Molecular and Cellular Mechanisms Responsible for Low-Grade Stress and Inflammation Triggered by Super Low-Dose Endotoxin

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ABSTRACT

The gram-negative endotoxin, lipopolysaccharide (LPS), has been extensively researched in high doses (10-200ng/ml) and is well-documented in the literature for its ability to result in devastating effects such as multi-organ failure, sepsis, and septic shock. In high doses, LPS signals through Toll-like-receptor 4 (TLR4) and triggers a cascade of events culminating in the release of pro- and anti-inflammatory cytokines and the activation of NF-κB. In contrast, super-low doses of LPS (1-100pg/ml) are able to trigger the persistent release of pro-inflammatory mediators while evading the compensatory activation of NF-κB. This mild yet persistent induction of inflammation may lie at the heart of numerous inflammatory diseases and disorders and warrants studies such as this to elucidate the novel mechanisms. In this study, we explored the novel mechanisms utilized by super-low dose LPS in cellular stress and low-grade inflammation.

In the first study, the molecular mechanisms governing the role of super-low dose LPS on cellular stress and necroptosis were examined. We show that in the presence of super-low dose LPS (50pg/ml), the key regulators of mitochondrial fission and fusion, Drp1 and Mfn1 respectively, are inversely regulated. An increase in mitochondrial fragmentation and cell death which was not dependent on caspase activation was observed. In addition, super-low dose LPS was able to activate RIP3, a kinase

responsible for inducing the inflammatory cell death, necroptosis. These mechanisms were regulated in an Interleukin-1 receptor-associated kinase 1 (IRAK-1) dependent manner.

In the second study, the molecular mechanisms governing the role of super-low dose LPS on cellular stress and endosome/lysosome fusion were examined. In the presence of low-dose LPS (50pg/ml), endosomal-lysosomal fusion is inhibited and a loss of endosomal acidification required for the successful clearance of cellular debris and resolution of inflammation was observed. Additionally, super-low dose LPS induced the accumulation of p62 indicative of the suppression of autophagy. Tollip and Interleukin-1 receptor-associated kinase 3 (IRAK-M) appear to be critical regulators in this process.

Collectively, these studies show that low-dose endotoxemia is capable of causing persistent cellular stress, not observed in the presence of high-dose LPS (10-200ng/ml), and that it promotes necroptotic cell death while suppressing mechanisms necessary for the resolution of inflammation such as endosome-lysosome fusion. This research reveals novel mechanisms utilized by low-dose endotoxemia which could aid future efforts to develop prevention and treatment for various debilitating inflammatory diseases.

Dedication

I would like to dedicate this work to my son and my parents. To my dear, sweet Zyon, I hope that one day you will be able to look over what your mother has accomplished in life and know that nothing is impossible! Any dream that you may have, I hope that you know it is obtainable and with God and family you are more than equipped for any battles you may face. Thank you for being my hero and motivation and reminding me everyday how blessed I am!

To my parents, thank you doesn't even begin to describe my gratitude for all you have done and continue to do in my life. Thank you for being amazing role models and the backbone of my support system. I would not be here and able to do what I have done without you in my corner to keep my eyes on the prizes when times got tough and I doubted myself. You are the most amazing and selfless people I know and I hope that I continue to make you proud.

Last but not least, I would like to dedicate this to my Lord and Savior, Jesus Christ. You have walked with me even in times when I was too stubborn to know that you were there. Thank you for your grace, mercy, and undeserved favor over my life. I know that I am what I am because of you and I ask that you continue to order my steps.

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Preface/Attribution

The work presented in this dissertation is provided in the manuscript format and will be submitted for publication. This preface will highlight the contributions to each manuscript. In Chapter 2, I designed the study, developed the methodology, collected the data, performed the analysis, interpreted the results, and wrote the manuscript. Dr. Urmila Maitra and Dr. Shuo Geng contributed to data collection and analysis. Dr. Liwu Li provided invaluable intellectual contributions in experimental design and interpretation of results as well as writing the manuscript. In Chapter 3, I designed the study, developed the methodology, collected the data, performed the analysis, interpreted the results, and wrote the manuscript. Dr. Shuo Geng, Dr. Keqiang Chen, and Ruoxi Yuan contributed to in vivo data collection and analysis. Dr. Liwu Li provided intellectual contributions in experimental design and interpretation of results as well as writing the manuscript.

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Chapter 1: Introduction

Introduction

1.1 Framework

In the circulating blood plasma of individuals living a variety of chronic lifestyles, low levels of the bacterial endotoxin lipopolysaccharide (LPS) can be found. Overwhelming evidence in the literature implicates this low dose endotoxemia in the pathogenesis and progression of numerous debilitating diseases resulting in increased mortality worldwide. Low dose LPS (1-100pg/ml) is capable of inducing pro-inflammatory mediators at a subclinical level allowing cellular stress and inflammation to go undetected by the immune system. This phenomenon is in contrast to the robust yet transient expression of pro-inflammatory mediators induced by high dose LPS (10-200ng/ml). The mechanisms utilized by low dose LPS in causing the persistence of unregulated subclinical inflammation are poorly understood and warrant studies such as this to begin to unravel the mystery surrounding low-dose endotoxemia. In this review, we will cover the sources and pathological consequences of low as well as the current understanding of LPS signaling in the innate immune system.

1.2 Origins of Low-Dose Endotoxemia

1.2.1 Obesity/High Fat Diet

Obesity is an increasingly prevalent pandemic in the United States. Each day thousands of Americans are consuming diets high in fat. In addition to causing obesity, consumption of a high fat diet has been linked to numerous pathological conditions including heart disease, stroke, and type 2 diabetes. It has been shown that feeding mice a high fat diet for a short period is capable of increasing the circulating concentration of

plasma LPS 2-3 times above control levels and shifting the intestinal microbiota from predominantly Gram-negative to a Gram-positive environment, although reduction in the levels of Gram-positive bacteria were also observed. Specifically, there is a reduction seen in the number of the Gram-positive species, bifidobacteria [1]. That finding is of particular interest due to the involvement of these bacteria in the improvement of mucosal barrier function and the reduction of intestinal LPS [2, 3]. This research group went on to show further that by specifically increasing the gut bifidobacterial content of high fat diet fed mice through prebiotic supplementation they were able to restore normal endotoxemia levels, improve glucose tolerance, and decrease inflammatory tone [4].

The currently accepted theories for intestinal absorption of LPS from the gut into the circulatory system are chylomicron-facilitated transport and leakage through tight junctions in the epithelium. Chylomicrons function to transport dietary lipids to peripheral tissues and are synthesized by enterocytes in the intestinal epithelium. Interestingly, LPS is absorbed by enterocytes and transported to compartments where chylomicrons are stored before secretion [5, 6]. It has been shown that inhibition of chylomicron formation blocks LPS absorption in vivo while stimulating cells to promote chylomicron formation resulted in increased LPS secretion [7]. In high-fat diet fed mice, increased levels of inflammation and gut permeability have been observed in addition to increased levels of circulating endotoxin [8, 9]. Tight-junction integrity was able to be improved or restored by supplementing the mice with prebiotics or antibiotics indicating that the shift in gut microbiota and tight junction permeability could play a role in the intestinal absorption of LPS [4, 9]. Also, genetically obese mice have been shown to exhibit significant redistribution of their tight junction proteins as well as reduced

electrical resistance in the intestinal epithelium [8]. Taken together these data show that obesity or high-fat diet is capable of shifting the intestinal environment towards promoting and sustaining endotoxemic conditions and represents one of the most common origins of subclinical endotoxemia.

1.2.2 Alcohol Consumption

Chronic alcohol consumption in the United States is a growing concern with the number of self-reported drinkers constantly increasing. It is commonly known that frequent consumption of alcohol can result in alcohol-induced hepatic disease or alcoholic liver disease in which scarring of the liver and poor liver function can occur. Research has shown that chronic alcohol consumption is capable of diminishing mucosal integrity, allowing for vascular leakage of endotoxin into circulation. The damage caused to the mucosal membrane increases gut permeability to macromolecules facilitating the transport of LPS to the blood and increasing the livers exposure to the toxin [10-13]. By prolonging the liver exposure to endotoxin, endotoxemia plays a pivotal role in the pathogenesis and progression of alcoholic liver disease.

It has been shown that in patients with alcoholic liver disease, the concentration of circulating endotoxin was significantly elevated in comparison to patients with non-alcoholic liver disease and that the same was true when comparing healthy individuals to alcoholics. Interestingly, these elevated endotoxin levels were able to be reduced when alcohol was no longer consumed. A significant increase in gut permeability was also observed in alcoholics as compared to healthy individuals [14-16]. This further illustrates the role of alcohol consumption in predisposing an individual to endotoxemic conditions.

Chronic alcohol consumption has also been shown to cause bacterial overgrowth or changes in the bacterial populations of the gut further supporting the theory that gut-derived endotoxins may play a role in the initiation of this pathology [10, 11]. Collectively, these data show that the chronic consumption of alcohol is capable of allowing for vascular leakage and gives rise to endotoxemia and alcohol-induced hepatic disease.

1.2.3 Smoking

In today's society, it is now widely known and accepted that smoking has devastating effects on the lungs and immune system. It has been linked to numerous diseases and disorders including asthma, cancer, and cardiovascular disease just to name a few. Research has begun to show that there is a strong link between bacterial endotoxin and active and passive smoking. In 1999, it was shown that LPS is an active component of cigarette tobacco and that 75-120ng/cigarette of biologically active LPS could be derived from cigarette smoke [17]. It is possible that the LPS acquired in the lungs via cigarette smoking or environmental tobacco smoke, also known as second-hand smoke, is capable of being absorbed into systemic circulation in low doses by breaking down the barrier defense provided by the lungs [17, 18]. Building on this foundation, Larsson et al went on to show that individuals who inhaled environmental tobacco smoke consumed LPS in amounts up to 120 times greater than those in tobacco-free indoor environments. Indoor exposure to cigarette smoke and its ability to increase the air concentrations of bacterial endotoxin highlight the need for limited exposure to indoor environmental tobacco smoke and hence the nation has seen an increase in the number and types of locations which now prohibit smoking [19, 20]. In addition to increasing LPS

concentrations in circulation and in the air, smoking and second-hand smoke have been linked to increased colonization by potentially pathogenic bacteria in humans [21, 22]. The bacterial metagenome of commercially available cigarettes showed that cigarettes themselves harbored a diverse range of gram-negative and gram-positive bacteria including several of which are potentially pathogenic to humans including *Bacillus, Klebsiella, and Pseudomonas aeruginosa* [23]. While there is limited information available concerning whether bacteria could definitively survive the smoking and burning process long enough to be inhaled and colonize the lungs of active and passive smokers it has been shown that *Mycobacterium avium* from smoked cigarette filters could be recovered [24]. This indicates that this particular bacterium was able to withstand the aerosolization and high temperatures of cigarette smoking and lends the question of what other bacteria may be capable of the same. Conjointly, this shows that smoking, whether actively or passively, exposes individuals to increased subclinical levels of LPS and may alter their immune functions through bacterial colonization.

1.2.4 Aging

In 1957, Denham Harman was the first to purpose a theory explaining the aging process known as the 'Free Radical Theory of Aging'. According to this theory, reactive oxygen and nitrogen species are generated in our bodies which induce pro-inflammatory mediators and chronic inflammation. In addition, chronic inflammation itself triggers the generation of reactive oxygen and nitrogen species creating a positive feedback loop which sustains and exacerbates the pro-inflammatory environment resulting in damage to cells and organs thus the act of aging [25]. Recent studies have shown that aging is accompanied by dysregulation of the immune response as well as chronic inflammation

resulting in various age-related diseases like atherosclerosis, Parkinson's and dementia manifesting as we become elderly [26-29]. This has led to the current belief that aging and age-related diseases are a result of persistent low-grade inflammation. In the macrophages of aged mice, decreased levels of Toll-like receptor (TLR) expression and function have been observed in comparison to those of young mice which could explain the subclinical levels of pro-inflammatory mediators able to go undetected by the immune response in elderly individuals [30, 31]. Also, increased levels of circulating endotoxin and elevated secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α, are observed in the elderly as opposed to young people further contributing to the loss of functional plasticity in macrophages [30, 32, 33]. Macrophages are not the only cell line believed to be affected by the dysregulation of the immune response observed in aging. In addition to the skewed pro-inflammatory environment created by the decline in the innate immune response, aged individuals are also deficient in their ability to initiate inhibitory feedback loops to limit pro-inflammatory cytokine production. Dendritic cells from aged individuals show a significant reduction in the production of the potent anti-inflammatory cytokine, IL-10 [34]. Research clearly supports the idea that persistent low-grade inflammation, like that caused by chronic low-dose LPS exposure, may be attributed to aging and thus the primary underlying cause of age-related diseases. The development of treatments or interventions which can prevent or delay the effects of low-grade inflammation may hold promise in improving the lifespan and quality of life for mankind.

1.2.5 Periodontal Disease

Periodontal disease is highly prevalent throughout the world affecting 50-90% of adults with varying severity. This disease causes inflammation in the tissues which support and surround the teeth. The mild form of periodontal disease known as gingivitis is found in almost all adults and is easily reversible with proper oral hygiene while severe periodontal disease may result in impaired mastication and loss of teeth. Periodontal disease is caused by pathogenic microflora in dental plagues or biofilms formed adjacent to or on the teeth respectively [35]. Research has shown that mechanisms, such as gentle mastication and brushing of the teeth, which can cause small legions in the mouth release bacterial endotoxin into the bloodstream and that these concentrations increase with the severity of periodontal disease [35-37].

A study completed in 2012 showed that even periodontal therapy was capable of elevating the levels of LPS in circulation of women with periodontitis. A group of women with chronic periodontal disease were selected and the levels of circulating endotoxin were measured pre- and post-scaling and root planing, which is an accepted treatment for periodontal disease. The results showed that there was a significant increase of LPS in circulation immediately following this intervention [38]. Together these results show that periodontal disease is capable of causing sustained release of bacterial endotoxin into the blood stream and highlight its role as a major source of persistent low-grade inflammation. Interestingly, tobacco and alcohol use, which have previously been discussed as origins of circulating LPS, are capable of causing periodontal disease further exacerbating endotoxemia and promoting the pathology of various diseases and disorders [35]. In support of this theory, periodontal disease has been implicated in the pathogenesis of numerous life-threatening conditions such as diabetes, atherosclerosis,

arthritis, and preterm delivery and low birth weight [39-43]. Given these points, it is quite clear that periodontal disease plays a significant role in low-grade inflammation and endoxtemia as well as the maintenance of systemic infections.

1.3 Pathological Consequences of Low-Dose Endotoxemia

1.3.1 Parkinson's Disease

Millions of people worldwide are living with Parkinson's disease with alarming numbers of new diagnoses being given each year. This debilitating disease is an age-related neurodegenerative disorder distinguished by loss of movement, rigidity, tremors at rest, and postural instability accompanied by several non-motor features [44, 45]. Numerous famous faces battling the disease, including Michael J. Fox, Muhammad Ali, and Johnny Cash, have advocated and raised funds for research into the mechanisms and possible interventions of Parkinson's disease improving our understanding of the causes and consequences of this disorder. Research has shown that inflammation in the brain may play a role in the pathogenesis of Parkinson's disease. Microglia are specialized macrophages which play a crucial role in neuroinflammation by clearing cellular debris and becoming activated in response to perturbations in the microenvironment thus providing a defense mechanism for cells of the central nervous system. Activated microglia are responsible for the release of various pro-inflammatory cytokines including IL-6, TNF-α, and IL-1β and their sustained activation is believed to contribute to the significant loss and degeneration of dopaminergic neurons in the substantia nigra observed in the progression of Parkinson's disease [44, 46, 47].

Interestingly, of all the regions in the brain, the substantia nigra, which is located in the mesencephalon or midbrain, has the highest microglia density and it has been

shown that this region of the brain undergoes the highest degree of morphological change after LPS exposure [48]. In rats injected with LPS into the hippocampus, cortex, and substantia nigra, neurodegeneration and a significant loss of dopaminergic neurons were only observed in the substantia nigra. Also, mixed neuron-glia cultures from the hippocampus, cortex, and mesencephalon treated with LPS showed that the mesencephalic cultures were sensitive to LPS at levels of 10ng/ml and released pro-inflammatory mediators in a dose-dependent manner. In contrast, the cortical and hippocampal culutures remained insensitive to LPS at concentrations as high as 10μg/ml [49, 50] . This stark contrast could be attributed to the fact that mesencephalic culutures have significantly more microglia than cultures from other regions allowing for heightened LPS sensitivity and sustained microglia activation. The substantia nigra being targeted for neuronal degradation in Parkinson's disease coupled with the fact that it is the region densest in microglia and highly susceptible to immunological insult clearly indicate a role for LPS in inducing sustained microglia activation resulting in the neurodegeneration and clinical features observed in Parkinson's disease. Further understanding the mechanisms utilized by LPS to facilitate the degradation of dopaminergic neurons in the substantia nigra will aid in the development of treatments which can target the pro-inflammatory trigger in individuals suffering for this disease.

1.3.2 Obesity

As technology advances in today's society, we are living in an age where it is common for individuals to rarely be required to engage in physical activity. Couple the increasing conveniences of modern technology with the excessive fat-enriched portion sizes served in many homes and restaurants and there is the ideal recipe for obesity, an

increasing epidemic in the United States. Obesity is the result of an accumulation of excess body fat and the number of adults in America currently diagnosed as such is steadily increasing. Cardinal features of obesity are insulin resistance and low-grade inflammation which in tandem can contribute to the development of type 2 diabetes, hypertension, stroke, heart attack, and various other cardiovascular diseases [51, 52]. High fat diets have been shown to cause an increase in the concentration of circulating LPS due to increased vascular permeability. This rise in circulating LPS triggers the release of pro-inflammatory mediators which results in low-grade inflammation [1]. Adipocytes are the primary cell in the composition of adipose tissue or fat and this tissue is traditionally accredited for its role in energy storage. Adipose tissue acts as an energy reservoir releasing fatty acids for oxidation by other organs when food intake is minimal or scarce [53]. However with the discovery of the obese protein leptin and that it was secreted from adipocytes, the view of adipose tissue switched from solely energy storage to dual-roles including that of an endocrine/secretory organ [54]. In addition to leptin, adipose tissue was found to secrete a variety of cytokines and growth factors/hormones, including adiponectin, IL-6 and TNF-α [53, 55].

