study. The authors of this report concluded that IgG containing ICs inhibit inflammasome activation by LPS in BMDMs. These experiments were performed by priming BMDMs with LPS in the presence of ICs containing sheep's red blood cell, OVA, or *C. albicans.* Under these conditions, IL-1β production was decreased compared to priming with LPS alone (123). However, in my studies I observed that similar to BMDCs, BMDMs also exhibit enhanced IL-1β secretion in response to oxLDL-ICs (Figure 2.2). One possible explanation for the discrepancy between this work and our current study is that the specific antigen contained in the immune complex plays an important role in the immune response. My data indicate that oxLDL-ICs activate the inflammasome by binding to TLR4, CD36, and FcγR on DCs. There is no precedent for either sheep's red blood cell or OVA binding to pattern recognition receptors although *C. albicans* can bind both TLR2 and TLR4. Interestingly, LPS has been shown to upregulate the expression of the inhibitory receptor FcγRIIb on the surface of cells downregulating inflammation (124). Therefore, it is possible that pre-treatment with LPS decreases IC-mediated inflammatory cytokines due to increased FcγRIIb expression. Given that high levels of LPS are not found in sterile inflammation, the experimental system used in my studies may be more clinically relevant to diseases such as atherosclerosis, SLE and RA which are all associated with ICs containing molecules that can signal through both TLRs and FcγRs.

In this study I show that intravenous (IV) injection of oxLDL-ICs causes increased atherosclerotic burden *in vivo* (Figure 2.9). However, I do not provide evidence that this increase in atherosclerosis is a direct result of oxLDL-IC mediated inflammasome priming. Past studies by Finbloom and Plotz have shown that over 80% of IV injected immune

complexes are deposited in the liver as early as 1 hr after injection whereas approximately 2% stay in the blood up to 48hours (125, 126). Given that inflammasome activation is a very rapid innate immune response, it is feasible that this occurs in the blood prior to deposition of the immune complexes in the liver or that the small amount of IC that stays in the blood is able to perpetuate this immune response. The studies by Finbloom and Plotz were completed using heat aggregated IgG and ova-ICs, so it is important to determine if oxLDL-ICs are deposited throughout the body in a similar fashion. It is also possible that an adaptive immune response is responsible for the increase in atherosclerosis. A 2012 study showed that DCs in the liver travel to the liver draining lymph nodes (portal and coeliac) to activate CD4 and CD8 T cells, both of which have been implicated in atherosclerosis disease progression (127, 128). OxLDL-IC modulation of T cell responses will be discussed in chapter 3.

In conclusion, the current study demonstrates that oxLDL-ICs have the potential to enhance inflammation by priming the Nlrp3 inflammasome, and the molecular mechanisms by which this occurs are similar to those utilized pathogens and/or ICs formed during bacterial infections (62, 106). Collectively, the data suggest that while such responses may be beneficial during acute septic inflammation, IC-mediated production of cytokines such as IL-1β during chronic sterile inflammation are likely pathogenic (summarized in Figure 2.12). Finally, this study shows that oxLDL-ICs increase atherosclerosis *in vivo*. These findings identify an important contribution of oxLDL-ICs to both innate and adaptive immune responses that go beyond its previous recognition as a biomarker for atherosclerosis disease severity.

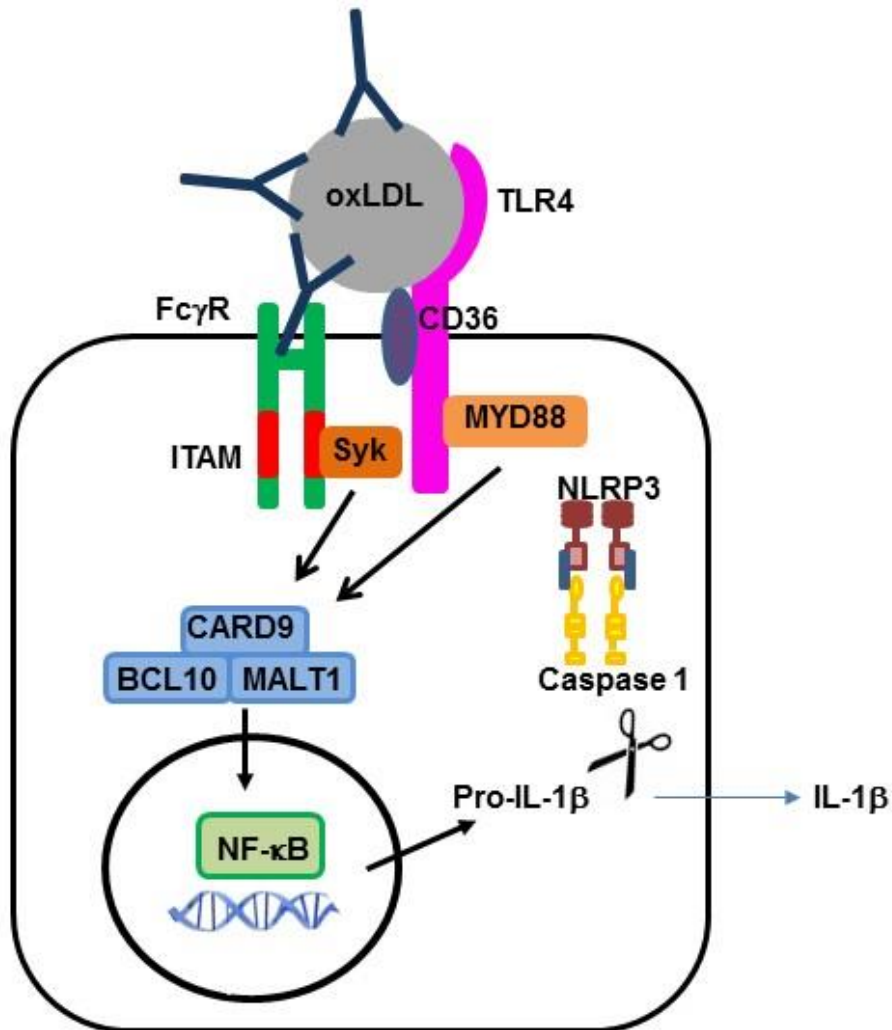

Figure 2.12. A proposed model for oxLDL-IC mediated inflammasome priming via CARD9-Bcl10-MALT1 (CBM) Complex formation. Briefly, oxLDL-ICs bind to multiple receptors on the suface of DCs. Signaling converges on the adaptor protein CARD9 resulting in formation of the CBM complex, resulting in nuclear translocation of Nfκb. This allows for transcription of pro-IL1b which is subsequently cleaved by caspase-1 following inflammasome activation.

CHAPTER 3

OxLDL Immune Complexes Promote Th17 Differentiation while Inhibiting IFNγ Responses.

Abstract

OxLDL-ICs are a prominent feature of atherosclerosis with important pathogenic potential. In Chapter 2, I discussed data supporting that oxLDL-ICs cause innate inflammation by priming the inflammasome and eliciting increased levels of inflammatory cytokines from macrophages and dendritic cells. However, it is currently unknown how oxLDL-ICs modulate the adaptive immune response. In this study, I provide evidence that oxLDL-ICs induceTh17 polarization via inflammasome mediated IL-1α and IL-1β. While enhancing IL-17 production, oxLDL-ICs simultaneously inhibit IFNγ responses through a Syk-dependent IL-23 mechanism. These findings are important given the important role of Th1 and Th17 T cells in atherosclerosis pathogenesis.