The production of pro-inflammatory mediators has been shown to be elevated in the adipose tissue of obese individuals and associated with the development of insulin resistance in obesity [56]. While protein secretion from adipocytes plays a major role in the inflammatory changes observed in adipose tissue, research has also shown that in obese individuals there is a significant infiltration of macrophages in the adipose tissue [57, 58]. This is of particular interest because of the role macrophages play in innate immunity and in skewing the microenvironment towards a pro- or anti-inflammatory

phenotype. Weisberg et al showed that adipose tissue macrophages are the primary source of TNF-α and other pro-inflammatory cytokines found in fat [58]. This drew attention to the pivotal role that TNF-α plays in promoting low-grade inflammation in obesity. In 2005, it was shown that co-culturing macrophages and adipocytes resulted in an upregulation of TNF-α and monocyte chemoattractant protein-1 (MCP-1) while downregulating the anti-inflammatory cytokine adiponectin [59]. Knowing that macrophages are the primary source of TNF-α in adipose tissue and that TNF-α stimulates the expression and secretion of MCP-1 can explain the significant infiltration of macrophages in adipose tissue as well as the sustained levels of pro-inflammatory mediators. This suggests a dangerous feedback loop where TNF-α is able to aggravate inflammatory changes in adipose tissue while simultaneously triggering the recruitment of additional macrophages to that site. Circulating levels of LPS found in obese individuals or those who consume a high fat diet are also capable of triggering the release of pro-inflammatory mediators particularly IL-6 and TNF-α which could exacerbate the inflammatory response and further sustain low-grade inflammation [1].

Additionally, free fatty acids (FFA) have been shown to be significantly increased in the circulation of obese individuals. FFA are known to induce inflammatory pathways, such as NF-κB and JNK, and potently induce expression of MCP-1 in adipocytes triggering increased macrophage recruitment to adipose tissue [60, 61]. This could further explain the low-grade inflammation observed in obesity. Interestingly, Lee et al discovered that a fatty acid component of LPS (lauric acid) was able to initiate TLR4 signaling in macrophages sparking a great debate about whether increased levels of FFA like those observed in obesity are able to induce TLR4 signaling and thus inflammation

[62, 63]. In 2006 it was shown that in isolated adipocytes from C57BL/6 mice, FFA induced the release of IL-6 and TNF-α however the secretion of these pro-inflammatory cytokines was not induced by FFA in *TLR4*-knockout mice [64]. TLR4 is the receptor for LPS where LPS binding activates the innate immune response via NF-κB culminating in the release of pro-inflammatory mediators. Taken together, this illustrates yet another dangerous feedback loop generated in obese individuals. Elevated levels of LPS and FFA in obese individuals are both capable of engaging TLR4 and triggering low-grade inflammation and the recruitment of macrophages to adipose tissue. This mechanism allows for sustained low-grade inflammation and can further aggravate the immune response.

1.3.3 Diabetes

Obesity is one of the most significant risk factors for developing Type 2 diabetes mellitus (T2DM). Type 2 diabetes is a chronic disease altering an individual's ability to properly utilize food as an energy source and it represents a rapidly growing problem across the United States costing our economy an estimated $245 billion in 2012. With T2DM being the most commonly diagnosed form of diabetes and the substantial economic burden it places on the nation, understanding the mechanisms which contribute to its development and pathogenesis is crucial [65, 66]. After consuming a meal, the hormone insulin is secreted by the β-cells of islets of Langerhans in response to increased levels of glucose in circulation. Insulin is responsible for regulating glucose homeostasis within the body by decreasing glucose output by the liver and increasing the uptake of glucose into muscle and adipose tissue. When the typical circulating concentrations of insulin are inadequate to maintain glucose homeostasis this is termed insulin resistance

[67, 68]. The early development and progression of insulin resistance has been shown to be triggered by obesity and high fat diet [1, 51]. For this reason many of the mechanisms utilized to induce low-grade inflammation are the same in this pathology as previously discussed. For example, adipose tissue secretes IL-6, TNF-α, and MCP-1 at levels which increase with adiposity while the opposite is true of the anti-inflammatory cytokine, adiponectin all of which contribute to the development of insulin resistance and persistent low-grade inflammation [56, 57, 59]. In addition to pro-inflammatory cytokines, obesity activates JNK and IKKβ/NF-κB and the inhibition of these pathways has been shown to effectively result in improved insulin sensitivity [69-71].

Obese individuals or those who consume high fat diets have increased levels of circulating LPS in their blood plasma and these elevated levels are capable of activating JNK and NF-κB, triggering the release of pro-inflammatory mediators and contributing to the sustained inflammation observed in obesity and T2DM [1, 72]. Reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress is also capable of activating the JNK and NF-κB pathways. Increases in adiposity have been linked to elevations in systemic markers for oxidative stress [73]. To further confirm the role of JNK and oxidative stress in insulin resistance, research has shown that use of the ROS inhibitor *N*-acetyl cysteine caused decreases in ROS levels and improved insulin sensitivity [74]. LPS has the ability to activate the JNK and NF-κB pathways via TLR4 and induces cellular stress and the generation of ROS [75, 76]. Collectively, this highlights the role of inflammation in obesity and the development of insulin resistance and displays how LPS plays a role in the initiation and exacerbation of the pro-inflammatory environment observed in those pathologies.

1.3.4 Atherosclerosis

Atherosclerosis is a prevalent disease characterized by the buildup of plaques in the arteries and represents one of the leading causes of death in the United States. The progression of this disease can result in numerous life-threatening complications including stroke, heart attack, and death. Low-density lipoproteins accumulate in the artery wall where they are modified by oxidative products to form oxidized LDL (oxLDL). The modified lipoprotein triggers the recruitment of macrophages which further oxidize LDL. The compound oxidation of LDL elicits a change in the way it is recognized, from recognition via the LDL receptor to recognition by the scavenger receptors [77-79]. This shift in receptor recognition allows for LDL to be taken up by receptors which are not mediated by the cholesterol levels within a cell and thus gives rise to cholesterol-rich macrophages known as foam cells, a hallmark feature of atherosclerosis [77, 80].

Innate immune signaling and macrophages plays a central role in the progression of atherosclerosis as early on as the initiation of the lesion. The accumulation of LDL triggers the immune response to release inflammatory cytokines and chemokines, such as MCP-1 which elicits the recruitment of macrophages to the site of the lesion that will ultimately uptake cholesterol and transform into foam cells [81]. Also, oxLDL itself will induce MCP-1 production by activated endothelium and therefore amplify the number of macrophages present. Macrophage foam cell formation causes a disruption in homeostatic cholesterol metabolism which includes the uptake and efflux of cholesterol. In healthy individuals, excesses of cholesterol are transported from the cells of peripheral tissues to the acceptors in the plasma, such as ApoE, for degradation by the liver in a

process known as reverse cholesterol transport [79, 82]. The conversion of macrophages into foam cells severely stifles this essential function of macrophages. The ATP-binding cassette transporter (ABC) ABCA-1 is responsible for trafficking cholesterol during reverse cholesterol transport and thus contributes to the prevention of foam cell formation [78, 79, 83]. Highlighting its importance in the progression of atherosclerosis, humans with a mutation in the ABCA-1 gene develop Tangier disease where an accumulation of cholesterol in macrophages is observed predisposing them to risk for cardiovascular disease [84]. It has been shown by our lab that subclinical doses of LPS, comparable to those observed in people who live chronic lifestyles or are obese, are capable of suppressing the expression of ABCA-1 and cholesterol efflux in macrophages via an IRAK1 dependent mechanism and thus promoting atherogenesis [85].

Atherosclerosis and its inflammatory links have also been extensively studied in obese individuals. In the obese state elevated levels of circulating LPS are observed which induce cytokine secretion in the adipocytes and infiltrating macrophages of adipocytes in adipose tissue [55]. One of these cytokines is transforming growth factor-beta (TGF-β) and it elicits the production of plasminogen activator inhibitor 1 (PAI-1). PAI-1 is the primary physiologic inhibitor of tissue-type plasminogen activator (t-PA) and this inhibition contributes to the development of cardiovascular disease and coronary thrombosis by promoting increased fibrin deposition within atherosclerotic lesions [86-89]. Interestingly, TNF-α, a pro-inflammatory cytokine whose secretion is induced by LPS, stimulates the synthesis of TGF-β which in turn triggers an increase in the production of PAI-1 observed in obese individuals.

The low levels of circulating LPS observed during high fat diet are also able to independently induce PAI-1 production as well as trigger the release of TNF-α generating a vicious cycle which maintains the elevated levels of LPS, TNF-α, and PAI-1 in circulation [88, 90]. Additionally, when adipocytes and macrophages were cultured together an increase in the expression of TNF-α and a decrease in the expression of adiponectin was observed [59]. Adiponectin is a cytokine secreted exclusively by adipocytes and has been shown to decrease lipid accumulation in foam cells as well as increase cholesterol efflux from macrophages [91, 92]. With increased LPS in circulation provoking the release of TNF-α and MCP-1 and thus promoting macrophage infiltration in adipose tissue, this emphasizes another role for LPS in the progression of atherosclerosis by down regulating the anti-inflammatory function of adiponectin. There are a variety of mechanisms used by LPS and the innate immune response in advancing and sustaining the progression of atherosclerosis however, for the purposes of this literature review we cannot cover them all in detail. The mechanisms we have discussed collectively show that low-dose endotoxemia stimulates a pro-inflammatory environment and promotes atherogenesis.

1.4 LPS/TLR4 Signal Transduction

Up to this point in our review, we have clearly seen that there are numerous sources of LPS in those who live chronic or adverse lifestyles and that the induction of inflammatory pathways by LPS plays a critical role in the pathogenesis of a variety of diseases which all have chronic low-grade inflammation as an underlying cause. Despite the physiological significance of low-dose enodotoxemia, the mechanisms have been poorly studied and are not thoroughly understood. For this reason, the next section of this

review will focus on the mechanisms of LPS/TLR4 signal transduction as they have been comprehensively studied in high dose LPS (≥100ng/ml) due to its ability to cause sepsis, septic shock, and death in humans.

1.4.1 Lipopolysaccharide/LPS Structure

Gram-negative bacteria represent a group which encompasses a number of human pathogens including *Escherichia coli*, *Haemophilus influenzae*, and *Bordetella pertussis*. In the outer membrane of the envelope surrounding Gram-negative bacteria, which maintains the shape and integrity of the organism, lipopolysaccharide or LPS is the predominant component of the outermost layer. LPS consists of a core oligosaccharide region, O-antigen repeats, and a lipid component designated lipid A [93-95]. The core region of LPS is divided into two distinct regions, the outer and inner core. The outer core contains the frequently encountered hexoses such as glucose, galactose, and glucosamine while the inner core consists of the LPS specific elements, heptose and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) [96, 97]. The distinct function of the outer core is still largely unknown while KDO of the inner core is essential to the biological activity of LPS as it serves to bridge lipid A to the core oligosaccharide and can up regulate lipid A's endotoxic activity [96, 98]. The composition of the core oligosaccharide is typically consistent in contrast to that of the O-antigen side chain which expresses vast diversity.

In pathogenic gram-negative bacteria, the O-specific chains which encounter the host cells during infection are antigenic and for this reason are also commonly referred to as O-antigen chains. These chains are composed of repetitive oligosaccharide subunits that differ in their length and branching structure. Gram-negative bacteria which utilize

these unique side chains synthesize smooth-type lipopolysaccharides while those lacking O-antigens produce the rough-type [96, 99]. The O-antigens function as a protection against host defense mechanisms and have been shown to eliminate the efficacy of antibiotics however, when detached from the lipid A component of LPS these side chains display no virulent activity [100, 101]. Lipid A or endotoxin is a highly conserved phosphoglycolipid and the most critical component of bacterial LPS as it is the biologically active moiety responsible for initiating immunological responses [102]. There are approximately 10^6 lipid A moieties/LPS residues on the surface of an *Escherichia coli* cell accounting for three-quarters of its cell surface [97, 103, 104]. Bound lipid A anchors LPS to the outer membrane of gram-negative bacteria and is not biologically active however, when bacteria multiply or die and lyse, LPS is freed from the outer membrane and represents a formidable bacterial toxin [104, 105]. This endotoxin plays a crucial role in signaling the immune system to eliminate local infections but, when high levels are found circulating systemically sepsis and septic shock can occur and research has focused on high dose LPS as a result [106, 107].

1.4.2 LPS Recognition

The recognition of LPS by the innate immune system begins with it being bound to LPS-binding protein (LBP) in the plasma. LBP is a serum protein acute-phase reactant with the levels in circulation significantly increasing after inflammatory challenge. Although the majority of LBP is derived from the liver, it can be synthesized locally and could explain the aggravated immune response in the presence of high doses of bacterial endotoxin [108-111]. LBP binds to LPS and catalyzes its transport from the plasma to membrane-bound CD14 (mCD14), soluble CD14 (sCD14), and phospholipids

culminating in the activation of the immune response or neutralization of LPS by high density lipoprotein (HDL). LPS is preferentially transported to CD14 prior to being moved to HDL for neutralization thus offering a compensatory mechanism to keep inflammation tightly regulated [112-114]. It has been shown that CD14 on macrophages bound red blood cells coated with both LPS and LBP but, not when individually coated with one or the other. Also, monocytes were able to respond to lower concentrations of LPS in a CD14-dependent manner when it was bound to LBP [115-117]. This highlights the role LBP plays in enhancing the sensitivity of cells to LPS and initiating its recognition by the immune response as well as the crucial role of CD14 as the receptor which contributes to facilitating this response. CD14 was one of the first recognized pattern recognition receptors (PRRs), which are responsible for recognizing pathogen associated molecular patterns (PAMPs) from Gram negative and Gram positive bacteria. CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein and as a result lacks transmembrane and intracellular domains. With its inability to traverse the cell membrane, there was great debate in the scientific community over its inability to transduce a signal which would lead to LPS activation and thus researchers began to look for an 'LPS signal transducer' [112, 115, 118, 119]. It was found that the primary function of CD14 is to serve as a ligand-binding component of the LPS recognition complex chaperoning LPS to the TLR4-MD2 complex or 'LPS signal transducer' responsible for downstream signaling effects. In addition to this role, CD14 also amplifies cell sensitivity to LPS thus enhancing the immune response [112, 118, 120, 121]. Collectively, LBP and CD14 represent two players responsible for LPS recognition and transport and are therefore essential steps required for LPS signaling. However,

CD14 is not capable of induce LPS signaling alone and therefore requires additional components to form the LPS recognition complex.

1.4.3 TLR4 and MD2 Receptor Complex

With innate immunity constituting of our first line of defense against invading microorganisms, the ability of phagocytic cells, such as macrophages and neutrophils, to distinguish between pathogen and self is vital. Toll-like receptors (TLRs) are a unique group of PRRs which provide this ability through the recognition of PAMPs. Currently, there are 10 known TLRs which recognize a variety of ligands from pathogenic organisms including viral dsRNA, bacterial flagellin, CpG-rich unmethylated DNA, and most importantly LPS. TLRs are type I transmembrane proteins and can be found on cell surfaces or bound to endosomes. Each TLR consists of 3 distinct domains each serving a unique purpose, the leucine rich repeat (LRR) domain, the transmembrane domain, and the cytoplasmic Toll/IL (interleukin)-1 receptor (TIR) domain [122-125]. The LRR domain is an extracellular domain consisting of multiple repeats of the LRR motif. This domain is important for the specificity of a TLR in that it is responsible for the recognition of the ligand while the transmembrane domain has been shown to be important for oligomerization [126, 127]. The TIR domain, which draws its name for the significant homology discovered between the cystolic domains of IL-1 receptor (IL-1R) and TLR, plays an essential role for TLRs as it is accountable for the downstream signaling effects necessary to induce an immune response through the interaction with adaptor proteins [128, 129]. It was found that LPS signals through TLR4 and it was originally thought to be the only receptor required for provoking the potent inflammatory reaction associated with LPS exposure.

However, Shimazu showed that transfection of TLR4 cDNA did not confer LPS responsiveness to cell lines with mutant TLR4 and therefore hyporesponsive to LPS. This study showed that a novel molecule, termed myeloid differentiation factor-2 (MD-2), restored LPS-responsiveness to recipient cell lines [130]. Additionally, mice lacking MD-2 have been shown not to respond to LPS and are able to survive endotoxin shock further supporting the idea that MD-2 links TLR4 to LPS signaling [131]. MD-2 acts a co-receptor of TLR4 binding to both LPS and TLR4 and facilitating the downstream signaling events. The discovery of MD-2 and its intimate interaction with TLR4 helped to solidify the TLR4/MD-2 complex as the LPS signal transducer responsible for the dramatic effects of LPS exposure [121, 132, 133].

1.4.4 Classical TLR4 Signaling

Upon LPS exposure, TLR4 undergoes oligomerization and begins to recruit adaptor proteins to facilitate the downstream immune response. Myeloid differentiation primary response gene 88 (MyD88) was originally isolated as a gene induced during IL-6 stimulated terminal differentiation of myeloid precursors into macrophages [134]. It was later discovered that MyD88 was an essential adaptor in the IL-1R signaling pathway and due to the homology between the TIR domains of IL-1R and TLR debate began about whether MyD88 could play a role in LPS-TLR4 signaling pathways [135, 136]. In 1999, it was shown that MyD88-deficient mice were resistant to septic shock and failed to induce pro-inflammatory mediators after LPS stimulation demonstrating the essential role of MyD88 as an adaptor for TLR4 mediated inflammatory responses [137]. MyD88 consists of an N-terminal death domain (DD), a C-terminal TIR domain, and a short connecting linker. The TIR domain of MyD88 interacts with the TIR domain of TLRs

and facilitates downstream signaling events [138, 139]. Additionally, the TIR-domain containing adaptor protein MyD88-adapter-like (Mal; also known as TIRAP) associates with MyD88 to further promote downstream signaling [140, 141]. Upon LPS stimulation, MyD88/Mal recruits IL-1 receptor-associated kinase (IRAK) 4 to TLR4 via a DD-DD interaction. This recruitment elicits IRAK4 to phosphorylate IRAK1 thus inducing its kinase activity. The activation of IRAK4 allows it to undergo sequential autophosphorylation on residues in its N-terminus. The hyperphosphorylation of this region serves dual purposes in that it allows for the protein TNF receptor associated factor (TRAF) 6 to bind to IRAK1 as well as for the disassociation of the IRAK1-TRAF6 complex from the upstream adaptor MyD88 [142-144]. The IRAK1-TRAF6 complex then interacts with a unique complex at the plasma membrane consisting of TGF-β-activated kinase 1 (TAK1) and TAK1-binding protein 1 and 2 (TAB1, TAB2). TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) which is crucial for LPS-induced activation of NF-κB. TAK1 has two adaptors proteins that are important for facilitating its activation. TAB1 functions as an activator of TAK1 and thus enhances its kinase activity while TAB2 functions as an adaptor linking TRAF6 and TAK1 [122, 145]. In TLR4 signaling, once the IRAK-1TRAF6 complex engages the TAK1-TAB1-TAB2 complex at the plasma membrane IRAK1 is left in the membrane for degradation and TRAF6 is translocated into the cytoplasm for TAK1 activation [146, 147]. Once in the cytoplasm a large complex is formed with the ubiquitin-conjugating enzyme (E2) ligases Ubc13 and Uev1A. Together with TRAF6 functioning as an E2 ligase via its Really Interesting New Gene (RING) finger domain, this super complex catalyzes the synthesis of Lys 63-linked polyubiquitin chains which are necessary for inhibitor of

kappa B (IκB) kinase (IKK) degradation and thereby induce TRAF6-mediated activation of TAK1. Activated TAK1 phosphorylates the IKK complex thus activating it [148-150]. This activation results in the phosphorylation of the inhibitory protein IκB and its disassociation from NF-κB. With IκB no longer bound to NF-κB and maintaining its inactive state, it migrates to the nucleus and induces the expression of pro-inflammatory mediators [122, 143, 151]. In addition to the activation of NF-κB, LPS can also signal through the MyD88-dependent or classical pathway to induce the activation of c-jun N-terminal kinase (JNK), which is responsible for the activation of transcription factors, such as activator protein 1(AP-1) and activating transcription factor 2 (ATF2), that are involved in a variety of cellular process including differentiation, proliferation, and apoptosis [149, 152, 153].

1.4.5 Alternative TLR4 Signaling

Much of the attention on TLR4 signaling originally focused on its dependence on MyD88 as the adaptor protein necessary for the facilitation of downstream signaling and this became known as the classical or MyD88 dependent pathway. However, it was shown that in MyD88-deficient mice LPS-induced activation of NF-κB and JNK was delayed but not ablated. Additionally, LPS-stimulation of MyD88-deficient cells did not result in the production of pro-inflammatory cytokines but, in the production of interferon (IFN) -inducible genes, such as IP-10, through the activation of the transcription factor, IFN-regulatory factor 3 (IRF-3) [137, 143, 154]. This highlighted the fact that there must be a pathway which does not utilize MyD88 for the activation of NF-κB, JNK, and IRF-3 and this pathway became commonly referred to as the MyD88-independent or alternative TLR4 signaling pathway. MyD88-independent TLR4 signaling functions in a sequential

order with MyD88-dependent signaling and requires two additional TIR-domain adaptors, TIR-domain-containing adaptor protein inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). TRIF and TRAM are recruited to activated TLR4 on early endosomes after its endocytosis [155-157]. TRIF recruits TRAF3 where it associates with TRAF family member-associated NF-κB activator (TANK), TANK binding kinase 1(TBK1) and IKKε to regulate downstream signaling and activate IRF-3. This activation leads to the induction of a variety of IFN-inducible genes responsible for eliciting anti-viral response against invading pathogens [143, 158, 159].

1.4.6 Missing Links

All of the fundamental research done on LPS and the mechanisms it utilizes to induce inflammation via TLR4 has focused on high doses of LPS due to its involvement in the pathogenesis of sepsis and septic shock. However research has shown that low dose endotoxemia is capable of skewing the microenvironment to a mild yet persistent pro-inflammatory state [160]. This chronic low-grade inflammation is commonly associated with a variety of inflammatory diseases including the ones discussed in this review and highlights the necessity to further understand the mechanisms and phenotypes associated with exposure to low-dose LPS.

Detailed mechanistic studies of the origins of low dose LPS leakage and possible interventions as well as the signaling pathways utilized in low dose endotoxemia will prove crucial to our understanding of the role it plays in the progression and pathogenesis of many diseases and disorders. With the complexity of tissue-specific responses, studying low-dose LPS exposure in various cell lines will be vital to developing a complete picture of how low dose endotoxemia functions throughout the body. Also,

addressing the issues of low dose LPS threshold and chronicity will help us to recognize the doses of LPS capable of causing persistent low-grade inflammation and what allows this inflammation to persist unregulated. Understanding the mechanisms utilized by low dose LPS to induce low-grade inflammation could hold promise in the development of therapeutics for the prevention or reversal of numerous inflammatory diseases and thus bears the importance and significance of research such as that presented in this dissertation.

1.5 References

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Chapter 2: Molecular and Cellular Mechanisms Responsible for Low-Grade Stress and Inflammation Triggered by Super-Low-Dose Endotoxin

2.1 Abstract

Super-low dose endotoxemia in experimental animals and humans are linked with low-grade chronic inflammatory diseases. However, the underlying molecular and cellular mechanisms are not well understood. In this study, we examined the effects of super-low dose LPS on low-grade inflammation in macrophages as well as the underlying mechanisms. We found that super-low dose LPS induces mitochondrial fission and cell necroptosis in primary murine macrophages, which was dependent upon interleukin-1 receptor associated kinase (IRAK-1).

Mechanistically, our study revealed that super-low dose LPS causes protein ubiquitination and degradation of mitofusin 1 (Mfn1), a molecule required for maintaining proper mitochondrial fusion. Super-low dose LPS also leads to dephosphorylation and activation of Drp1, a molecule responsible for mitochondrial fission and cell necroptosis. We demonstrated that super-low dose LPS activates receptor interacting protein 3 kinase (RIP3K), a key molecule critical for the assembly of necrosome complex, the initiation of Drp1 dephosphorylation and necroptosis. The effects of super-low dose LPS are abolished in macrophages harvested from IRAK-1 deficient mice. Taken together, our study identified a novel molecular pathway that leads to cellular stress and necroptosis in macrophages challenged with super-low dose endotoxin. This may reconcile low-grade inflammation often associated with low-grade endotoxemia.

2.2 Introduction

Individuals with adverse health conditions and lifestyles tend to maintain mildly elevated levels of circulating endotoxin lipopolysaccharide (LPS), a phenomenon referred to as subclinical low-grade endotoxemia [1]. This may contribute to chronic low grade-inflammation as manifested in chronic diseases such as diabetes, Parkinson's disease, and atherosclerosis [2-6]. Mechanistically, super-low dose endotoxin results in a mildly sustained activation of pro-inflammatory mediators without the activation of anti-inflammatory mediators, allowing low-grade non-resolving inflammation to persist [7]. In contrast, higher dosages of LPS induce a robust yet transient immune response where both pro- and anti-inflammatory mediators are activated, providing the host with a compensatory mechanism to resolve inflammation and maintain homeostasis [8].

Propagation of non-resolving low-grade inflammation can be potentially achieved by cell necroptosis instead of apoptosis [9-10]. Although apoptosis may facilitate the resolution of inflammation and homeostasis, necroptosis may contribute to prolonged inflammation through the release of inflammation-propagating DAMPs (damage-associated molecular patterns) [11-13]. Necroptosis is initiated through the activation of receptor-interacting protein 3 kinase (RIP3) and the assembly of a complex 'necrosome" near mitochondrial membranes [11]. RIP3 necrosome leads to the activation of a critical phosphatase PGAM5 that subsequently activates Dynamin-related protein 1 (Drp1) through its de-phosphorylation [14]. Drp1 dephosphorylation triggers mitochondrial fission and the generation of reactive oxygen species (ROS) and other un-identified events that ultimately lead to necroptosis and chronic inflammation [14].

In contrast to Drp1, mitofusins (Mfn1/2) act to prevent mitochondrial fission and facilitate mitochondrial fusion. Both Mfn1 and Mfn2 are believed to be regulated via post-

translational modifications such as ubiquitination, and proteasome inhibition can stabilize Mfn1 and prevent mitochondrial fission [16].

Higher dosages of LPS are known to trigger compensatory tolerance in innate immune cells, as reflected in the reduced expression of pro-inflammatory cytokines as well as increased mitochondrial bioenergetics and function [8]. However, the mechanisms responsible for the non-resolving low-grade inflammation initiated by super-low dose LPS are not well understood. To address this critical question, we examined the effects of super-low dose LPS on cellular necroptosis as well as key upstream signaling pathways. We demonstrate that super-low dose LPS selectively induces necroptosis through an interleukin-1 receptor-associated kinase 1 (IRAK-1) dependent pathway that leads to selective activation of Drp1 and degradation of Mfn1.

2.3 Methods

Reagents

LPS (*Escherichia coli* 0111:B4) and z-VAD-FMK (V116) were purchased from Sigma Aldrich. Anti-Mfn1(H-65) antibody was obtained from Santa Cruz Biotechnology. Anti-Drp1(#5391S), Anti-phospho-Drp1(Ser637)(#4867), Anti-Ubiquitin(#3933), Anti-phospho-JNK (#9251S), Anti-JNK (#9252S), and Anti-β-actin(#4967) antibodies were obtained from Cell Signaling Technology. Anti-RIP3K antibody was kindly provided by Dr. Jiahuai Han. Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology. MitoTracker Red (M7512) was purchased from Invitrogen.

Mice and cell culture

Wild type (WT) C57BL/6 mice were purchased from the Charles River laboratory. IRAK-$1^{-/-}$ mice from C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. All mice were housed and bred at Virginia Tech animal facility in compliance with approved Animal Care and Use Committee protocols of Virginia Tech. BMDMs were isolated from the tibia and femur of WT and IRAK-$1^{-/-}$ mice by flushing the bone marrow with Dulbecco's modified Eagle's medium (DMEM). The cells were cultured in untreated tissue culture dishes with 50 mL DMEM containing 30% L929 cell supernatant. On the third day of culture, the cells were fed with an additional 20 mL fresh medium and cultured for another additional 3 days. Cells were harvested with phosphate-buffered saline (PBS), resuspended in DMEM supplemented with 1% fetal bovine serum, and allowed to rest overnight before further treatments.

Confocal and electron microscopy

WT and IRAK-1$^{-/-}$ BMDM were plated in 35 mm glass bottom petri dishes (MatTek). For staining of mitochondria, the cells were incubated with 75 nM Mito Tracker Red (Invitrogen) for 20 min at 37ºC in darkness. After washing three times with PBS, the cells were fixed with paraformaldehyde (4%) in PBS for 15 min at room temperature and then washed three times with PBS. The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were obtained with a laser-scanning confocal microscope Zeiss LSM510. Mito Tracker Red was excited with a 543-nm laser line and its emission was collected between 590 and 640 nm. For the purpose of electron microscopy, WT and IRAK-1$^{-/-}$ BMDM were harvested after specified treatments and washed with PBS. The cells were pelleted in a microcentrifuge tube and 1mL of a 2% Glutaraldehyde with 0.1M cacodylate buffer [pH 7.4] fixative was placed on top of the cells. Samples were sliced and prepared on grids for visualization on a JEOL JEM 1400 transmission electron microscope.

Assays for cell viability, proliferation and death

Cell viability was determined using the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen). Briefly, cells were cultured in a 96 well plate in phenol red free media with at least three empty wells that serve as the background. The cells were then treated with or without super-low dose LPS for the specified time periods and the media was replaced with 100ul fresh media containing 10 ul of 12mM stock MTT. The plates were incubated for 4 hours at 37ºC covered in aluminum foil. 50 µL of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 minutes and the absorbance was measured at 540.

BMDM cell proliferation was detected using the BrDU cell proliferation assay kit (Cell signaling) as per manufacturer's recommendations. Assays were performed in triplicate for each experiment; mean cell viability was compared with vehicle-treated controls. Cell death was assessed by multiple assays that include Annexin V, release of cytochrome C, and DAPI staining through either microscopy or flow cytometry. For the Annexin V staining, cell death was detected using the Annexin V apoptosis assay kit (BioVision). BMDM cells were treated with super-low dose LPS followed by labeling with Annexin-Cy3 for 5 min at room temperature. Cells were then washed with annexin V binding buffer and counterstained with DAPI to stain the nuclei. The fluorescent images were captured using the scanning confocal microscope Zeiss LSM510 with a HeNe-argon laser as a light source. Each image was collected with excitation at 488 nm and emission at 520 nm for annexin V. For the quantitative analyses of PI staining, cells stained with 50nm PI were treated according to experimental group and analyzed by FACSCanto flow cytometer and the mean fluorescent intensities (MFI) of PI staining were collected.

Cyctochrome c release

The release of cytochrome c was measured using a mitochondrial/cytosol fractionation kit (Biovision). Cells at 1×10^7 with or without different treatments were harvested by centrifugation at 700 × *g* for 5 min and washed twice with cold phosphate-buffered saline. Afterward the cells were resuspended in a 250 µl of extraction buffer containing protease inhibitor mixture and dithiothreitol (Biovision). After incubation on ice for 30 min, the cells were and centrifuged at 700 × *g* for 10 min at 4 °C, and the supernatant was collected. Then the collected supernatant was centrifuged again at 10,000 × *g* for 30 min at 4 °C. The resulting supernatants were harvested and designated as cytosolic fractions, and the pellets were resuspended in an appropriate buffer and designated as mitochondrial fractions. Cytochrome *c*

release in isolated mitochondria was monitored after treatment with super low-dose LPS. The cytochrome *c* distributed in fraction, the cytosol/media or the mitochondria, in each experiment was analyzed using Western blotting with anti-cytochrome *c* monoclonal. Signals were detected using the horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1,000) and enhanced chemiluminescence substrate kit (Thermo Scientific).

Cellular protein extraction, immune-precipitation, and immune-blot analysis

Cells were harvested after specified treatments and washed with cold PBS. The cells were resuspended in a SDS lysis buffer containing protease inhibitor cocktail (Sigma) and subjected to SDS-PAGE. The protein bands were transferred to an immunoblot PVDF membrane (BioRad) and subjected to immunoblot analysis with the indicated antibodies. For immuno-precipitation analysis, cells were lysed in TBS buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl) supplemented with 1% Triton-X 100, 1 mM PMSF and a protease inhibitor cocktail (Roche). After pre-clearing for 1 hour, lysates were incubated with the appropriate antibody for four hours to overnight at 4°C. Two hours after adding protein A/G agarose, the immuno-precipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 3 min. For immuno-blot analysis, the samples were resolved by SDS-PAGE and transferred to a PVDF membrane (Biorad). Immunoblotting was probed with indicated antibodies. The proteins were visualized by using a chemiluminescence ECL kit (Pierce).

Statistical analysis

Results are expressed as mean ± SD. Statistical significances between groups were determined using a two-tailed Student's *t* test and indicated by an asterisk in figures; *p* values <0.05 were considered statistically significant.

2.4 Results

Super-low dose LPS induces low-grade stress and necroptosis in macrophages

We first examined the effect of super-low dose LPS on the activation status of stress kinase JNK. Wild type (WT) bone marrow-derived macrophages (BMDMs) were treated with either a super low-dose (50 pg/mL) or a higher dose (100ng/mL) LPS. As shown in Fig 1, 100ng/mL LPS induced a robust yet transient activation of JNK phosphorylation, which peaked at 30 minute post stimulation and quickly diminished by 1 h post LPS stimulation. In contrast, super-low dose LPS induced a mild and sustained phosphorylation of JNK that lasted throughout the 2 hr treatment period.

Low grade inflammation induces cellular stress and death that in turn may sustain persistent inflammation [17]. Next, we tested the effect of super-low dose LPS on macrophage viability and death. As shown in Fig 2A and B, super-low dose LPS (50 pg/mL) significantly reduced cell viability and proliferation as measured by MTT and BrDU incorporation assays respectively. We further performed Annexin V and DAPI staining in macrophages treated with super-low dose LPS. As shown in Fig 3, super-low dose LPS resulted in the elevated Annexin V staining in WT BMDMs. To examine the potential mechanisms, we determined the selective contribution of apoptosis and necroptosis. WT BMDMs were treated with LPS 50 pg/mL for 18 h followed by subcellular mitochondrial fractionation. As shown in Fig 4, super-low dose LPS failed to induce significant release of cytochrome c from mitochondria to the cytosol. Caspase assays also failed to show noticeable difference between control and LPS treated cells (data not shown). These data indicate that super-low dose LPS is unlikely to cause programmed cell death. Our DAPI staining also revealed intact nuclei in cells treated with super-low dose LPS, further

suggesting that necroptosis, instead of apoptosis may be involved (Fig 3). As caspase activity generally suppresses necroptosis, we subsequently studied the effect of caspase inhibitor Z-VAD. As shown in Figure 5, application of Z-VAD exacerbated cell death induced by super-low dose LPS as measured by PI staining and flow cytometry. Collectively, these data suggest that super-low dose LPS leads to cellular stress and necroptosis in macrophages.