Introduction

OxLDL-ICs represent an important biomarker for atherosclerosis, as circulating titers of these ICs correlate with disease severity(25). While it has been shown previously that oxLDL-ICs enhance innate inflammatory responses in macrophages and DCs (32 and Chapter 2), it is currently unknown whether oxLDL-ICs can modulate adaptive immunity, and if so, what might be the mechanism(s). $CD4^+$ T cell responses are a critical component of adaptive immunity. Activation of these cells occurs by antigen presentation in the context of MHCII by an APC. The cytokine milieu generated by the APC in which antigen presentation occurs drives the $CD4^+$ T cell to a certain lineage (reviewed in 125).

IFNγ producing Th1 cells and IL-17 producing Th17 cells have both been implicated in the pathogenesis atherosclerosis. In general, Th1 cell differentiation is driven by the production of IL-12 from the APC, whereas Th17 differentiation occurs in the presence of IL-1, IL-6, IL-23, and TGFβ.

A pathogenic role for IFNγ in atherosclerosis is well established. IFNγ receptor (IFNγR) null mice crossed to the ApoE$^{-/-}$ background have a significant reduction in atherosclerotic lesion size accompanied by decreased lipid accumulation and lesion cellularity(130). Similarly, mice treated with a monoclonal antibody to IFNγ are protected from atherosclerosis(127). The contribution of Th17 cells to atherosclerosis pathogenesis is less clear. Studies have shown that there is enhanced expression of IL-17 secreting T cells both in the *in situ* in the aorta and systemically during early atherosclerosis development(132). Blockade of IL-17 in hyperlipidemic mouse models using both an adenoviral vector and a monoclonal antibody significantly improved atherosclerotic outcomes; however, genetic deletion of IL-17 enhanced lesion size(133–135). Studies by Taleb *et al.* and Gistera *et al.* also suggest a regulatory role for Th17 cells by enhancement of lesion stability and formation of a solid fibrous cap (136, 137).

Free oxLDL has been shown to mature dendritic cells, enhance T cell proliferation, and promote both IFNγ and IL-17 production *in vitro*(105, 138, 139). Clues from studies in FcγR$^{-/-}$ mice implicated a potential role for oxLDL-ICs in T cell differentiation and cytokine production, as well. Experiments performed in ApoE$^{-/-}$ mice deficient in the activating FcγRs reported increased levels of IFNγ and decreased IL-17, whereas ApoE$^{-/-}$ mice lacking the inhibitory receptor FcγRIIb have increased levels of IL-17(57, 140).

Given the pathogenic potential of IFNγ and IL-17 in atherosclerosis and the fact that that majority of oxLDL in circulation is complexed to antibody in immune complexes, it is important to determine how oxLDL-ICs modulate T cell polarization.

Materials and Methods

Mice. C57BL/6J (B6), B6N.129-Nlrp3$^{tm1Hhf/J}$ (*Nlrp3*$^{-/-}$), and B6.Cg-Tg (TcraTcrb) 425Cbn/J (OT-II) mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained and housed at Vanderbilt University. All mice used in these studies were on the C57BL/6J background. Procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

OxLDL and oxLDL-ICs. Human native low density lipoprotein was purchased from Intracel Resources (Frederick, MD) or Sigma-Aldrich (St. Louis, MO). OxLDL was made by dialyzing human LDL for 24 hrs against 0.9 M NaCl at 4°C with two buffer changes, followed by dialysis against 0.9 M NaCl containing 20 μM $CuSO_4$ for 4 hrs at room temperature. Oxidation was terminated by dialysis against 1 mM EDTA in 1X PBS for 16 hrs with two buffer changes. Extent of oxidation was determined by TBARS assay (Cell Biolabs, Inc., San Diego, CA). OxLDL-ICs were generated by incubating polyclonal rabbit anti-human apoB-100 (Alfa Aesar, Ward Hill, MA) with oxLDL at a ratio of 10:1 (500 μg of antibody, 50 μg of oxLDL) overnight at 37°C. Unbound antibody and antigen were removed by size exclusion filtration. For all experiments, immune complex concentrations were normalized based on oxLDL concentration to ensure that equal amounts of oxLDL were used in both the oxLDL and oxLDL-IC conditions. Fab_2 fragments were made using the Pierce Fab Fragmentation Kit (Thermo Fisher Scientific, Waltham, MA) according to

manufacturer's protocol. OxLDL enriched immune complexes were obtained from the serum of *Apoe*$^{-/-}$ mice fed Western diet (21% saturated fat, 0.15% cholesterol) for 12 weeks. Whole blood was obtained by retro-orbital bleeding. Serum was incubated with protein G beads for 1 hr at room temperature. Immune complexes were eluted from protein G beads and protein concentration was calculated by BCA assay according to manufacturer's instructions (Thermo Fisher Scientific).

Cell Culture. BMDCs were generated as previously described (102). Briefly, bone marrow from hind legs was flushed with RPMI-1640 (Corning, Corning, MA) supplemented with 10% FBS (Gibco, Grand Island, NY), 10 mM HEPES (Corning), and 1× Penicillin/Streptomycin/L-glutamine (Sigma-Aldrich) (hereafter referred to as TCM). Cells were plated in 100 mm^2 petri dishes at 2×10^5 cells/mL in TCM containing 20 ng/mL recombinant GM-CSF (R&D Systems, Minneapolis, MN). Media was replaced on days 3 and 6 and cells were harvested on day 9. To make BMDCs from various transgenic strains femurs were shipped overnight. *Cd11c*^{cre}/*Syk*$^{flox/flox}$, *IL1b*$^{-/-}$, and *IL1a*$^{-/-}$ femurs were obtained from Dr. John Lukens (University of Virginia, Charlottesville, VA).

Real-Time Quantitative PCR. BMDCs were treated with indicated stimuli for two hrs. Total RNA was isolated from cells using Norgen Total RNA Isolation Kits (Norgen Biotek Corporation, Thorold, Ontario, Canada). RNA concentrations were normalized and RNA was reversed transcribed with a high capacity RNA to cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). The reverse transcription product was used for detecting mRNA expression by quantitative real time PCR using the QuantStudio 6-flex System (Life Technologies, Grand Island, NY). The cycling-threshold (C_T) value for each

gene was normalized to that of the house keeping gene *Ppia*, and relative expression calculated by the change in cycling threshold method ($\Delta\Delta C_T$).

T cell Assays. For T cell assays, 50,000 BMDCs were incubated overnight with the indicated stimuli in round bottom 96 well plates. $CD4^+$ T cells were isolated from spleens of OT-II mice using the $CD4^+$ T cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's protocol and labeled with Cell Tracker Violet (Invitrogen, Gran Island, NY). 100,000 labeled $CD4^+$ T cells were added to pre-treated dendritic cells in the presence of 100 ng/mL $OVA_{323-339}$ peptide (Invivogen, Grand Island, NY) for 72 hours. Culture supernatants were collected for cytokine measurement and T cells were analyzed for proliferation and activation by flow cytometry.

Flow Cytometry. To measure T cell activation, cells were incubated for 15 minutes at room temperature with Fc-block (BD Bioscience) diluted 1:200 in FACS buffer containing HBSS, 1% BSA, 4.17mM sodium bicarbonate, and 3.08mM sodium azide. The following antibodies were diluted 1:200 and incubated with the cells for 30 minutes on ice: CD11c-FITC, CD62L-PE, CD4-PECY7, TCRβ-CD4, and CD44-APCCY7 (BD Bioscience). Cells were washed and re-suspended in 2% PFA for analysis on a MACSQuant seven color flow cytometer (Miltenyi Biotech) and data were analyzed using FlowJo Single Cell Analysis Version 7.6.5.