Induction of mitochondria fission by super-low dose LPS in WT macrophages

Mitochondrial dynamics play a key role in the modulation of cellular stress, with mitochondrial fission leading to necroptosis and chronic low-grade inflammation [18]. We thus analyzed the effect of super-low dose LPS on mitochondrial fission and fusion. WT BMDMs were either untreated or treated with 50 pg/mL LPS and then labeled with the mitochondria specific MitoTracker dye. Cell nuclei were counter stained by DAPI and the mitochondrial morphology was observed by laser-scanning confocal microscope. As shown in Fig 6A, super-low dose LPS induced the conversion of elongated mitochondria morphology to fragmented ones as indicated in the magnified panel, indicative of mitochondrial fission. To further confirm mitochondrial fragmentation in response to super-low dose LPS at the ultra-structural level, we performed electron microscopy analyses using WT BMDMs. As shown in Fig. 6B, consistent with our observation using confocal microscopy, WT BMDMs treated with super-low dose LPS exhibited fractionated mitochondria potentially due to increased fission as compared to the untreated cells.

Super-low dose LPS mediated cellular stress is dependent upon IRAK-1

Based on previous studies that IRAK-1 is preferentially responsible for mediating the cellular responses of super-low dose LPS, we tested the effect of super-low dose LPS on cell stress in

IRAK-1 deficient macrophages. As shown in Fig 7, JNK1 phosphorylation induced by super-low dose LPS was reduced in IRAK-1 deficient cells. Furthermore, the effect of super-low dose LPS on cell death was alleviated in IRAK-1 deficient cells. (Fig 8-9).

We further tested the effect of IRAK-1 on mitochondrial dynamics. In contrast to WT cells, super-low dose LPS failed to induce mitochondrial fragmentation in the absence of IRAK1 (Fig 10).

Super-low dose LPS activates Drp1 and degrades Mfn1 through an IRAK-1 dependent manner

Cellular necroptosis and mitochondrial fission is regulated by the phosphorylation status of dynamin-related protein 1 (Drp1) [18-19]. Phosphorylation of Drp1 at Ser637 inhibits both cell necroptosis as well as mitochondrial fission [20-21]. On the other hand, de-phosphorylation of Drp1 is critical for the initiation of necroptosis and mitochondrial fission. We therefore tested the phosphorylation status of Drp1 in BMDMs from WT and IRAK-1 deficient mice treated with super-low dose LPS. As shown in Fig 11, super-low dose LPS significantly reduced the phosphorylation of Drp1 at Ser637 in WT BMDMs, corresponding to elevated cell necroptosis and mitochondrial fission. This effect was ablated in IRAK1-deficient cells. Besides Drp1, Members of the dynamin family of protein, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), are involved in fusion between mitochondria by tethering adjacent mitochondria. We determined the effect of super-low dose LPS on the levels of Mfn1 in macrophages. We observed that Mfn1 levels were drastically reduced in response to super-low dose LPS in WT but not in IRAK-1 deficient BMDMs (Fig 12). There was no change in the levels of Mfn2 upon super-low dose LPS treatment (data not shown). These results suggest that super-low dose LPS may induce

necroptosis and cellular stress by modulating key regulatory molecules such as Drp1 and Mfn1 through an IRAK-1 dependent mechanism.

Super-low dose LPS activates RIP3 responsible for the dephosphorylation of Drp1 through the IRAK-1 dependent pathway

RIP3 kinase (RIP3) pathway has been closely associated with the initiation of cellular necroptosis [11,19]. RIP3 activation triggers the activation of phosphatase PGMS5, which subsequently dephosphorylates Drp1 [14]. To test whether super low dose LPS may induce cellular stress and necroptosis through the RIP3 pathway, we examined the phosphorylation status of RIP3. As shown in Fig 13, 50 pg/mL LPS induced rapid phosphorylation of RIP3K, indicative of RIP3 activation. In contrast, this effect was abolished in IRAK-1 deficient cells.

Super low dose LPS causes the ubiquitination and degradation of Mfn1 dependent upon IRAK-1

With regard to the regulation of Mfn1, protein stability and degradation play a key role [16]. We further tested whether protein degradation is responsible for the decreased Mfn1 levels in cells challenged with super-low dose LPS. As shown in Fig 14, the proteasomal inhibitor MG132 blocked the reduction of Mfn1 in response to super-low dose LPS. To further test whether Mfn-1 undergoes ubiquitination, immunoprecipitation experiments were performed to detect the ubiquitination status of Mfn-1 in the presence of super-low dose LPS. We detected ubiquitinated Mfn1 in the presence of super-low dose LPS (Fig 15). In contrast, the ubiquitination of Mfn1 was completely abolished in IRAK-1 deficient cells, indicating that IRAK-1 is responsible for super-low dose LPS-mediated Mfn1 ubiquitination and degradation.

Taken together, our current study reveals a novel molecular pathway in innate macrophages

that is preferentially induced by super-low dose LPS and is responsible for triggering cellular stress and necroptosis (Fig 16).

2.5 Discussion

Our current study revealed novel mechanisms that underlie cell necroptosis and low-grade inflammation preferentially induced by super-low dose LPS. Our findings suggest that super-low dose LPS selectively induces cell stress and necroptosis through an IRAK-1 mediated modulation of mitochondrial fission. This conclusion is corroborated by various molecular and cellular evidence. First, super-low dose LPS activates RIP3, a necrosome kinase responsible for the downstream dephosphorylation of Drp1 and the initiation of mitochondrial fission and necroptosis, dependent upon IRAK-1. Second, super-low dose LPS degrades Mfn1, a protein involved in maintaining proper mitochondrial fusion and antagonizing the function of Drp1, in an IRAK-1 dependent manner. Third, super-low dose LPS triggers low-grade cellular stress, mitochondrial fission and necroptosis in wild type, but not in IRAK-1 deficient macrophages.

Our data complement and extend recent studies that support an intriguing link between low-grade endotoxemia, cell necroptosis, and chronic inflammatory diseases [4,18,22,23]. Subclinical dosages of circulating endotoxin in experimental mice and humans (1-100pg/mL) are associated with chronic inflammatory conditions [24-25]. Despite its significance, most mechanistic studies regarding low-dose endotoxin used dosages in the ng/mL range, 10-100 fold higher then the pathologically relevant super-low dose [26]. Low to higher dosages of endotoxin (>100 ng/mL) can trigger both robust inflammatory responses as well as compensatory anti-inflammatory responses in innate immune cells and tissues [27-28]. To our knowledge, this is the first report that reveals a novel role of super-low dose endotoxin. Our data indicate that super-low dose endotoxin is highly potent in inducing macrophage necroptosis instead of apoptosis. Unlike programmed apoptotic cell death that resolves inflammation, necroptosis is associated with non-

resolving inflammation and persistent activation of stress kinases such as JNK [29-30]. As a consequence, cell necroptosis is associated with a multitude of inflammatory complications such as atherosclerosis, reduced wound repair, inflammatory bowl diseases, and ischemia injury [10,30-34]. Our finding that super-low dose endotoxin elicits cell necroptosis potentially explains the detrimental effect of super-low grade endotoxemia in humans.

Biochemically, our study provides a new perspective regarding the novel connection between IRAK-1 and necroptosis pathway. Conventionally, IRAK-1 is primarily examined in the context of NFkB signaling and transcriptional regulation of inflammatory gene expression [35]. Our data reveal that IRAK-1 is also critically involved in mediating the activation of RIP3, a key component of the necrosome complex, in cells challenged with super-low dose endotoxin. RIP3 pathway eventually leads to the dephosphorylation and activation of Drp1, leading to mitochondrial fission and necroptosis [14,18].

In addition to Drp1 activation and mitochondrial fission, a reduction in the compensatory mitochondrial fusion process may further perpetuate non-resolving inflammation and necroptosis. Our data indicate that super-low dose LPS not only activates Drp1, but also reduces the protein levels of Mfn1 through an IRAK-1 dependent pathway. We documented that IRAK-1 is required for Mfn1 ubiquitination and degradation induced by super-low dose LPS. Future studies are needed to further define the biochemical process responsible for Mfn1 degradation and RIP3 activation mediated by IRAK-1.

Taken together, our study reveals a novel IRAK-1 mediated pathway that is responsible for low-grade inflammation and necroptosis induced by super-low dose LPS. Given recent findings that selective inhibition of necroptosis may hold promise in treating chronic inflammatory diseases [34-36] our current study suggests that IRAK-1 may be a viable target in the potential

intervention of chronic inflammation mediated by low-grade endotoxemia.

2.6 Figures

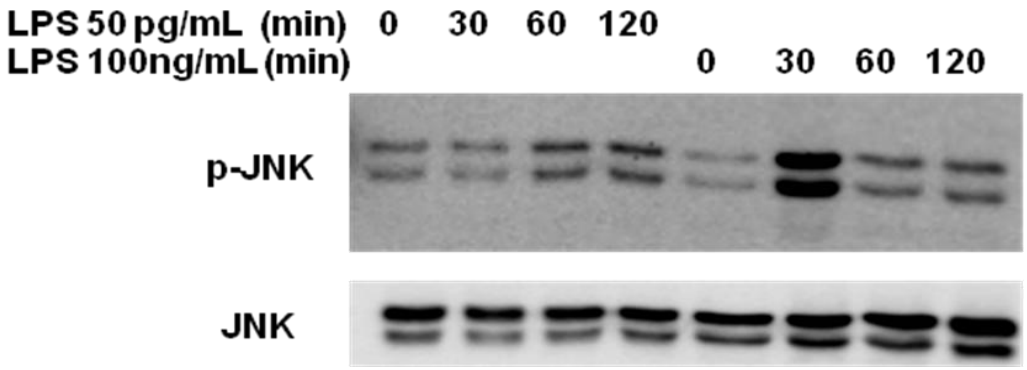

Figure 1: Super- low dose LPS induces low-grade stress

WT macrophages were treated with either 50pg/mL or 100ng/mL LPS for the indicated times. The phosphorylation levels of JNK were determined by western blot. The same blots were probed for total levels of JNK as a loading control. All data are representative of three independent experiments.

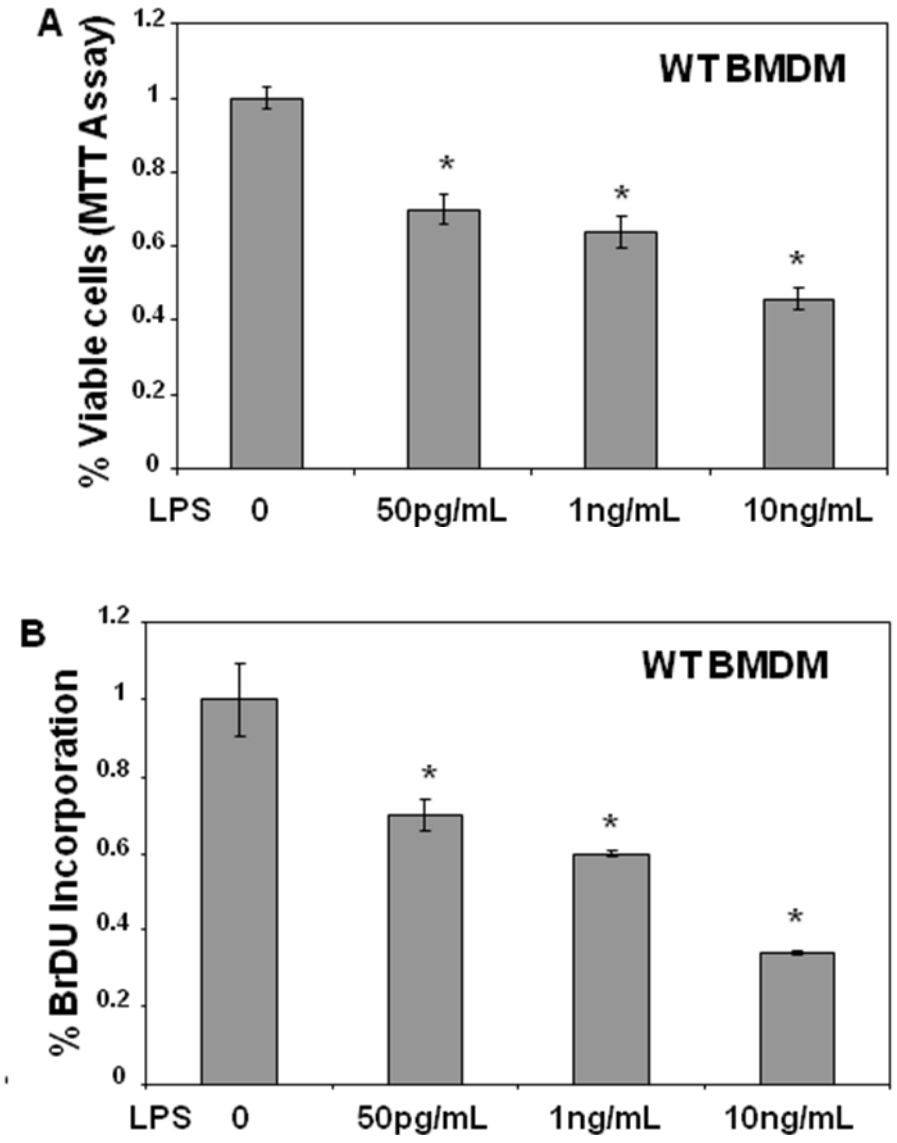

Figure 2: Super-low dose LPS reduces cellular proliferation and viability

WT macrophages were treated with the specified doses of LPS for 18hr. The effect of LPS on cell death was measured by (A) MTT assay and cellular proliferation was examined by (B) BrDU incorporation assay. Absorbance was read at 540 on a SpectraMax M5 microplate reader. All data are representative of three independent experiments and represented as the mean value ± SD. * $P<0.05$.

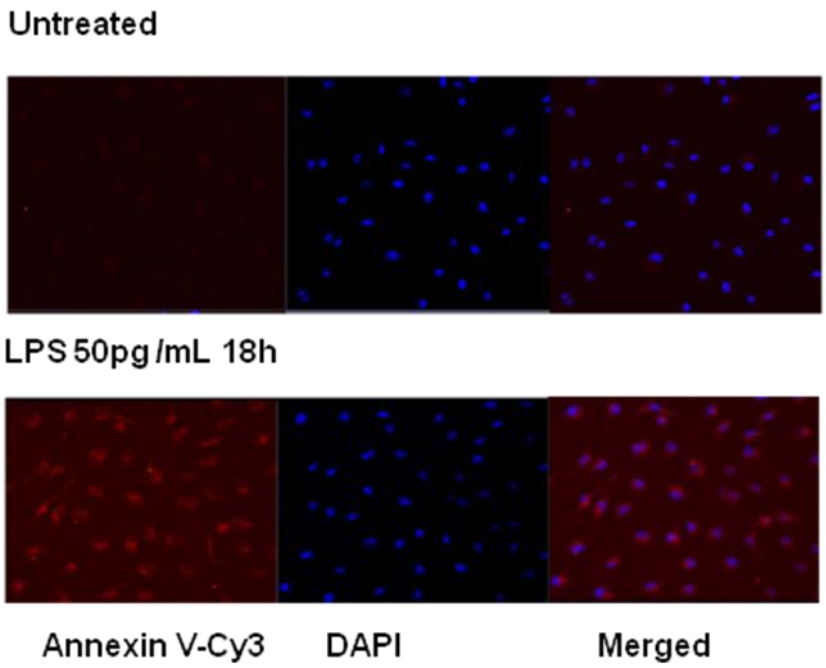

Figure 3: Super-low dose LPS induces cell death

WT macrophages were untreated or treated with 50pg/mL LPS for 18hr and then labeled with AnnexinV-Cy3. DNA was stained with DAPI, and induction of cell death was analyzed by visualizing the cells under fluorescence microscope. All data are representative of three independent experiments.

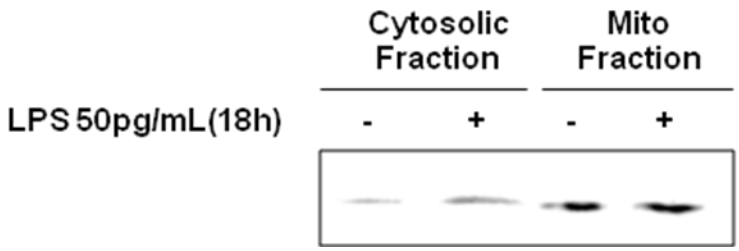

Figure 4: Super-low dose LPS fails to induce cytochrome c release

WT macrophages were stimulated with 50pg/mL LPS for 18hr followed by mitochondrial fractionation. The mitochondrial and cytosolic fractions were separated on a 10% SDS-PAGE gel and probed with cytochrome c specific antibodies.

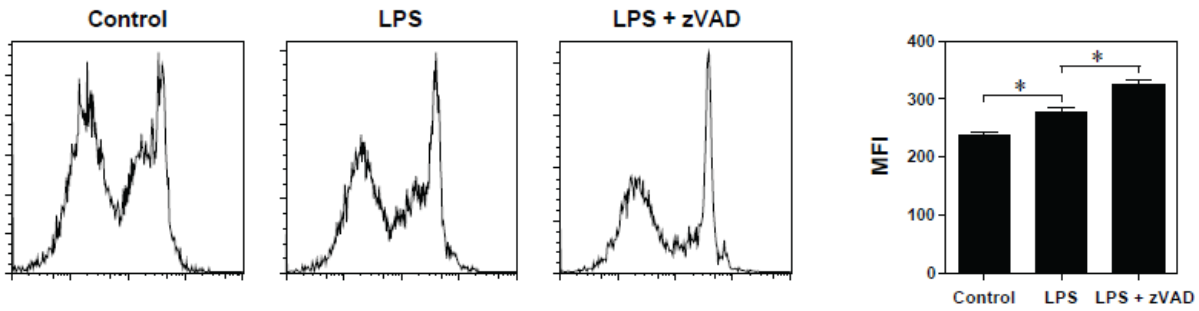

Figure 5: Caspase inhibition exacerbates super-low dose LPS induced cell death

WT macrophages were treated with 50pg/ml LPS, 20 µM z-VAD-FMK, or LPS plus z-VAD-FMK overnight, stained with PI, and analyzed by FACSCanto flow cytometer. All data are representative of three independent experiments and represented as the mean value ± SD. * $P<0.05$.