ELISA. IFNγ (BD Biosciences, San Jose, CA), and IL-17 (eBioscience, San Diego, CA) ELISAs were performed according to manufacturer's instructions.

Statistical Analyses. Where appropriate statistical significance was determined using a Student's *t* test. If more than two groups were compared, a one way Analysis of Variance (ANOVA) was used. In all cases $p<0.05$ was considered statistically significant.

Results

OxLDL-IC treated BMDCs skew T cells to a Th17 phenotype. In order to determine whether oxLDL-IC treatment of BMDCs affected antigen-specific T cell responses, BMDCs were treated for 24 hours with oxLDL or oxLDL-ICs then co-cultured with bead-purified splenic OT-II $CD4^+$ T cells in the presence of $ova_{323-339}$ peptide for an additional 72 hours. While there were no observed differences in T cell activation (Figure 3.1A) or proliferation (Figure 3.1B), analysis of cytokines in culture supernatants showed that oxLDL-IC treatment of BMDCs induced increased production of IL-17 from T cells but decreased IFNγ compared to oxLDL alone (Figure 3.1C). To test the effects of oxLDL-ICs on T cell responses to oxLDL itself, we utilized the T cell hybridoma clone 48.5 (a kind gift of Dr. Goran Hansson, Karolinska Institute, Stockholm, Sweden) that recognizes native apoB100 on LDL. Similar to Hermansson *et al.*, we observed that oxLDL induces less IL-2 production from the hybridoma compared to native LDL. Interestingly, oxLDL-ICs induced IL-2 secretion to nearly the level of native LDL (Figure 2D). This result suggests that perhaps oxLDL-ICs may be facilitating increased uptake and presentation of oxLDL-associated apoB-100 by DCs.

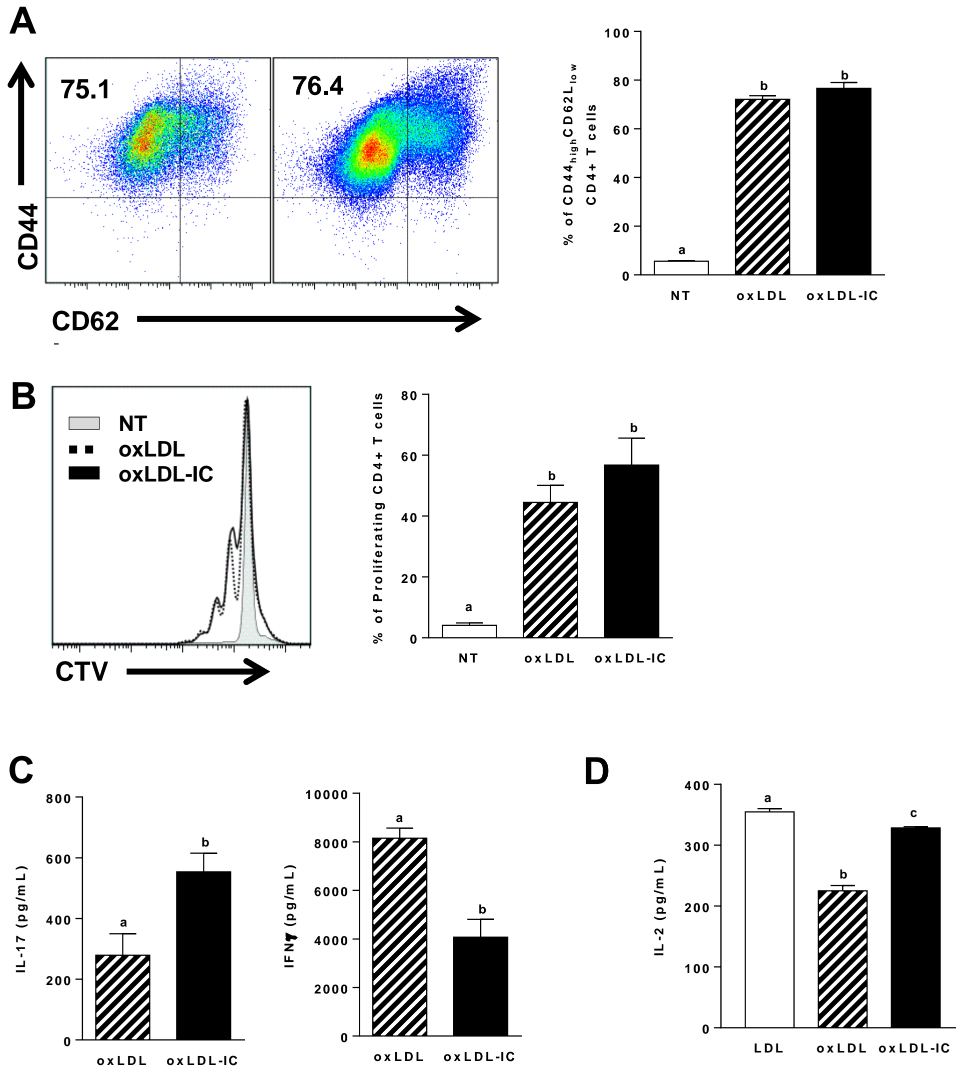

Figure 3.1. OxLDL-ICs elicit different T cell cytokine responses. (A-C) 10^4 BMDCs were treated with oxLDL or oxLDL-ICs for 24 hours followed by co-culture with 10^5 MACs sorted OT-II CD4$^+$ T cells and OVA peptide (50μg/mL) for 72hrs. (A) T cell activation was measured by expression of CD44 and CD62L on CD4$^+$TCRβ$^+$ cells. Representative dot plots are shown (left) and percent of Cd44^{hi}CD62L^{lo} cells are quantitated (right). (B) T cell proliferation was determined by Cell Trace Violet dilution. Shown is a representative histogram (left) and percent of proliferating cells is graphed (right). (C) IL17 (left) and IFNγ (right) were measured in culture supernatants by ELISA. n= 3 mice. All experiments were conducted 3 times. Unlike letters denote significance p<0.05 by One-way ANOVA for comparisons of more than two groups and Student's *t* test for comparisons of two groups. Error bars represent SEM. (D) D) 4x10^5 BMDCs were co-cultured with 10^5 T cell hybridoma cells (clone 48.5) for 24 hrs in the presence of 20ug/mL native LDL (positive control), oxLDL, or oxLDL-ICs. IL-2 in culture supernatants was used as a measure of T cell activation and was quantified by ELSA. All Experiments were conducted a total of 3 times. Unlike letters denote significance (p<0.01) by Student's *t* test. Error bars indicate SD.

OxLDL-ICs induce quantitatively and qualitatively different cytokine secretion from BMDCs compared to oxLDL alone. To determine the mechanism for differences in induced T cell response to oxLDL-ICs, cytokine levels in BMDCs following different stimuli were measured by qRT PCR. Results show that while oxLDL-ICs increased the expression of *il12* by two-fold over the no treatment group, *il23* mRNA levels were enhanced by an impressive 8-fold (Figure 3.2). This was approximately 2-times the amount of message elicited by oxLDL alone which seemed to favor *il12* expression (Figure 3.2). As expected, oxLDL-IC treatment caused a robust increase of IL-1β expression compared to the no treatment and oxLDL groups.