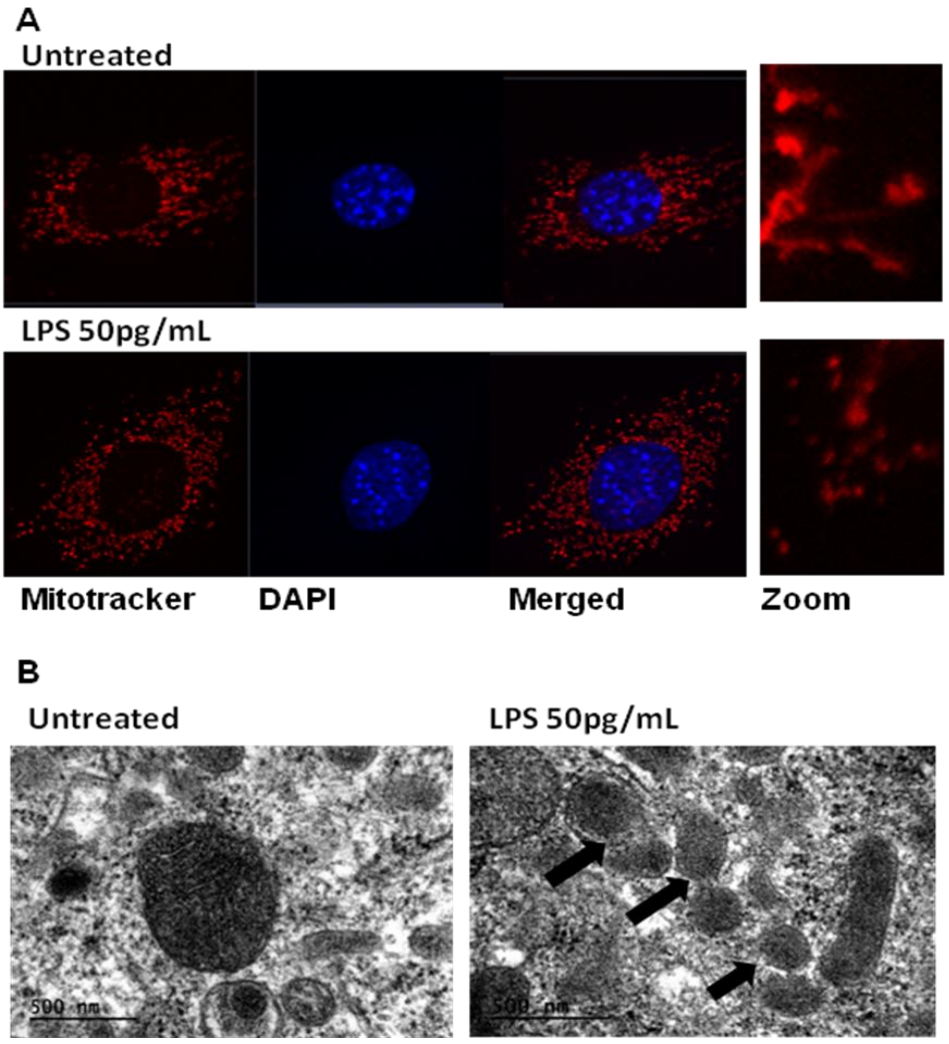

Figure 6: Super-low dose LPS induces mitochondrial fission

(A) WT BMDM were treated with 50pg/mL LPS for 1hr and then labeled with MitoTracker red to stain the mitochondria. The nuclei were stained using DAPI (blue). The cells were visualized under a ZEISS LSM 510 laser-scanning confocal microscope (original magnification ×400). The merged images were magnified and shown on the right panel. (B) WT BMDMs were treated with super-low dose LPS (50 pg/mL) for 1hr. Cells were prepared and visualized under a JEOL JEM 1400 transmission electron microscope. All data are representative of three independent experiments.

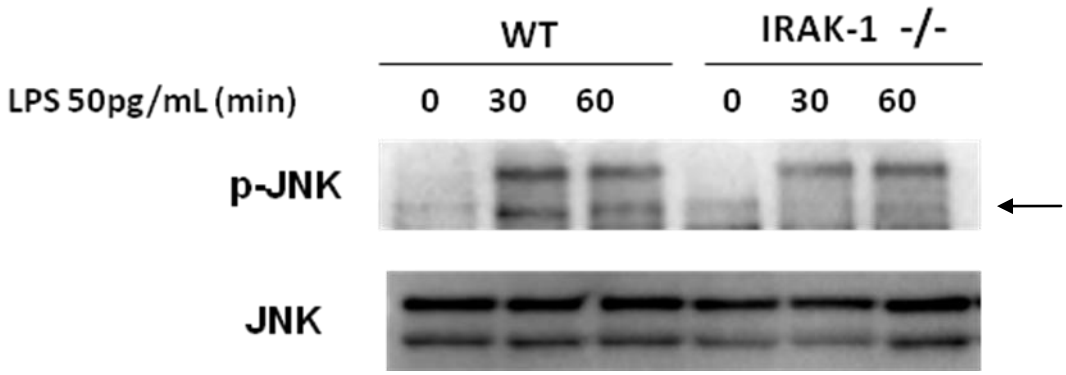

Figure 7: Super-low dose LPS promotes cellular stress through IRAK-1 dependent mechanism

Murine macrophages derived from WT and IRAK-1 deficient mice were treated with 50pg/mL LPS for the indicated times. The levels of JNK phosphorylation were determined via western blot and total JNK levels were detected as a loading control. All data are representative of three independent experiments.

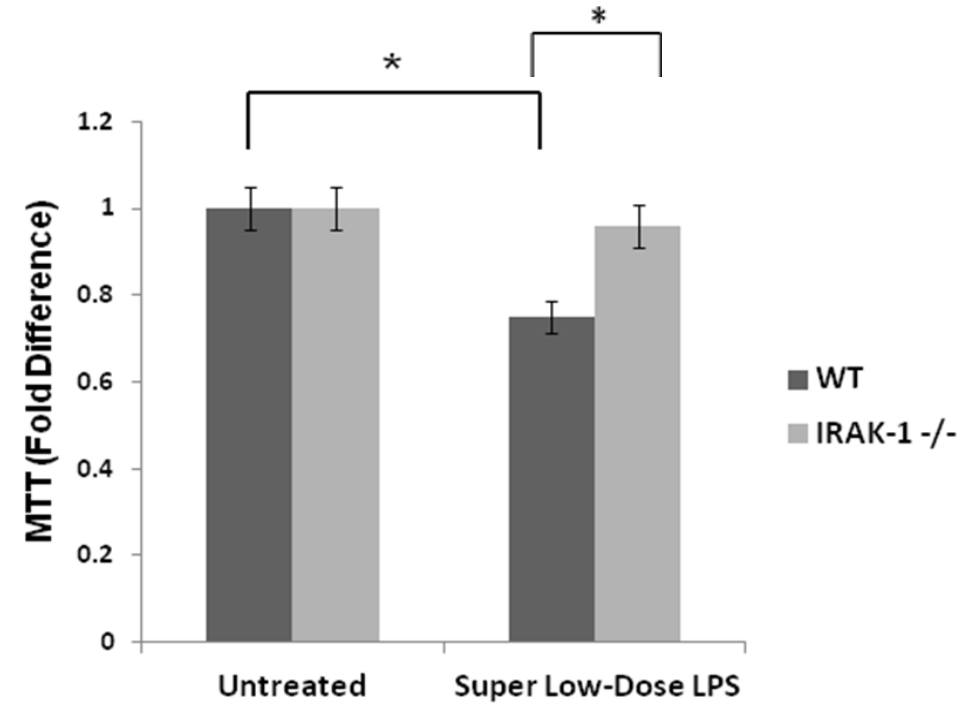

Figure 8: Super-low dose LPS reduces cellular viability through an IRAK-1 dependent mechanism

Macrophages derived from WT and IRAK-1 deficient mice were treated with super-low dose LPS (50pg/mL) for 18hr. The effect of LPS on cell death was measured by MTT Assay. All data are representative of three independent experiments and represented as the mean value ± SD. * $P<0.05$.

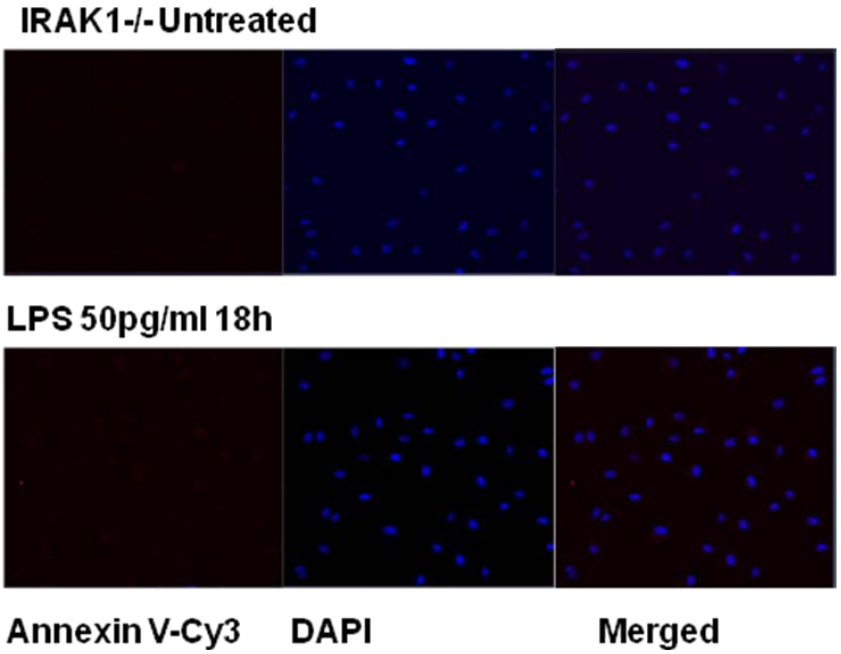

Figure 9: IRAK-1-/- macrophages fail to promote super-low dose induced cell death

IRAK-1 deficient macrophages were treated with 50pg/mL LPS for 18hr and then labeled with AnnexinV-Cy3. DNA was stained with DAPI, and the induction of apoptosis was analyzed by visualizing the cells under fluorescence microscope. All data are representative of three independent experiments.

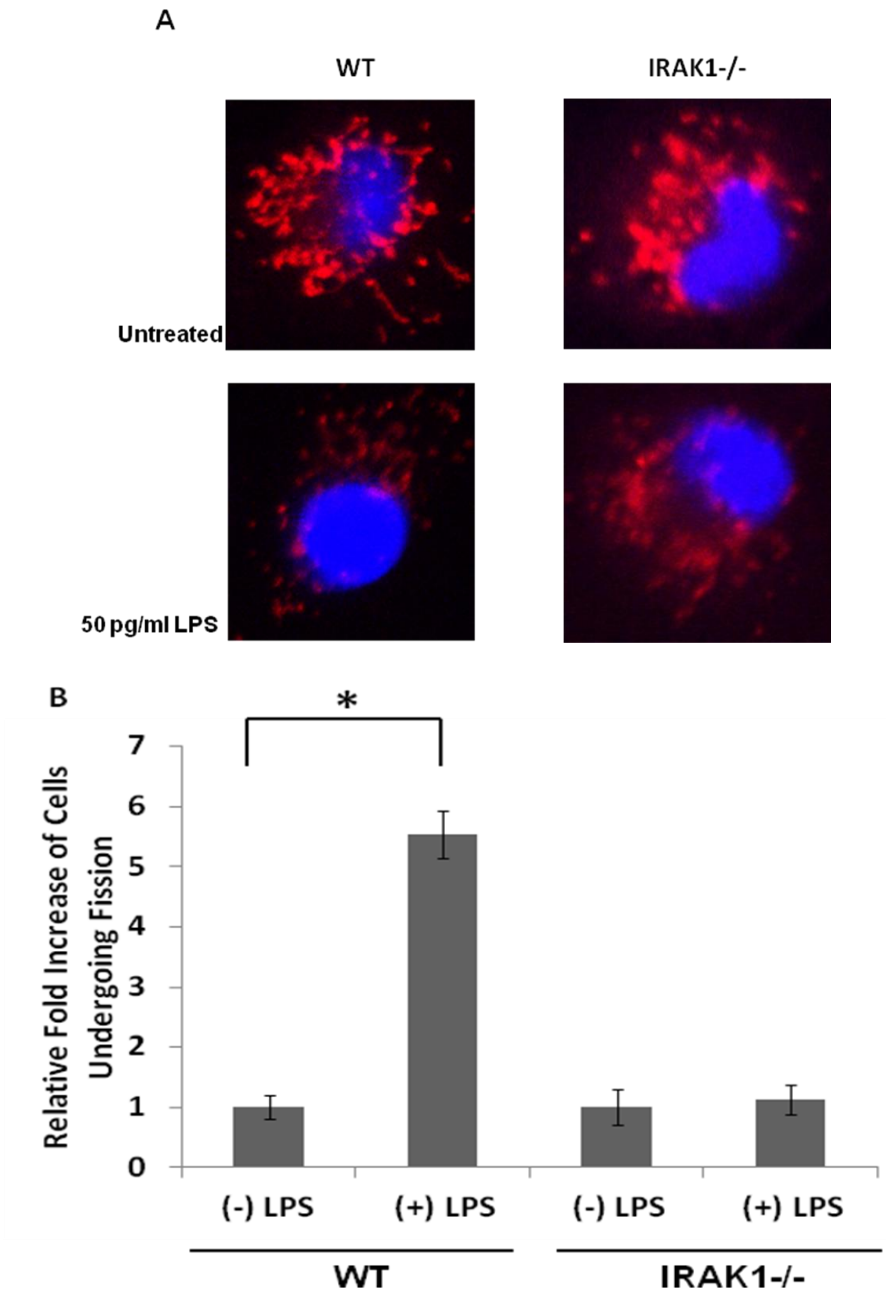

Figure 10: Super-low dose LPS promotes mitochondrial fission through an IRAK-1 dependent mechanism

WT and IRAK-1 deficient BMDMs were treated with 50pg/mL LPS for 1hr. Cells were stained with MitoTracker Red to stain the mitochondria. The nuclei were counterstained using DAPI (blue). The cells were visualized under a laser-scanning confocal microscope (original magnification ×400). Quantification of the fission events in each cell type is represented in graphical format. All data are representative of three independent experiments and represented as the mean value ± SD. * $P<0.05$.

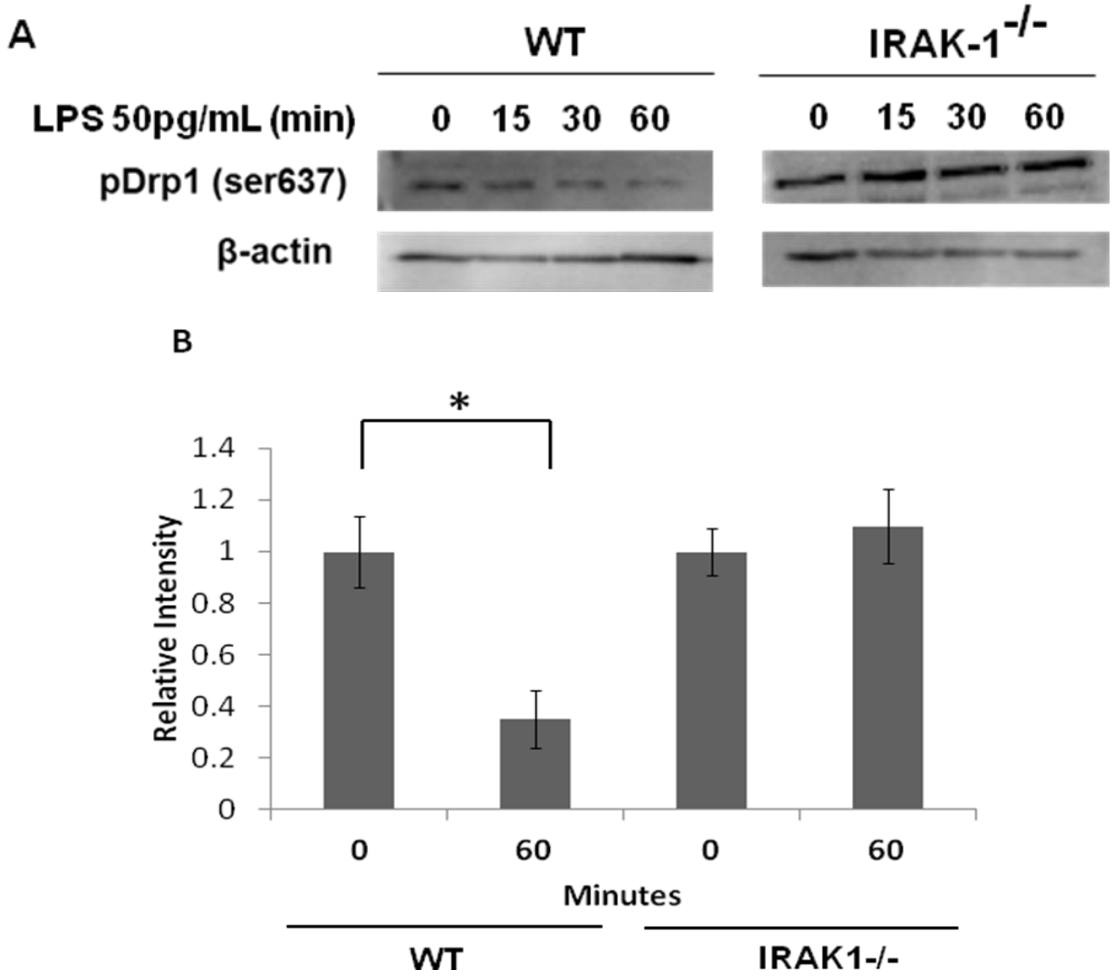

Figure 11: Super-low dose LPS promotes mitochondrial fission through Drp1 dephosphorylation

(A) WT and IRAK-1 deficient BMDMs were treated with super-low dose LPS (50pg/mL) for the indicated times. The levels of phosphorylated Drp1 (Ser637) were determined by western blot. The same blots were probed with β-actin as a loading control. (B) Band intensities of phospho-Drp1 were quantified and normalized relative to β-actin content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