OxLDL-IC polarize BMDCs to promote Th17 responses via inflammasome dependent IL-1. To determine if oxLDL-IC inflammasome activation and IL-1β production played a role in T cell polarization, OT-II $CD4^+$ T cells were incubated with $Nlrp3^{-/-}$ and IL-$1\beta^{-/-}$ BMDCs pretreated with oxLDL or oxLDL-ICs. Loss of Nlrp3 resulted in abolished IL-17 production (Figure 3.3A, left panel), and absence of IL-1β reduced IL-17 by two-fold (Figure 2.3B, left panel). Interestingly, IL-$1\alpha^{-/-}$ BMDCs treated with oxLDL or oxLDL-ICs were not able to elicit strong Th17 responses (Figure 3.3C, left panel). $Nlrp3^{-/-}$ IL-$1\beta^{-/-}$, and IL-$1\alpha^{-/-}$ BMDCs pretreated with oxLDL-ICs induced similar levels of IFN-γ compared to B6 BMDCs (Figure 3.3, right panels). While these experiments provide evidence that IL1α and/or β mediates oxLDL-IC enhancement of Th17 responses, they do not provide an explanation for the suppression of IFNγ.

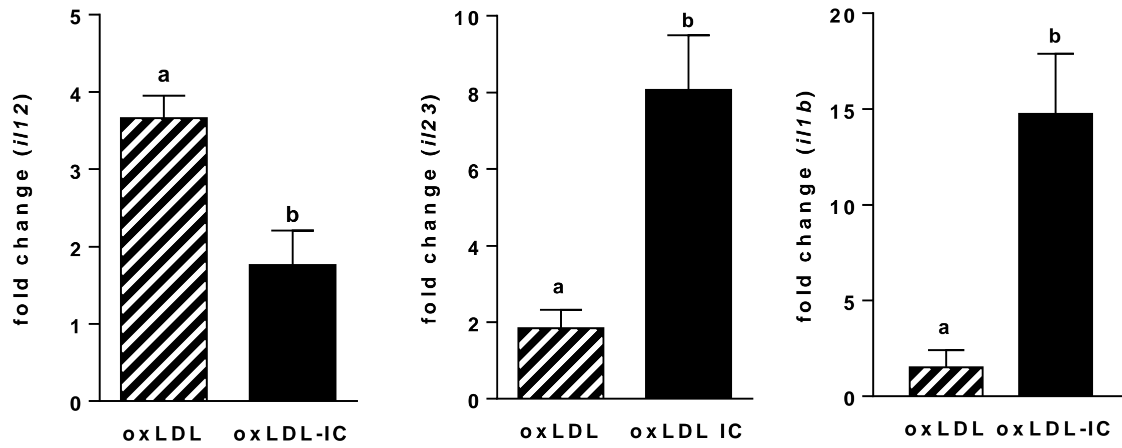

Figure 3.2. OxLDL-ICs induce production of Th17 polarizing cytokines. Quantitative real-time RT-PCR was used to measure the expression of *il1b, il23, and il12* and mRNA and quantification was completed using the ΔΔCT method. Shown is one representative of 3 experiments with three mice per experiment. Unlike letters denote significance ($p<0.01$) by Student's *t* test. Error bars represent SEM.

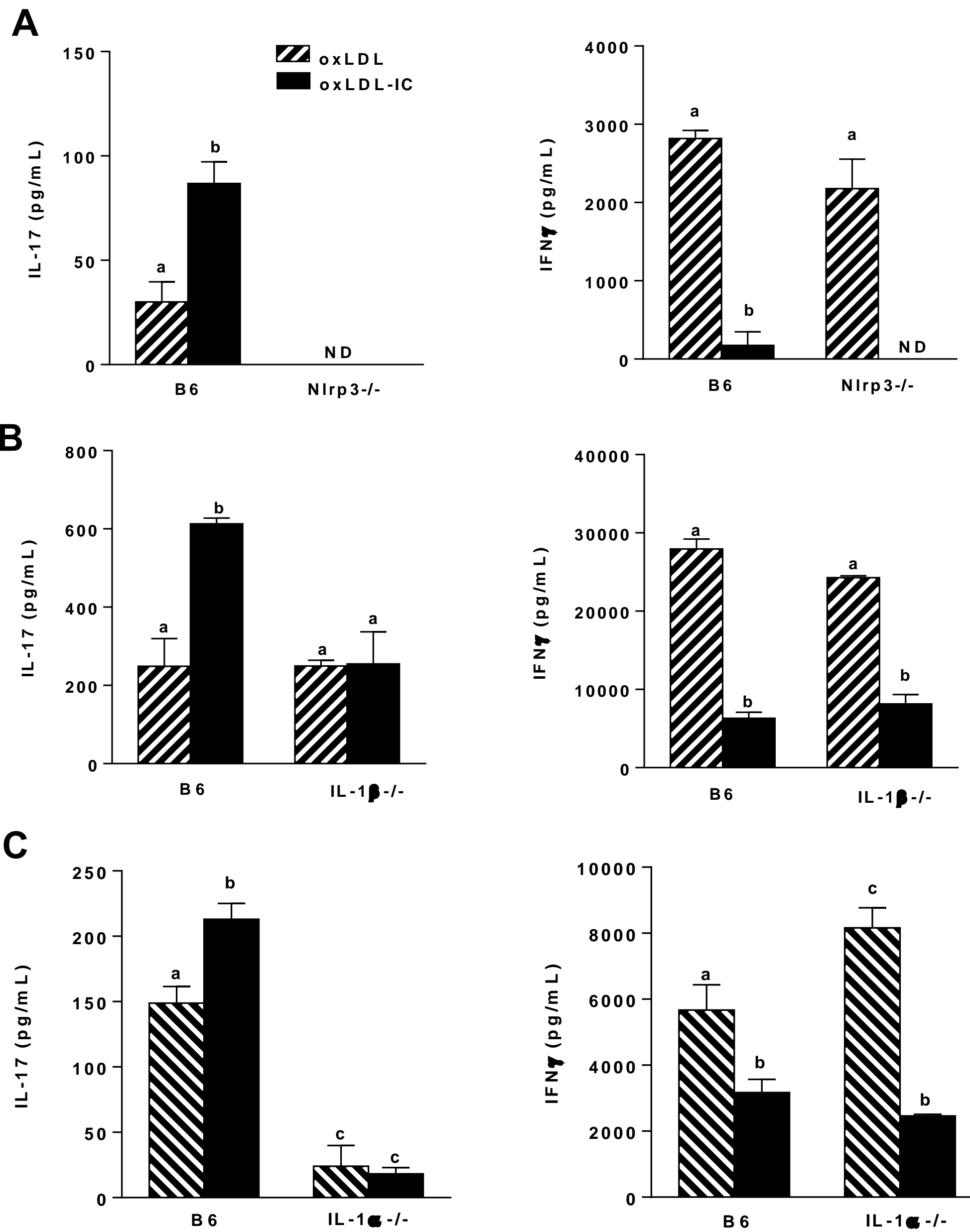

Figure 3.3. Inflammasome-mediated IL-1 regulates IL-17 production. 10^4 BMDCs were treated with oxLDL or oxLDL-ICs for 24 hours followed by co-culture with 10^5 MACs sorted OT-II $CD4^+$ T cells from (A) $Nlrp3^{-/-}$ (B) IL-$1\beta^{-/-}$ or (C) Il-$1\alpha^{-/-}$mice and OVA peptide (50μg/mL) for 72hrs. IL-17 (left) and IFNγ (right) were measured in culture supernatant by ELISA. Shown is one of three experiments with three mice per experiment. Error bars represent SEM. Unlike letters denote significance ($p<0.01$) by One-way ANOVA with Bonferroni post-test.

OxLDL-ICs signaling through FcγRs enhances IL-23 production in BMDCs via Syk phosphorylation. It was demonstrated in Chapter 2 that oxLDL-ICs signal through FcγRs and induce Syk phosphorylation. To determine if oxLDL-IC induced T cell responses were pSyk-dependent OT-II T cell experiments described in previous figures were repeated using $Syk^{-/-}$ BMDCs. Measurement of cytokines in culture supernatants indicated that pSyk plays a role in both IL-17 and IFNγ production as loss of Syk decreased oxLDL-IC elicited IL-17 production, while simultaneously increasing IFNγ production (Figure 3.4). Decreased induction of Th17 responses in the absence of Syk is likely due to decreased IL-1 production by BMDCs. This is supported by data demonstrating significantly decreased oxLDL-IC induced IL-1β production from $Syk^{-/-}$ BMDCs or BMDCs treated with a Syk inhibitor (Data shown in Figure 2.7D). A 2010 study by Sieve *et al.* defined a novel role for IL-23 as an inhibitor of IL-12 mediated IFNγ production in murine splenocytes leading to the hypothesis that Syk mediated IL-23 from DCs was suppressing IFNγ responses. Analysis of IL-23 and IL-12 expression in wild-type and $Syk^{-/-}$ BMDCs indicated that loss of Syk dampened oxLDL-IC mediated il23 expression (Figure 3.4B). To confirm this hypothesis, OT-II T cell experiments were repeated in the presence of an IL-23 neutralizing antibody. As expected, addition of the neutralizing antibody increased IFNγ production from T cells incubated with oxLDL-IC treated DCs (Figure 3.4C).

Discussion

This study identifies a novel role for oxLDL-ICs in the adaptive immune response. I have shown not only that oxLDL-ICs promote Th17 polarization in an IL-1 dependent manner, but also that these ICs inhibit IFNγ production from T cells through an IL-23

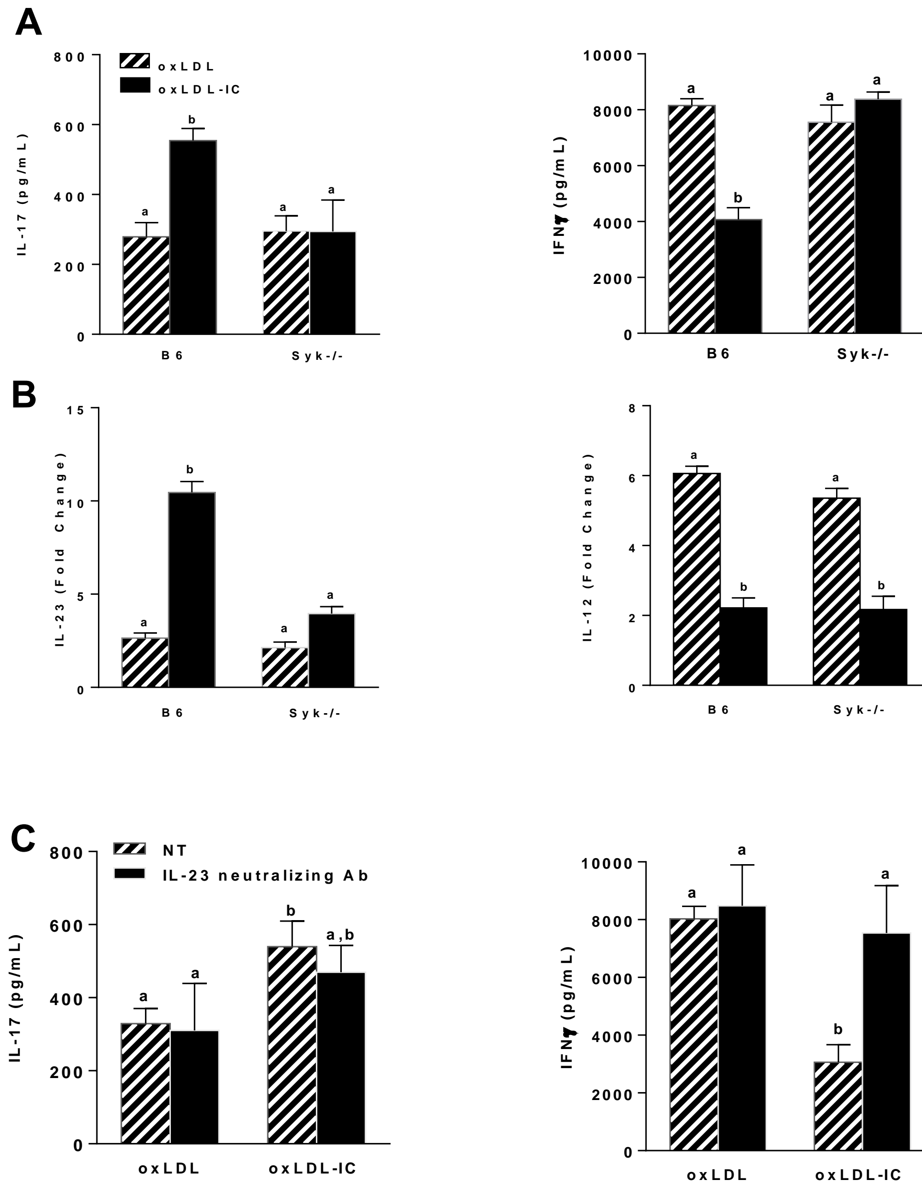

Figure 3.4. Syk Signaling mediates IFNγ suppression. (A) Wild type and Syk$^{-/-}$ BMDCs were treated with oxLDL or oxLDL-ICs for 24 hours followed by co-culture with MACs sorted OT-II CD4^{+} T cells and OVA peptide (50μg/mL) for 72hrs. IL-17 (top) and IFNγ (bottom) were measured in culture supernatants by ELISA. n=3 mice /experiment. Unlike letters denote significance ($p<0.01$) by Student's *t* test. Error bars represent SEM. (B) Wild-type and Syk$^{-/-}$ BMDCs were treated for 24 hours with oxLDL or oxLDL-ICs. Quantitative real-time RT-PCR was used to measure the expression of *il12* and *il23* mRNA and quantification was completed using the ΔΔCT method. n=3 mice/experiment. Unlike letters denote significance ($p<0.01$) by Student's *t* test. Error bars represent SEM.(C) Wild type BMDCs were treated as describe in (A) and co-cultured with MACs sorted OT-II CD4+ T cells in the presence or absence of an IL-23 neutralizing antibody. Shown is one experiment. Error bars represent SD and unlike letters denote significance ($p<0.05$).

mechanism. There is currently a large discrepancy in the field as to whether IL-17 is atherogenic or atheroprotective in nature. Although I have observed increased atherosclerosis in oxLDL-IC treated mice suggesting that Th17 polarization is pathogenic, I have not provided direct evidence that this adaptive immune response is the cause (133–137). However, given the prevalence of oxLDL-ICs in sterile inflammatory disorders and the importance of further understanding Th17 biology in the context of atherosclerosis, it is certainly an area that warrants further study.

A recent study by Ciraci *et al*. showed that ova IgG ICs suppress Th17 responses in an ova immunized mouse model through an IL-10 mechanism, begging the question as to whether the findings highlighted in this chapter would stand up *in vivo*. The authors noted that while the IL-17 production in their model required IL-1α, it was not dependent on inflammasome activation(141). However, in the studies presented herein, loss of the inflammasome gene *Nlrp3* completely abolished IL-17 production. This disparity is likely due to the antigen contained in the IC. I demonstrated in Chapter 2 that oxLDL-ICs have enhanced inflammasome activation due to the ability of the antigenic portion (oxLDL) to bind to multiple receptors on the cell surface. As OVA is not known to bind pattern recognition receptors, it is likely that ova-ICs elicit differential innate and therefore adaptive immune responses.