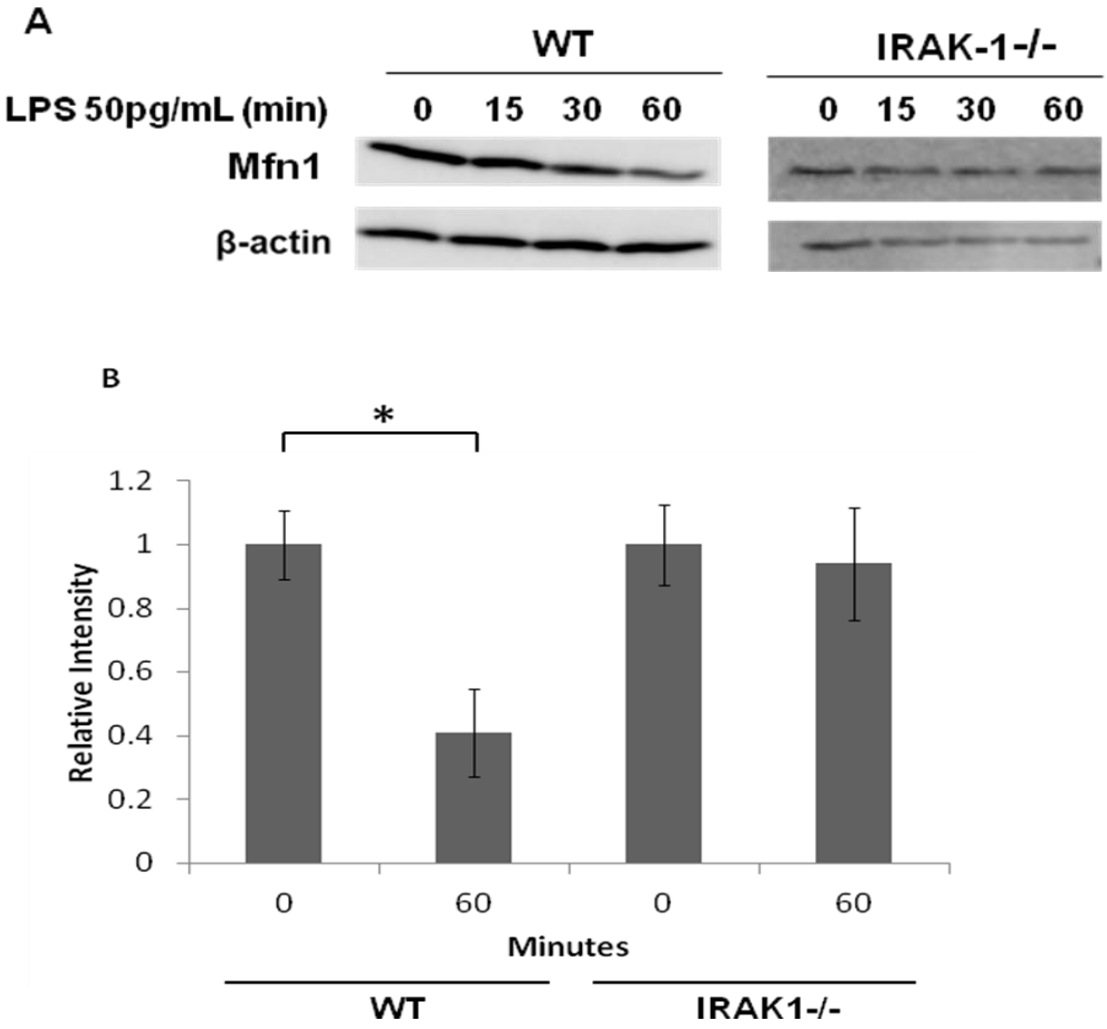

Figure 12: Super-low dose LPS suppresses mitochondrial fusion

(A) WT and IRAK1-deficient BMDMs were treated with super-low dose LPS (50pg/mL) for the indicated times. The levels of Mfn1 were determined by western blot. The same blots were probed with β-actin as a loading control. (B) Band intensities of Mfn1 levels at 0 and 60 minutes were quantified and normalized relative to β-actin content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

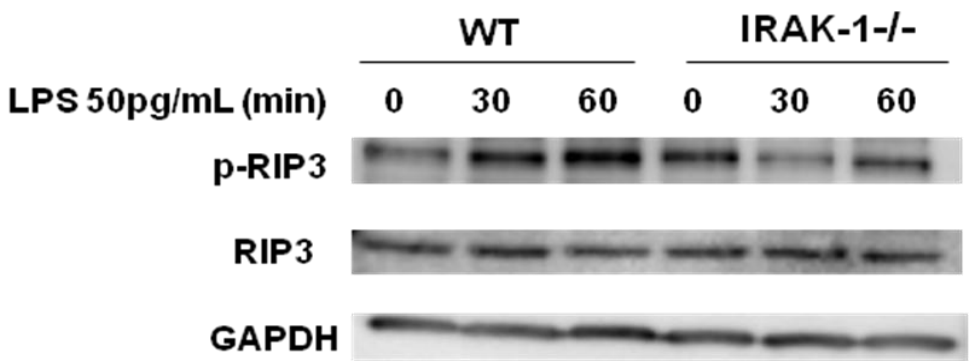

Figure 13: Super-low dose LPS mediation of Drp1 phosphorylation is dependent upon RIP3 kinase

WT and IRAK1-deficient BMDMs were treated with super-low dose LPS (50pg/mL) for the indicated times. The phosphorylation, and thus activation, of RIP3 was determined by western blot. The same blots were probed with total RIP3 and GAPDH as loading controls. All data are representative of three independent experiments.

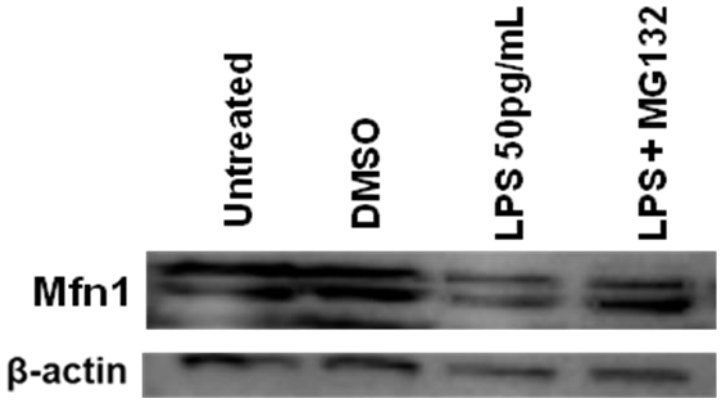

Figure 14: Super-low dose LPS induces proteasomal degradation of Mfn1

WT macrophages were treated with 50pg/mL LPS in the absence or presence of the proteasome inhibitor MG132 followed by immunoblot analysis of Mfn1 protein levels. The same blots were probed with β-actin as a loading control. All data are representative of three independent experiments.

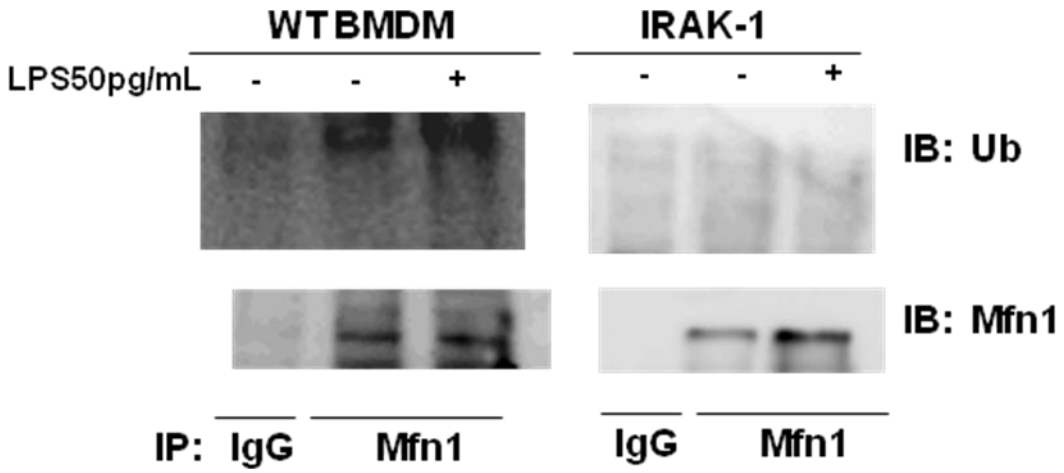

Figure 15: IRAK-1 is responsible for super-low dose LPS mediated Mfn1 ubiquitination and degradation

Immunoprecipitation (IP) analysis of whole cell lysates were derived from WT and IRAK1-deficient macrophages treated with or without 50pg/mL LPS with either IgG or Mfn1 specific antibodies as indicated on the blots. The blots were probed with either ubiquitin (top panel) or Mfn1 (bottom panel) specific antibodies. All data are representative of three independent experiments.

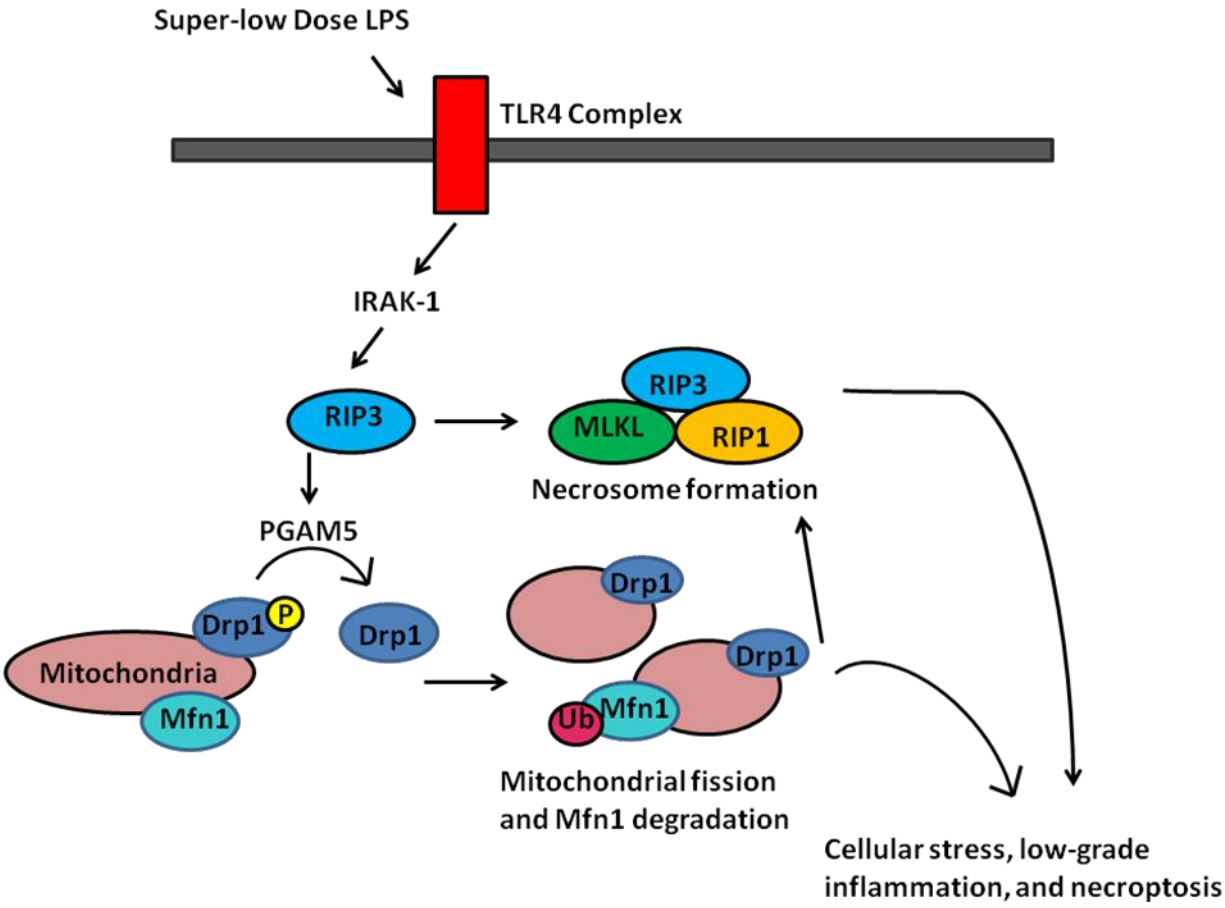

Figure 16: Super-low dose LPS induces low-grade inflammation and necroptosis

Schematic representation of the novel mechanism utilized by super-low dose LPS to induce cellular stress, low-grade inflammation, and necroptosis through an IRAK-1 dependent mechanism. Super-low dose LPS facilitates the activation of RIP3 kinase, triggering the formation of the necrosome and induction of necroptosis. Activated RIP3 kinase is also responsible for the dephosphorylation and activation of Drp1 which induces mitochondrial fission. Collectively, necroptosis and mitochondrial dysregulation via mitochondrial fission contribute to the non-resolving inflammation observed under super-low dose LPS exposure.

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Chapter 3: Low-grade inflammation through disruption of the endosome pathway mediated by super-low dose endotoxin

3.1 Abstract

Subclinical super-low dose endotoxin lipopolysaccharide (LPS) is a risk factor for the establishment of low-grade inflammation during the pathogenesis and progression of chronic diseases. However, the underlying mechanisms are not well understood. At the cellular level, a disruption of endosome-lysosome fusion may contribute to the potentiation of low-grade inflammation. In this report, we identified that subclinical super-low dose endotoxin lipopolysaccharide (LPS) can potently inhibit the process of endosome acidification and endosome-lysosome fusion in primary macrophages. Super-low dose LPS induces the inhibitory phosphorylation of VPS34, a key kinase controlling the fusion of endosome and lysosome. We documented that Tollip is responsible for the constitutive fusion of endosome and lysosome. Tollip deficient macrophages have constitutively elevated levels of VPS34 inhibitory phosphorylation, and disruption of endosome-lysosome fusion. By employing a skin excision wound model, we observed that Tollip deficient mice have significantly elevated levels of pro-inflammatory monocytes in the wound tissue and reduced wound repair. Taken together, our current study reveals a novel mechanism responsible for the modulation of endosome-lysosome fusion and low-grade inflammation in innate macrophages.

3.2 Introduction

Chronic low-grade inflammation is a common feature of numerous debilitating diseases and disorders such as cardiovascular disease, diabetes, impaired wound repair and Parkinson's diseases [1-6]. Subclinical super-low dose endotoxin in blood circulation is one of the emerging risk factors that could cause and sustain chronic low-grade inflammation in humans and animals [7-9]. Super-low dose endotoxin selectively induces mild inflammatory responses in innate leukocytes without triggering compensatory anti-inflammatory responses [10]. As a consequence, this may explain the non-resolving nature of low-grade inflammation that often accompanies low-grade endotoxemia.

At the cellular level, alterations of intra-cellular trafficking processes such as endosomal acidification, autophagy initiation and completion, as well as fusion of endosome and lysosome are shown to be critically involved in the propagation of low-grade inflammation [11-15]. Of particular interest, timely and orderly fusion of lysosomes with endosomes or autophagosome would enable efficient clearance of cellular debris as well as resolution of inflammation. Limited studies suggest that disruption of endosomal acidification and/or endosome-lysomsome fusion is correlated with cellular skewing into pro-inflammatory phenotypes in monocytes and macrophages [12, 16].

At the biochemical level, several distinct gate-keeping enzymes are responsible for the initiation of autophagy and the fusion of lysosomes with autophagosome and/or endosome. The cellular stress-sensing protein kinase adenosine monophosphate-activated protein kinase (AMPK) is responsible for autophagy initiation, through the activation of

unc-51 like autophagy activating kinase 1 (ULK1) [17]. Upon phosphorylation, AMPK becomes activated and is able to phosphorylate and thus activate ULK1, triggering the autophagic cascade [17-19]. Activated ULK1 is recruited to the phagophore where it binds to regulatory proteins and begins the assembly process of the autophagosome.

On the other hand, the activation of class III PI3K VPS34, also known as PIK3C3, plays a key role during completion of autophagy, as well as in the fusion of lysosome with endosome [20, 21]. VPS34 is responsible for the phosphorylation of phosphatidylinositol to generate phosphatidylinositol 3-phosphate [PI(3)P], an essential signaling lipid in the regulation of endosomal-lysosomal fusion event [20-23]. VPS34 is negatively regulated via phosphorylation by various kinases including cyclin-dependent kinase (Cdk) [24, 25]. The phosphorylation of VPS34 inhibits the proper fusion of endosome and/or autophagosome with lysosome [26].

Although subclinical low-grade endotoxemia has been associated with non-resolving low grade inflammation in humans and experimental animals, limited studies are available to clarify the underlying mechanisms. Previous studies suggest that the Toll-Interacting Protein (Tollip), one of the key signaling molecules downstream of the Toll-Like-Receptor 4 (TLR4) pathway, can interact with Tom1, and may be involved in endosomal trafficking [27, 28]. However, the physiological and pathological relevance of Tollip are poorly understood. We also reported in this study that the interleukin-1 receptor associated kinase M (IRAK-M) facilitates autophagy, although the underlying biochemical mechanisms are not understood. More importantly, the function of Tollip and IRAK-M in the context of super-low dose endotoxemia is poorly defined.

In this study, we tested the hypothesis that super-low dose endotoxin may modulate endosomal trafficking and low-grade inflammation in macrophages through Tollip and IRAK-M. We demonstrate that super-low dose LPS potentiates cellular stress in macrophages by hindering endosomal-lysosomal fusion and suppressing autophagy completion. Mechanistically, we show that super-low dose LPS suppresses the activation of AMPK and VPS34 in macrophages, critical regulators in the fusion of endosome and lysosome. Using macrophages harvested from IRAK-M and Tollip mice, we examined the role of these two signaling molecules during the process of endosome-lysosome fusion. We further examined the inflammatory phenotype by using an excision skin wound repair model in wild type and Tollip deficient mice. Collectively, our study defines a novel mechanism responsible for the potentiation of low-grade inflammation through the disruption of endosome-lysosome fusion.