Interestingly, although IL-23 is known to be one of the cytokines involved in Th17 polarization, inhibition of IL-23 with a neutralizing antibody did not inhibit oxLDL-IC elicited IL-17 production. Previous studies have shown that IL-1β is the only cytokine required for commitment to the Th17 lineage, whereas IL-23 and IL-6 are involved in maintenance of the population (142–144). Another surprising observation in this study is that IL-1α played

a greater role in Th17 polarization than IL-1β as evidenced by the data presented in Figure 3.3. Despite the fact that it is a unique and equally potent cytokine, very little is known about the inflammatory potential of IL-1α as it is traditionally just considered a partner of IL-1β (reviewed in 142). Many studies directed at evaluating the role of IL-1 in mediating Th17 driven diseases such as arthritis utilize the IL1 Receptor (IL1R) deficient mouse(146, 147). However, as both IL-1α and IL-1β signal through IL1R, these studies do not tease apart the individual contribution of each of these cytokines. However, a 1991 study by Jacobs *et al.* demonstrated that injections of soluble IL-1α exacerbated the symptoms of Experimental Autoimmune Encephalomyelitis (EAE), another Th17 driven disease(148). A more recent study on the role of IL-1 in EAE showed that IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ mice develop EAE similar to wild type mice, but double knockouts were resistant to developing EAE(149). These studies in combination with the data presented in this chapter suggest an important role for IL-1α in Th17 pathology and highlight an important area of future study.

It has been shown that uptake of ICs by FcγRs contributes to the pool of presented peptides in the context of MHCI and MHCII(87–89, 150). This observation has been exploited by the field of cancer biology as a potential therapeutic option for generating tumor specific T cell responses(151, 152). While this study determines how oxLDL-ICs polarize $CD4^+$ T cells in an antigen-dependent fashion, it does not address whether oxLDL-IC internalization directly contributes to the pool of peptides presented to T cells. As it is known that pathogenic antigen specific T cell responses can be made to oxLDL, this is an area of continued interest.

In conclusion, I have demonstrated that oxLDL-ICs enhance antigen dependent Th17 polarization via inflammasome mediated IL-1β and, perhaps more importantly, IL-1α. In addition, oxLDL-ICs elicit IL-23 production via Syk signaling which acts to suppress IFNγ production (Figure 3.5). These findings are important given the important role of Th1 and Th17 cells in atherosclerosis, and have broader implications to other diseases of sterile inflammation characterized by high levels of circulating ICs.

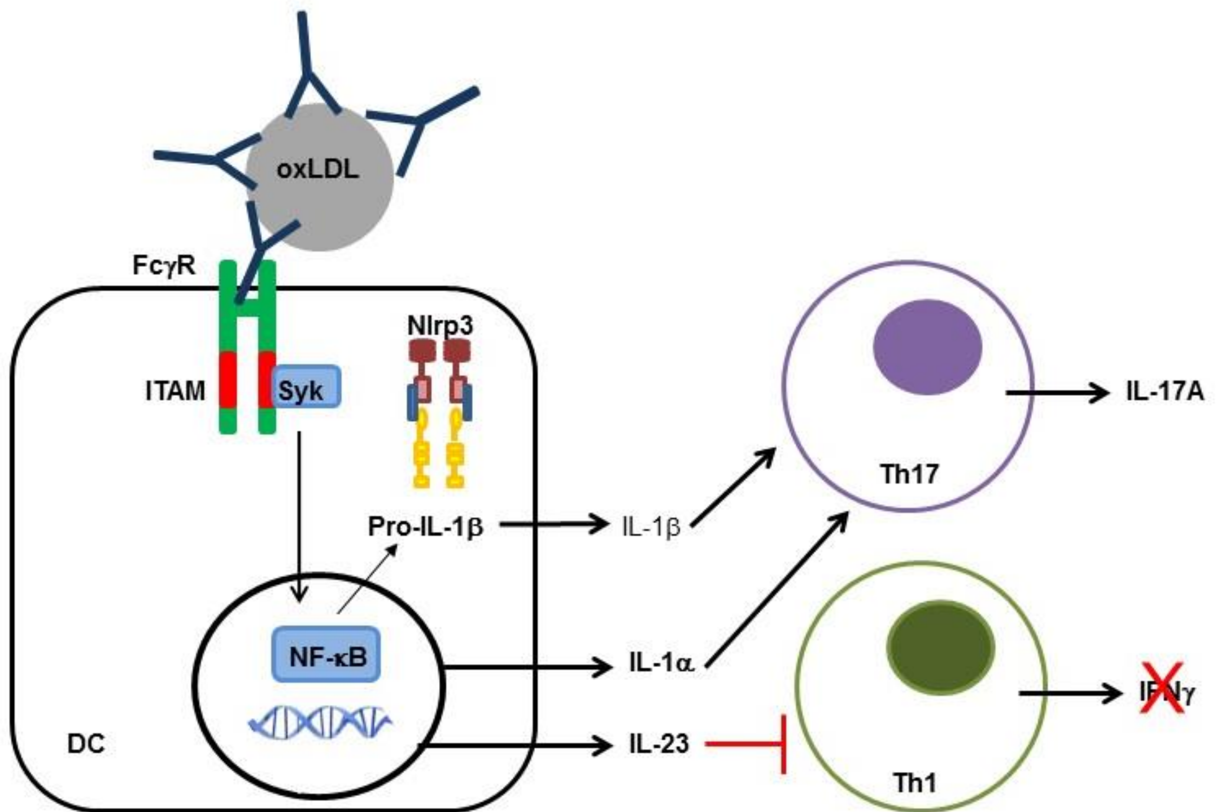

Figure 3.5. A proposed model for oxLDL-IC mediated Th17 polarization and IFNγ inhibition. OxLDL-ICs elicit the production of the Th17 polarizing cytokines IL-1band IL-23 from DCs. Inflammasome mediated IL-1 promotes Th17 responses, whereas IL-23 inhibits the production of IFNγ.

CHAPTER 4

General Discussion and Future Directions

Collectively, the data presented in this dissertation support the hypothesis that oxLDL-ICs play a mechanistic role in the immune response in atherosclerosis and are not simply a biomarker for disease severity. In Chapter 2 I demonstrate that oxLDL-ICs result in increased activation of DCs and elicit a differential cytokine profile than free oxLDL (Figure 2.1 and 2.2A). These findings are not unique to DCs, as oxLDL-IC treatment of BMDMs produced similar results (Figure 2.2B). I have provided strong evidence that oxLDL-ICs act as a potent priming signal for the Nlrp3 inflammasome as BMDCs treated with oxLDL-ICs prior to ATP treatment showed enhanced IL-1β production, and oxLDL-IC treatment induced transcription of inflammasome related genes *il1a*, *il1b*, and *nlrp3* (Figure2.4A and 2.5A). Interestingly, oxLDL-IC mediated inflammasome priming was unique of previously discovered mechanisms given that oxLDL-ICs did not cause cholesterol crystal formation (Figure 2.4B). These findings were confirmed as oxLDL-ICs did not elicit IL-1β production from *Nlrp3*$^{-/-}$ BMDCs and pretreatment with a caspase-1 or pan-caspase inhibitor significantly decreased oxLDL-IC mediated IL-1β (Figure 2.5B, D).

To prime the Nlrp3 inflammasome, oxLDL-ICs signaled through multiple receptors on BMDCs including FcγR, TLR4, and CD36. Contribution of these receptors was additive, as inhibition or absence of each one individually only partially decreased IL-1β production (Figure 2.6B, C). Analysis of untreated BMDCs demonstrated that

these cells expressed high levels of the activating FcγRs I and III (Figure 2.7A). Phosphoflow analysis of Syk following treatment with oxLDL-ICs but not oxLDL showed Syk phosphorylation (Figure 2.7A). Genetic and chemical inhibition of Syk decreased, but did not completely abolish oxLDL-IC mediated IL-1β production (Figure 2.7D). This finding not only confirmed a role for activating FcγRs in enhanced inflammasome activation, but also supports the necessity for multiple receptors. OxLDL-IC signaling through these receptors converged on the adaptor protein CARD9, a pathway previously implicated in fungal pathogenesis. Deletion of CARD9 in BMDCs drastically reduced oxLDL-IC mediated IL-1β production and transcription of *il1a*, *il1b* and *nlrp3*. OxLDL-IC signaling through CARD9 resulted in formation of the CBM complex that was not observed in oxLDL treated cells, and formation of the CBM complex resulted in nuclear translocation of Nfκb (Figure 2.9). Finally, in chapter 2 I provide evidence that oxLDL-ICs enhance atherosclerosis burden *in vivo*. Intravenous injection of oxLDL-ICs but not oxLDL or saline into LDLr$^{-/-}$ mice on Western diet increased total lesion area independent of serum cholesterol and triglyceride levels (Figure 2.10). Interestingly, oxLDL-ICs did not significantly increase lipid accumulation, indicating that the lesions are cellular in nature. These findings are summarized in Figure 2.12.

In chapter 3, I went on to demonstrate that oxLDL-ICs are also able to modulate the adaptive immune response. While oxLDL-IC treatment of BMDCs did not result in increased T cell activation or proliferation compared to oxLDL treated BMDCs, oxLDL-ICs treatment did result in different T cell cytokine profiles (Figure 3.1A, B). OT-II T cells incubated with oxLDL-IC treated DCs produced significantly more IL-17 than those co-cultured with oxLDL treated BMDCs (Figure 3.1C). However, while oxLDL-IC treatment

enhanced IL-17 production, it also resulted in decreased IFNγ production compared to treatment with oxLDL (Figure 3.1C). Consistent with this finding, BMDCs treated with oxLDL-ICs showed reduced levels of the Th1 polarizing cytokine IL-12 and increased levels of the TH17 polarizing cytokines IL-1β and IL-23 compared to oxLDL treated BMDCs (Figure 3.2).

Nlrp3 inflammasome mediated IL-1 was found to be responsible for enhanced Th17 polarization as BMDCs deficient in *Nlrp3*, *Il1b*, and *I1a* were not able to induce increased IL-17 production (Figure 3.3, left panels). However, IL-1 did not play a role in oxLDL-IC mediated IFNγ suppression as absence of these genes did not result in increased IFNγ production (Figure 3.3, right panels). Given that oxLDL-ICs, but not oxLDL signal through FcγR and Syk, I tested whether loss of Syk in BMDCs would result in increased IFNγ production when these cells were treated with oxLDL-ICs and cocultured with T cells. As expected, Syk deficient BMDCs treated with oxLDL-ICs elicited IFNγ levels comparable to oxLDL treatment (Figure 3.4A, right). Syk knock-out BMDCs showed decreased levels of IL-23 following oxLDL-ICs treatment (Figure 3.4B, left). Co-culture experiments in the presence or absence of an IL-23 neutralizing antibody confirmed that oxLDL-IC mediated IL-23 production was responsible for suppression of IFNγ responses. These findings are summarized in Figure 3.5.

Inflammasome priming is commonly associated with pathogens including both bacteria and fungi. LPS, a key component in the cell wall of many gram negative bacteria, is the canonical inflammasome priming signal by binding to TLR4(153). Fungal pathogens including *Candida albicans*, *Microsporum canis*, *Malassezia* spp,

Paracoccidioides brasiliensis, *Cryptococcus neoformans*, and *Aspergillus fumigatus* all prime the inflammasome via the ITAM-coupled dectin-1(96, 154–158). The findings presented herein are novel and important as oxLDL-ICs are the first sterile, endogenous ligands identified that are directly capable of priming the Nlrp3 inflammasome. Although TNF has been shown to license transcription of inflammasome related genes, this is not a direct effect as previous inflammatory events are required to initiate the production of TNF(159). Increased levels of oxLDL-ICs are observed in many sterile inflammatory disorders in addition to atherosclerosis including type 1 and type 2 diabetes, SLE, and RA, and many of these diseases are also associated with inflammasome hyperactivation (26, 42–44, 92–94). The observation that oxLDL-ICs can act as a priming signal for the Nlrp3 inflammasome identifies a novel mechanistic role for these ICs and provides an important link between oxLDL-IC titers and chronic inflammasome activation.

The studies presented in chapter 2 as well as those by Duffy *et al*. demonstrate that ICs containing an antigen capable of TLR binding elicit increased inflammasome activation compared to the TLR antigen alone due to the additive effects of FcγR and TLR signaling(62). Inflammatory diseases such as RA and SLE are also associated with ICs that have been shown to bind both to both FcγRs and TLRs(30, 60, 61). However, it is unclear whether these ICs are also able to act as a priming signal for the inflammasome. Because citrullinated fibrinogen containing ICs associated with RA were shown to bind both FcγR and TLR4 much like oxLDL-ICs, it is likely that they would evoke similar inflammasome responses. It is more questionable whether the double stranded DNA and single stranded RNA containing ICs would elicit robust

inflammasome responses. In the case of both oxLDL-ICs and citrullinated fibrinogen-ICs, TLR4 and FcγRs are both present on the cell surface, and it has been observed that these receptors cluster close together on glycoprotein microdomains allowing for concomitant ligation of these receptors by ICs. In the case of double stranded DNA and single stranded RNA, the cognate TLRs are found intracellularly within the endosome. Signaling through these TLRs would require prior endocytosis of the ligand containing ICs via FcγRs. Although both TLR7 and TLR9 have been implicated in inflammasome activation, it is unclear whether SLE associated ICs would induce the enhanced IL-1b responses seen with concomitant signaling through multiple receptors on the cell surface(160). Given the vast pathogenic potential of ICs in sterile inflammatory diseases, this represents an important area for future study.

The adaptor protein CARD9 has been well studied in fungal pathogenesis, however its role in sterile inflammation is unclear. Initial studies of the role CARD9 in fungal infection utilized zymosan, a β glucan found on the cell surface of many fungi that binds both TLR2 and the ITAM coupled receptor dectin-1(161, 162). These studies showed that innate immune responses to zymosan required CARD9 and formation of the CBM complex to promote Nfκb translocation to the nucleus and production of pro-inflammatory cytokines(109, 163). Follow up studies confirmed a role for CARD9 in Nlrp3 inflammasome priming and activation via Syk signaling downstream of the ITAM (106, 164). While initial studies indicated that FcγR crosslinking could not elicit inflammatory responses in the absence of CARD9, the focus of the Syk-CARD9 signaling axis has largely remained on dectin-1 due to its role in the immune response to fungal pathogens(109). To the best of my knowledge, mine is the first study to

provide direct evidence that ICs utilize this signaling pathway for inflammasome activation.

Activation of the Nlrp3 Inflammasome is required for development of atherosclerosis(101). Although I observed increased atherosclerotic lesion size in mice treated with oxLDL-ICs, it is currently unclear if this is a direct result of oxLDL-ICs signaling through CARD9 and resulting inflammasome activation. Given that oxLDL has also been shown to activate the inflammasome through an separate mechanism of cholesterol crystal formation and lysosomal disruption and my observations that oxLDL mediated inflammasome activation does not require CARD9, it is possible that CARD9 signaling is not required for atherogenesis *in vivo* (68 and Figure 2.7A). However, a recent study by Nemeth *et al.* demonstrated that neutrophil-specific deletion of CARD9 was protective against auto-antibody induced inflammation in a serum transfer model of arthritis, indicating that the IC signaling through CARD9 may play an important role in the pathogenesis of sterile immune diseases(165). Future studies evaluating the specific role of CARD9 in the development and progression of atherosclerosis are an important next step in these studies.