3.3 Methods

Reagents

LPS (*Escherichia coli* 0111:B4), Tunicamycin (T7765), and TUDCA (T0266) were obtained from Sigma Aldrich. Anti-GAPDH antibody (sc-25778) was obtained from Santa Cruz Biotechnology. Anti-phospho-JNK(9251), Anti-JNK (9252), Anti-phospho-ERK (4377), Anti-ERK (4372), Anti-phospho-p38 (4731), Anti-p38 (9219), Anti-SQSTM1/p62 (5114), Anti-phospho-AMPK (2535), and Anti-AMPK (2532) antibodies were obtained from Cell Signaling Technology. Anti-PI3KC3/VPS34 (OAAB14062) antibody was obtained from Aviva Systems Biology. Anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (7074) was purchased from Cell Signaling Technology. Autophagy Sensor GFP-LC3B (P36235), dextran, alexa fluor 546 (D22911), dextran, alexa fluor 647 (D22914), dextran, fluorescein and tetramethylrhodamine (D1951), Zymosan A Bioparticles (Z2841), and opsonizing reagent (Z2850) were obtained from Invitrogen.

Mice and cell culture

Wild type (WT) C57BL/6 mice were purchased from the Charles River laboratory. IRAK-M$^{-/-}$ mice from C57BL/6 background were kindly provided by Dr. Richard Flavell from Yale University. Tollip$^{-/-}$ mice from C57BL/6 background were provided by Dr. Jürg Tschopp from the University of Lausanne at Switzerland. All mice were housed and bred at the Virginia Tech animal facility in compliance with approved Animal Care and Use Committee protocols of Virginia Tech. BMDMs were isolated from the tibias and femurs of WT, IRAKM$^{-/-}$ and Tollip$^{-/-}$ mice by flushing the bone marrow with Dulbecco's

modified Eagle's medium (DMEM). The cells were cultured in untreated tissue culture dishes with 50 mL DMEM containing 30% L929 cell supernatant. On the third day of culture, the cells were fed with an additional 20 mL fresh medium and cultured for another additional 3 days. Cells were harvested with phosphate-buffered saline (PBS), resuspended in DMEM supplemented with 1% fetal bovine serum, and allowed to rest overnight before further treatments.

Visualizing autophagy via LC3 conversion

WT, IRAKM$^{-/-}$ and Tollip$^{-/-}$ BMDM were plated in 35 mm glass bottom petri dishes (MatTek). For LC3 visualization, the cells were incubated overnight with GFP-LC3 virus particles (Invitrogen) at 37ºC to allow for uptake. After washing three times with PBS, the cells were treated with LPS as specified followed by fixation with paraformaldehyde (4%) in PBS for 15 min at room temperature. LC3 conversion, and thus autophagy, was indicated by fluorescent puncta (green). The nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Data was collected using a laser-scanning confocal microscope Zeiss LSM510.

Analysis of endosomal–lysosomal fusion

BMDM from WT, Tollip-/-, and IRAKM-/- mice were incubated with AlexaFluor-546-tagged dextran (1 mg/ml) for 1 h, cells were then washed several times and reincubated in fresh medium for 3hr. Cells were further incubated with FITC-conjugated zymosan for an additional hour followed by washing and fixation with paraformaldehyde (4%) in PBS for 15 min at room temperature. Data was collected using a laser-scanning

confocal microscope Zeiss LSM510. Experiments were conducted at 37°C. Phagosomes (intracellular zymosan, green) fused with lysosomes (red) appeared yellow.

Electron microscopy

WT, IRAKM$^{-/-}$ and Tollip$^{-/-}$ BMDM were harvested after specified treatments and washed with PBS. The cells were pelleted in a microcentrifuge tube and 1mL of 2% glutaraldehyde with 0.1M cacodylate buffer [pH 7.4] fixative was placed on top of the cells without causing resuspension. Samples were sectioned and prepared on grids to be visualized utilizing a JEOL JEM 1400 transmission electron microscope.

Immunoblotting

Cells were washed with PBS and harvested in a SDS lysis buffer containing protease inhibitor cocktail (Sigma) and subjected to SDS-PAGE. The protein bands were transferred to an immunoblot PVDF membrane (BioRad). Blots were incubated overnight at 4 °C with specific antibodies as indicated in the figures and subsequently with goat anti-rabbit antibody conjugated to horseradish peroxidase (Cell Signaling) at a 1:1,000 dilution for 60 min at room temperature. Detection was by enchanced chemiluminesence (Pierce ECL Western Blotting Substrate).

Determining endosomal acidification

The effect of LPS on endosomal acidification was measured using dextran 70 kDa double-labeled with fluorescein (FITC) and tetramethylrhodamine (TMR) (Invitrogen). Cells were treated for the indicated times and then pulsed with labeled dextrans (1 mg/mL) for 10 minutes at 37°C. Cells were then extensively washed with cold PBS and immediately analyzed by FACS. The ratio of the mean fluorescence intensity (MFI)

emission between both dyes was determined. An increase in MFI represents a decrease in acidification. A control of dextran attached to cells was performed keeping cells at 4°C.

Wounding procedure

The mice were anesthetized with intra peritoneal injection of avertin (7.5 mg/kg, Tribromoethanol, Sigma). The backs of the mice were shaved with an Oster Mark II animal clipper (Sunbeam-Oster, Fort Lauderdale, FL) followed by treatment with cooling hair removal gel (Church & Dwight Co., NJ). After disinfection with 7.5% iodine followed by 70% ethanol to remove iodine, 4 full-thickness punch biopsies (Acu.Punch, 6 mm, Acuderm, FL) were created. The skin incision was immediately covered with transparent dressing (3.8 cm x 3.8 cm, Systagenix, UK). The mice were monitored and photographed twice a day. The area of wound was assayed with ImageJ (NIH, USA) and the results were expressed as ratio of initial (day 0) wound area.

Analyzing cell populations from skin cells

Skin was collected from mice after sacrifice with an approved method. To obtain single cell population, the skin was minced by scissors and then immersed in collagenase IV solution (1 mg/ml in DMEM with 10% FBS) for 1-2 hr at 37 °C followed by filtration (pore size 70μm) and washing with PBS. Cells were then resuspended in 10% FBS in PBS. Cells were aliquoted to 1.5mL microcentrifuge tubes and labeled with CD11b and Ly6C antibodies at a final concentration of 2ug/mL on ice for 30min. Cells were then washed with PBS and analyzed using a BD FACScanto II flow cytometer.

Statistical analysis

Results are expressed as mean ± SD. Statistical significances between groups were determined using a two-tailed Student's *t* test and indicated by an asterisk in figures; *p* values < 0.05 were considered statistically significant.

3.4 Results

Compromise in the endosome-lysosome fusion and low-grade inflammation in macrophages challenged with super-low dose LPS

We first tested the endosomal acidification process in primary macrophages harvested from wild type mice treated with super-low dose LPS. As shown in Fig. 21, a higher dose LPS (100 ng/mL) induced significant acidification of the endosome as observed through flow cytometry analyses of the phagocytosed pH-sensitive dye. In contrast, super-low dose LPS (50 pg/mL) failed to induce any noticeable endosome acidification. Next, we monitored the fusion of endosome and lysosome through sequential incubation of fluorescently labeled dextran and zymosan. As shown in Fig 22, macrophages challenged with 50 pg/mL LPS exhibited significantly reduced fusion of endosome and lysosome as compared to control macrophages. To further corroborate with our conclusion, we performed electron microscopy analyses of WT macrophages treated with and without 50 pg/mL LPS. As shown in Fig 20, macrophages treated with 50 pg/mL have elevated levels of densely-stained lysosome structures as compared to fused gray-colored endo-lysosomes. We also tested whether super-low dose LPS may affect autophagy initiation. To test this, we treated the immortalized macrophages with a constitutive expression of GFP-LCIII. As shown in Fig 18, 50 pg/mL LPS reduced the levels of GFP-LCIII, suggesting that super-low dose LPS may also affect the autophagy process. We further examined the cellular levels p62, whose accumulation is correlated with the disruption of endosome-lysosome fusion [29]. As shown in Fig 19, super-low dose LPS induced a rapid accumulation of p62. Next, we determined the activation status of key stress kinases such as JNK and p38. As shown in Fig 17, 50 pg/mL LPS induces

mild yet sustained phosphorylation of JNK and p38. In contrast, 100 ng/mL LPS triggers a robust and transient phosphorylation of JNK and p38. Intriguingly, 50 pg/mL LPS not only failed to induce the phosphorylation of ERK, but also caused a slight reduction in ERK phosphorylation.

In addition, when autophagy was induced in WT macrophages with tunicamycin (Tm), super-low dose LPS stimulation was able to abolish its effects as observed through the accumulation of p62 and endosomal-lysosomal inhibition (Fig 23-24).

Tollip and IRAK-M facilitate the process of endosome-lysosome fusion

IRAK-M has been shown to modulate intra-cellular processes such as autophagy [30-32]. Thus, we tested the process of endosome-lysosome fusion in IRAK-M deficient cells. As shown in Fig 28, IRAK-M deficient macrophages also have a constitutive defect in the acidification of endosome. Similarly, the endosome-lysosome fusion process is also constitutively disrupted in IRAK-M deficient macrophages, as visualized in the clear separation of dextran and zymosan in IRAK-M deficient cells under confocal microscopy (Fig 26). Electron microscopy analyses also revealed elevated lysosomes in IRAK-M deficient macrophages (Fig 27). Western blot analyses demonstrated that cellular p62 levels are also constitutively elevated in resting macrophages harvested from IRAK-M deficient mice (Fig 25). There is no noticeable difference between IRAK-M deficient cells treated with or without super-low dose LPS.

Given previous findings that Tollip interacts with PI3P, Tom1, and localizes at endosome [27, 28] we also tested the hypothesis that Tollip may be involved in the process of endosome-lysosome fusion. As shown in Fig 32, Tollip deficient macrophages have a constitutive defect in the acidification of endosome. Furthermore,

the endosome-lysosome fusion process is constitutively disrupted in Tollip deficient macrophages, as visualized in the clear separation of dextran and zymosan in Tollip deficient cells under confocal microscopy (Fig 30). Electron microscopy analyses also revealed elevated lysosomes in Tollip deficient macrophages (Fig 31). Western blot analyses demonstrated that cellular p62 levels are constitutively and significantly elevated in resting macrophages harvested from Tollip deficient mice. To test whether Tollip modulates the effects of LPS, we examined these cellular processes in Tollip deficient cells treated with 50 pg/mL LPS. We observed that super-low dose LPS has no further effects in Tollip deficient macrophages (Fig 29).

Modulation of AMPK and VPS34 by super-low dose LPS

To further examine the underlying molecular mechanisms, we determined the phosphorylation status of AMPK and VPS34. The phosphorylation of AMPK is required for the initial assembly of autophagosome [17,18], while VPS34 phosphrylation inhibits endosome-lysosome fusion [25,26]. As shown in Fig 33 and 35, WT macrophages treated with 50 pg/mL LPS exhibit a rapid and dramatic reduction of AMPK phosphorylation. On the other hand, 50 pg/mL significantly induced the inhibitory phosphorylation of VPS34 (Fig 34 and 36).

Since we identified that IRAK-M and Tollip are involved in the process of lysosome-endosome fusion, we then determined the phosphorylation status of AMPK and VPS34 in IRAK-M and Tollip deficient cells. As shown in Fig 35, IRAK-M deficient cells have constitutively reduced levels of AMPK phosphorylation as compared to WT cells. LPS failed to further alter the phosphorylation status in IRAK-M deficient cells.

There was no noticeable difference in the levels of VPS34 phosphorylation comparing WT and IRAK-M deficient cells (Fig 36).

With regard to Tollip deficient cells, we observed a similarly reduced phosphorylation of AMPK in resting Tollip deficient macrophages (Fig 33). Strikingly, the inhibitory phosphorylation of VPS34 was constitutively elevated in Tollip deficient macrophages as compared to WT macrophages (Fig 34). LPS failed to further alter the phosphorylation status of either AMPK or VPS34 in Tollip deficient cells.

Impaired wound healing and elevated inflammation in Tollip deficient mice

We next tested the *in vivo* relevance of our above observations, by using a full thickness skin-deep excision wound model in wild type and Tollip deficient mice. As shown in Fig 37, Tollip deficient mice have significantly impaired ability to heal the skin wound as compared to the wild type mice. To further determine the cellular status of inflammation, we performed flow cytometry analyses of the CD11b+Ly6C+ pro-inflammatory monocytes in the wound tissues. As shown in Fig 38, there were significantly elevated levels of CD11b+Ly6C+ cells in the wound tissues of Tollip deficient mice as compared to WT mice. In terms of the effect of super-low dose LPS, we observed that the percentage of CD11b+Ly6C+ population was significantly elevated in wound tissues of mice injected with 100 pg/mL LPS as compared to PBS controls. Intriguingly, super-low dose LPS failed to further induce, instead significantly reduced the population of CD11b+Ly6C+ cells in Tollip deficient mice (Fig 38).

3.5 Discussion

Our current study reveals novel mechanisms that underlie chronic inflammation preferentially induced by super-low dose LPS. Our findings suggest that super-low dose LPS selectively induces cellular stress and interferes with endosomal-lysosomal fusion and autophagy completion resulting in the propagation of non-resolving inflammation. This conclusion is corroborated by a variety of evidence. First, super-low dose LPS induces persistent cellular stress and impedes fusion of the endosome and lysosome. Second, super-low dose LPS inhibits endosomal-lysosomal fusion through the modulation of AMPK and VPS34. Third, IRAK-M and Tollip which have previously been shown to modulate the innate immune response to LPS, are shown to be involved in the mediation of this novel pathway.

In addition to its traditional role in promoting cell survival, autophagy is known to play an integral role in mediating the innate immune response [33-34]. For example, in autophagy-deficient cells levels of the adaptor protein p62 accumulate, as we observed in WT macrophages treated with super-low dose LPS. This accumulation of p62 has been shown to activate NF-κB, the pro-inflammatory transcription factor, leading to chronic inflammation [29, 35, 36]. Our data complement and extend recent studies that support an intriguing link between perturbations in autophagy and chronic inflammatory diseases [11, 13-15, 37-39]. Our data shows that super-low dose LPS is highly potent in hindering endosomal-lysosomal fusion and autophagy completion. Studies such as this which highlight novel mechanisms utilized by super-low dose LPS to cause perturbations in these intracellular processes are necessary to further our understanding of the cellular and molecular mechanisms utilized to induce chronic low-grade inflammation.

Biochemically, our study provides a new perspective regarding the connection between Tollip and endosomal-lysosomal fusion and super-low dose LPS induced inflammation. Tollip has been shown to associate with endosomes and recruit the protein Tom1 and ubiqutin-conjugated proteins to these compartments for endosomal trafficking. Tom1 has been linked to the promotion of the maturation of autophagosomes and their subsequent fusion with lysosomes while a loss of Tom1 results in the accumulation of p62 and autophagosomes which are unable to undergo lysosomal fusion signifying its importance in the clearance of cellular debris and resolution of inflammation [27, 28]. Our data shows that in Tollip-deficient cells, endosomal-lysosomal fusion is suppressed irrespective of exposure to super-low dose LPS indicating its requirement for this process. In addition, we have previously shown that super-low dose LPS induces the translocation of Tollip from the cytoplasm to the mitochondria where it induces ROS production and chronic inflammation [10]. These results indicate a unique mechanism utilized by super-low dose LPS to recruit Tollip to the mitochondria where it triggers low-grade inflammation as opposed to allowing Tollip to associate with endosomes and recruit Tom1 to initiate the clearance of cellular debris.

In addition, wound healing represents the body's physiological response to tissue trauma. In a variety of inflammatory diseases delayed or impaired wound healing is observed where wounds remain in a state of chronic inflammation generating a gateway for persistent infection and sepsis. It has been shown that LPS plays a significant role in wound healing impairment [6, 40-42]. Our study shows that in Tollip-deficient mice, wound healing is severely impaired in the absence of LPS. Additionally, the wound tissue of Tollip-deficient mice showed significantly increased levels of pro-inflammatory

monocytes which decreased after super-low dose LPS exposure. This could highlight a potentially bi-phasic effect of Tollip where its role at resting levels and its role upon LPS stimulation serve different functions to negatively or positively regulate the pro-inflammatory immune response.

Taken together, our study reveals a novel mechanism that is responsible for low-grade inflammation and endosomal-lysosomal fusion inhibition induced by super-low dose LPS. Through the potential suppression of IRAK-M and the recruitment of Tollip to the mitochondria, thus hindering its involvement in facilitating autophagy and endosome-lysosome fusion, super-low dose LPS could promote the propagation of non-resolving inflammation. Understanding the unique molecular and cellular mechanisms utilized by super-low dose LPS to induce persistent low-grade inflammation may hold promise in the identification of targets for the development of therapeutics to treat and/or prevent chronic inflammatory diseases.

3.6 Figures

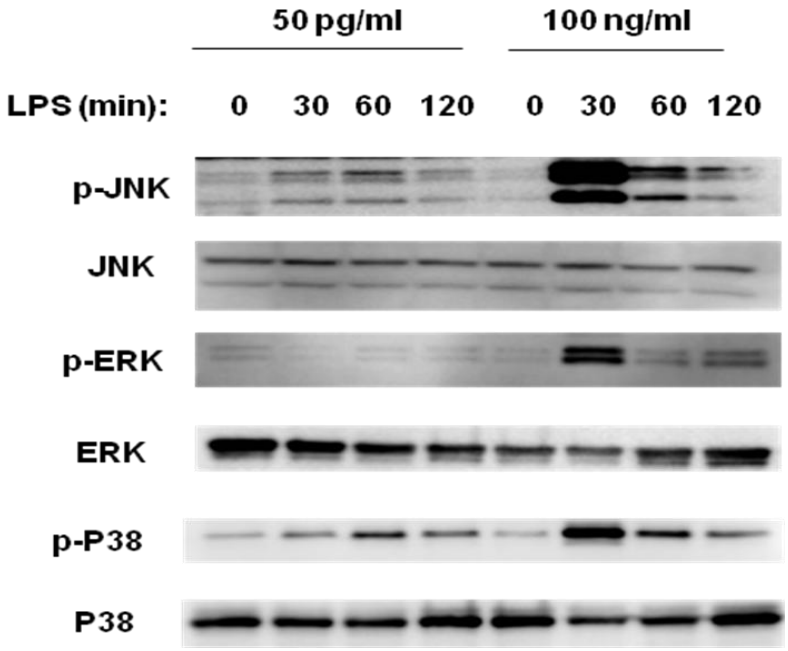

Figure 17: Super-low dose LPS induces mild inflammatory reaction
WT macrophages were treated with either 50pg/mL or 100ng/mL LPS for the indicated times. The phosphorylation levels of JNK, ERK, and p38 were determined by western blot. The same blots were probed for total levels of JNK, ERK, and p38 as a loading control. All data are representative of three independent experiments.