In addition to playing a direct role in innate immunity, CARD9 signaling in myeloid cells has been found to be important in adaptive immune responses, as well. Ligation of the c-type lectin receptor dectin-1 on DCs by the fungal pathogen *Candida albicans* has been shown to result in Syk-CARD9 signaling that leads to the production of robust levels of IL-6 and IL23 and promote Th17 responses(166). Binding of the same pathogen to dectin-2 on DCs also results in a Th17 immune response via Syk-CARD9 dependent production of IL-2 and IL-10(108). A glycolipid adjuvant for Mycobacterium

tuberculosis subunit vaccination has been shown to promote protective Th17 responses by activating macrophages and dendritic cells using the Syk-CARD9 pathway and formation of the CBM complex(167). An interesting follow up to this study determined that development of the protective Th17 response required inflammasome activation and IL-1β production(168). Thus, CARD9 represents an important link between the innate and adaptive immune response. Much like the aforementioned studies, in chapter 2 I demonstrate that oxLDL-ICs enhance IL-1 production through CARD9 mediated inflammasome priming and in chapter 3, I provide evidence that oxLDL-ICs enhance Th17 polarization in an inflammasome dependent fashion. Although it seems likely that CARD9 is a key intermediary given that levels of IL-1β were barely detectable in CARD9$^{-/-}$ BMDCs treated with oxLDL-ICs, direct evidence is required to confirm this hypothesis (Figure 2.7A).

The *in vivo* studies presented in figure 2.10 and 2.11 show that intravenous injection of oxLDL-ICs results in increased atherosclerotic lesion area compared to injection with saline or oxLDL, and these observed changes are independent of serum cholesterol or triglycerides. This finding is very exciting as oxLDL-ICs have long been considered a biomarker for atherosclerosis rather than playing an active role in disease progression. However, questions still remain including: 1.) Is oxLDL-IC enhanced atherosclerosis a direct result of inflammasome activation and/or Th17 polarization? 2.) Is the immune response to oxLDL-ICs occurring in the lesion or at a systemic site? A set of complementary studies by Finbloom *et al.* in 1979 demonstrated that nearly 80% of intravenously injected polyclonal IgG ICs deposited in the liver within 2hrs following injection(125, 126). Liver resident DCs have been shown to take up antigen with in the

liver and migrate to liver draining lymph nodes to elicit both $CD4^+$ and $CD8^+$ immune responses(127). Much like the *in vitro* observations with oxLDL-ICs, the parasitic pathogen *Schistosoma mansoni* elicits increased inflammasome mediated IL-1β production and antigen specific Th17 responses *in vivo*. This immune response occurs in the liver as that is the lodging site for *Schistosoma mansoni* eggs(110). The liver and the heart have long been linked, as Fatty Liver Disease greatly enhances the risk of cardiovascular complications(169). Although a direct connection has not been made between the immune responses in Fatty Liver Disease and atherosclerosis, patients with Fatty Liver Disease have increased levels serum levels of inflammatory cytokines, and it has been observed that liver resident macrophages called Kupffer cells enhance hepatic inflammation following up-take of oxLDL (170, 171). Taken together, this information strongly suggests that oxLDL-ICs may generate innate and adaptive immune responses within the liver that result in systemic inflammation and increased atherosclerosis. Future studies will determine the definitive site of oxLDL-IC deposition and how this directly contributes to atherosclerosis.

Figure 2.11 demonstrates that oxLDL-IC treatment causes aortic dissection Vascular remodeling in atherosclerosis is typically associated with Matrix Metalloproteinases (MMPs)(172). These proteins degrade extracellular matrix, and are involved in normal tissue turnover. However, over expression of MMPs results in poor cardiovascular outcomes. In humans, high levels of MMPs both in the lesion and in the serum as associated with plaque instability and likelihood of heart attack or stroke(173–175). Additionally, single nucleotide polymorphisms in MMP genes represents an independent risk factor for poor cardiovascular outcomes(176). In mice, MMP2 and

MMP9 are associated with advanced atherosclerotic lesions(177). Studies have shown that IL-1β can stimulate the production of MMP9 from a variety of cell types including macrophages (178–180). Furthermore, it has been demonstrated that Nlrp3 inflammasome activates MMP9 in smooth muscle cells(181). Given the potent ability of oxLDL-ICs to activate the Nlrp3 inflammasome, IL-1β mediated MMP9 activation represents a likely mechanism by which oxLDL-ICs are inducing aortic dissection. Future studies will determine the ability of oxLDL-ICs to stimulate MMP9 activity and prevalence of MMP9 within the atherosclerotic lesion of oxLDL-IC treated mice.

The studies presented in this body of work suggest that oxLDL-ICs increase atherosclerosis by inducing the production of IL-1 via an inflammasome and CARD9 dependent mechanism and oxLDL-IC mediated IL-1 skews T cells towards a Th17 phenotype. Thus, it stands to reason that IL-1 inhibition represents a potential therapeutic option in atherosclerosis. The soluble IL-1R agonists anakinra (Kineret) is currently approved to treat the disease RA (100). As previously mentioned, in addition to having ICs containing self-antigens, RA patients also have increased titers of oxLDL-ICs and enhanced cardiovascular disease (44). Thus it is possible that the enhanced IL-1β observed in RA patients are a result of circulating oxLDL-ICs, and that anakinra provides protective effects by inhibiting this immune response. A clinical trial using anakinra following myocardial infarction showed that this treatment improved cardiac remodeling decreased the prevalence of new onset heart failure (182). A similar study in mice using a monoclonal antibody to IL-1β produced similar results (183). Given that anakinra is already approved by the Food and Drug Administration for treatment of RA, it represents a potential therapy for atherosclerosis that is not years in the making. Longitudinal studies

on the cardiovascular outcomes of RA patients that have and have not received this therapy could provide insights as to whether it may protect against atherosclerosis. While much less established, CARD9 represents a novel therapeutic target for the treatment of atherosclerosis. In addition to the findings presented in this dissertation, it has been shown that genetic deletion of CARD9 in mouse models protects against cardiac fibrosis and high fat induced myocardial dysfunction (184, 185). In the case of cardiac fibrosis, protection was correlated with decreased macrophage infiltration and IL-1β production in the heart. Although the role of CARD9 in humans is little understood outside of fungal infections, it represents an important area of future study in sterile inflammation as well as a promising therapeutic target. One major barrier to the treatment of atherosclerosis is that many individuals do not know that they have it until they have a major cardiovascular event. It is important to determine whether IL-1β or CARD9 interventions can provide reversal of disease.

Overall, the work presented in this dissertation highlights an important pathogenic role for a molecule that was previously believed to be only a biomarker for atherosclerosis disease severity. I have shown that oxLDL-ICs are a sterile ligand for the Nlrp3 inflammasome and signal through multiple receptors on the cell surface. To prime the inflammasome, oxLDL-ICs utilize the CARD9 signaling pathway that has not previously been implicated in sterile inflammation. OxLDL-ICs also modulate the adaptive immune response by skewing T cells towards a Th17 phenotype while simultaneously inhibiting IFNγ responses. Finally, oxLDL-ICs increase atherosclerotic lesion burden *in vivo*. Future studies will focus on the direct contribution of CARD9 to atherosclerotic outcomes and

developing an understanding of exactly how these immune responses are playing into atherosclerosis development.

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