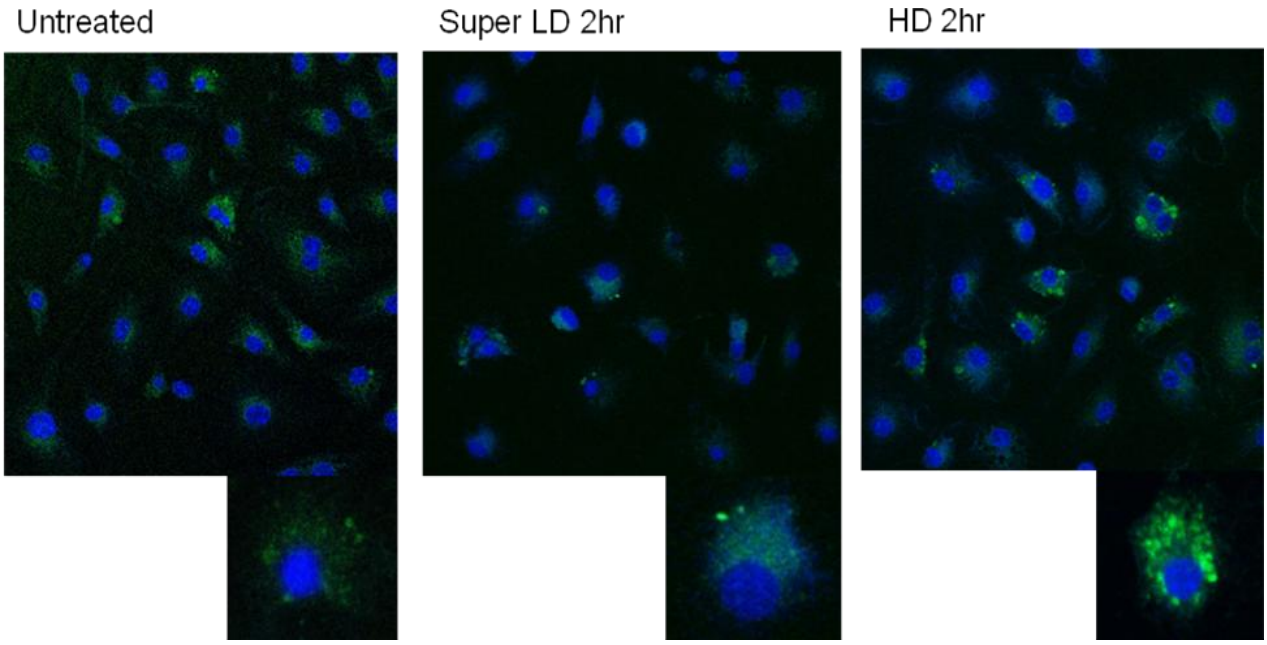

Figure 18: Super-low dose LPS suppresses LC3 conversion observed during autophagy

WT macrophages were incubated overnight with GFP-LC3 viral particles to allow for transfection. Cells were then treated with either 50pg/mL or 100ng/mL LPS for 2hr and the nuclei were stained with DAPI. GFP-LC3 puncta were visualized under a ZEISS LSM 510 laser-scanning confocal microscope. The merged images were magnified and shown on the bottom panel. All data are representative of three independent experiments.

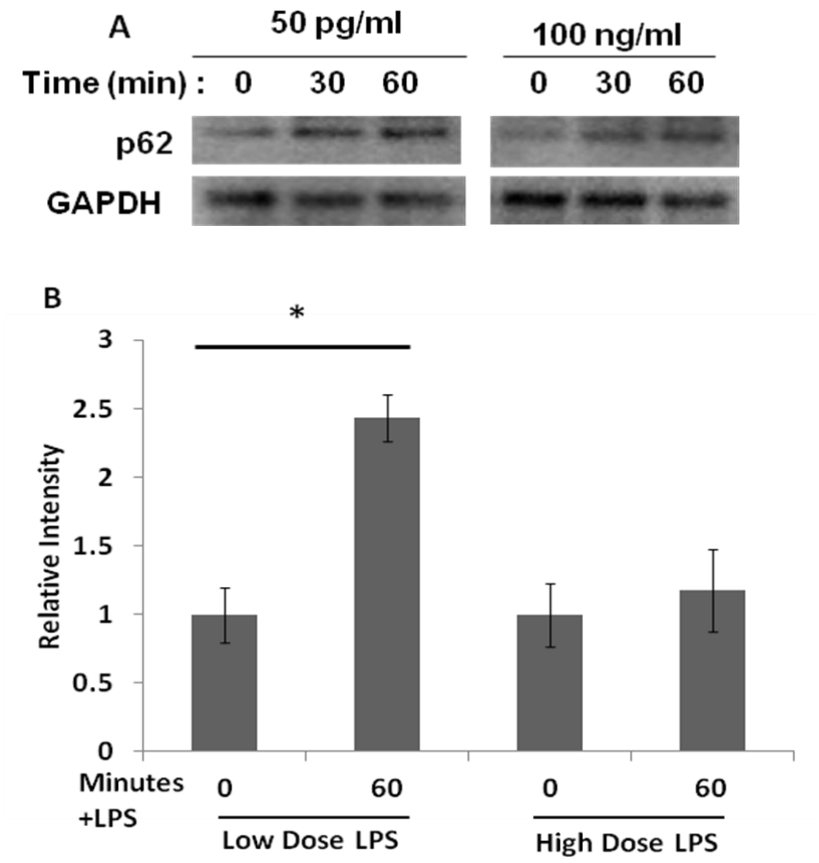

Figure 19: Super-low dose LPS induces p62 accumulation

(A) WT macrophages were treated with either 50pg/mL or 100ng/mL LPS for the indicated times. The levels of p62 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of p62 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

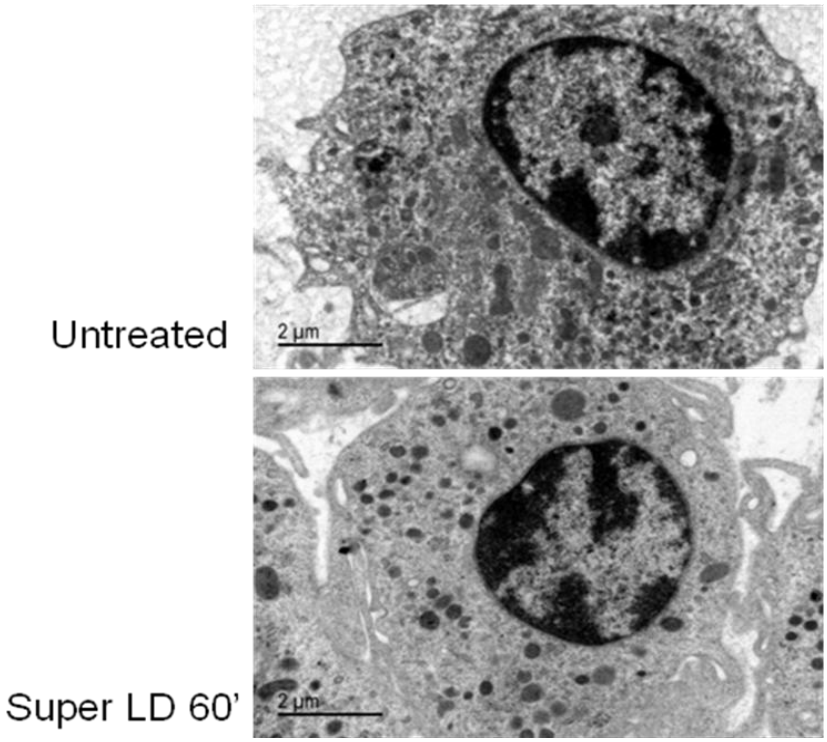

Figure 20: Super-low dose LPS induces the accumulation of lysosomes

WT macrophages were treated with super-low dose LPS (50pg/mL) for 1hr. Cells were prepared and visualized under a JEOL JEM 1400 transmission electron microscope. Lysosomes were identified as small dark bodies. All data are representative of three independent experiments.

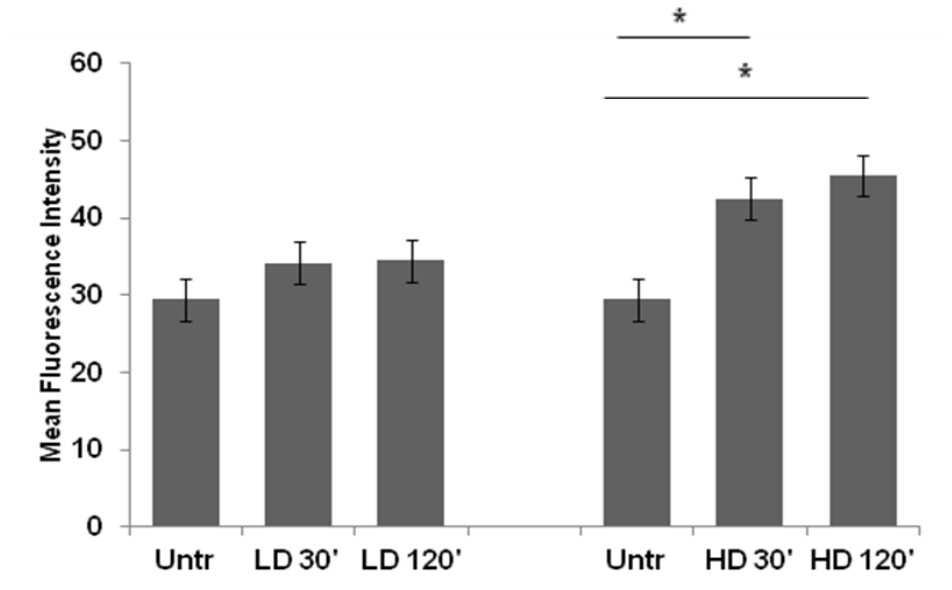

Figure 21: Super-low dose LPS fails to induce endosomal acidification

WT macrophages were treated with super-low dose (50pg/mL) or higher dose (100ng/mL) LPS for the indicated times. Cells were harvested and incubated in media containing 1 mg/ml fluorescein and tetramethylrhodamine double-labelled dextran (70 kDa) for 10min at 37°C. Cells were extensively washed and analyzed using a FACScanto flow cytometer. An increase in fluorescence intensity indicated a decrease in endosomal pH. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

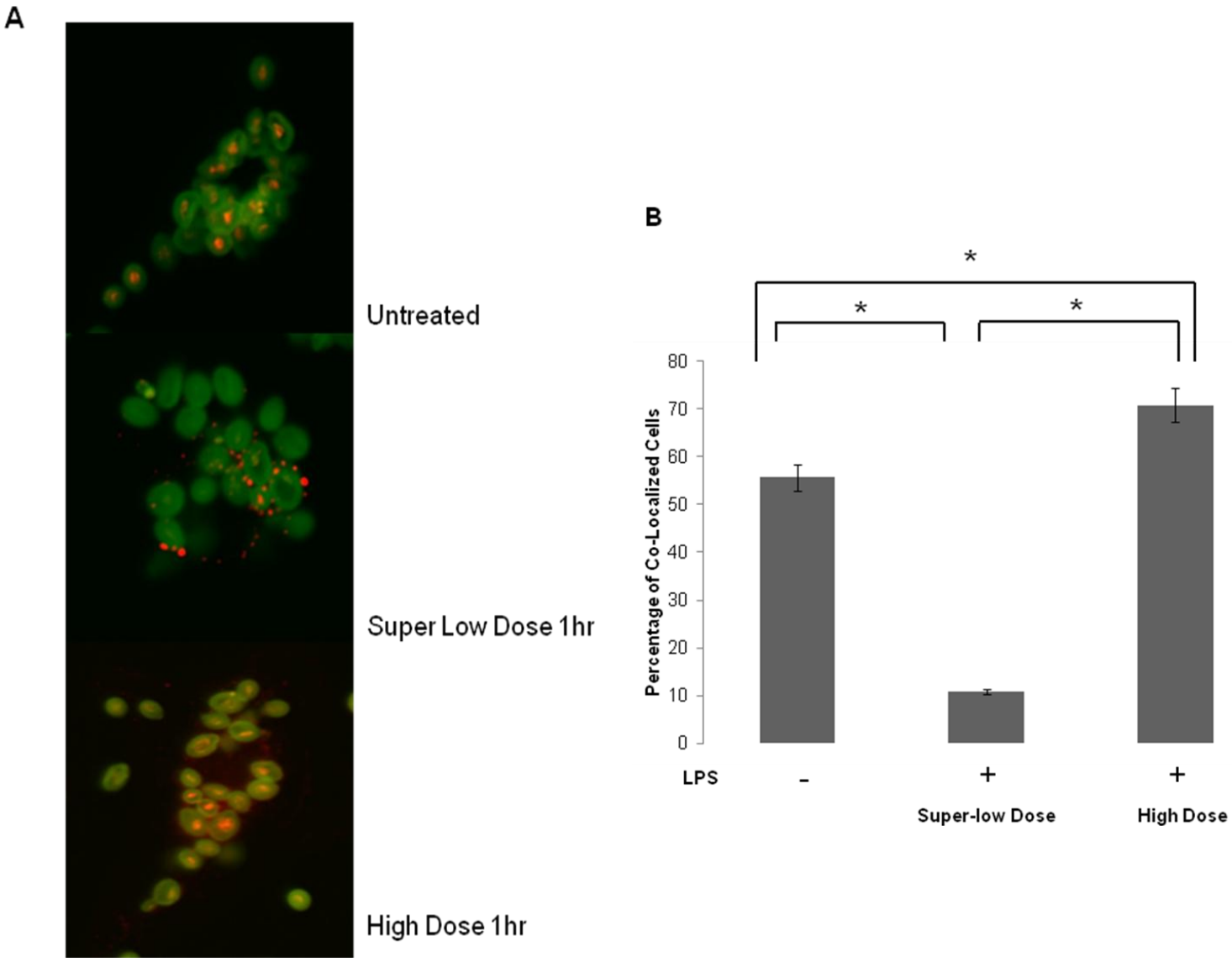

Figure 22: Super-low dose LPS hinders endosomal-lysosomal fusion

(A) WT macrophages were treated with super-low dose (50pg/mL) or higher dose (100ng/mL) LPS for 1hr followed by 1hr incubation with dextran-alexa fluor 546. Cells were challenged with zymosan-FITC bioparticles for 1hr and then fixed with 4% PFA. Fluorescence images were obtained with a laser-scanning confocal microscope Zeiss LSM510 to observe lysosome (red)–endosome (green) fusion. (B) Graph represents quantification of endosomal-lysosomal fusion. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

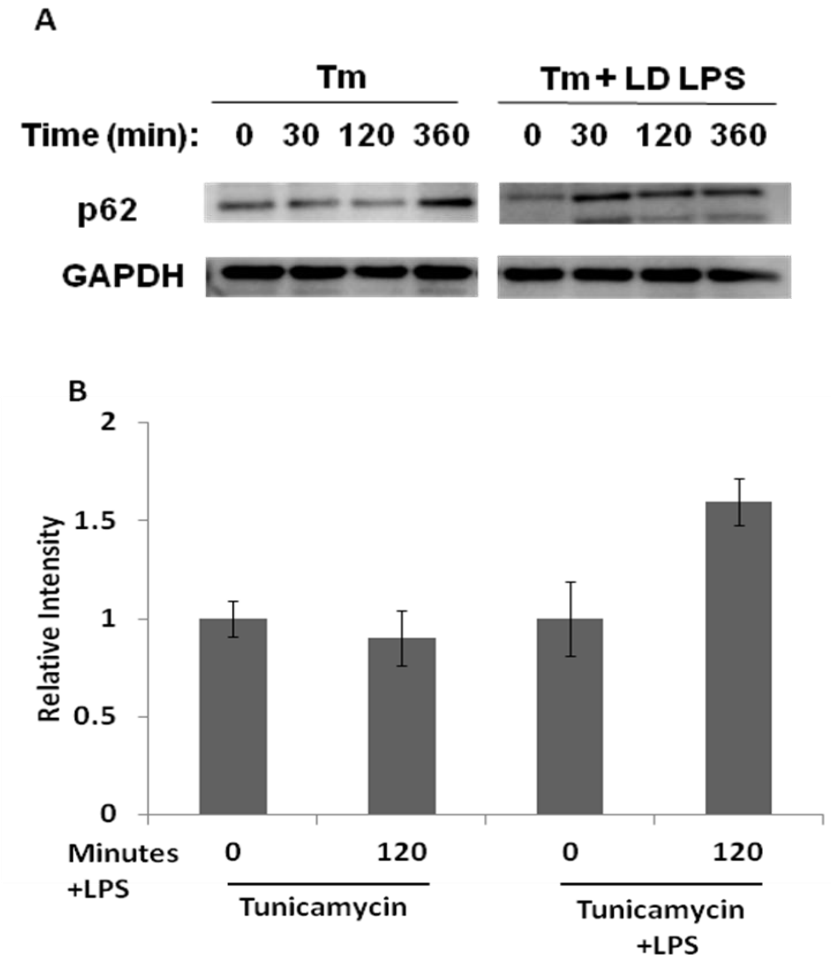

Figure 23: Super-low dose LPS prevents tunicamycin-induced p62 degradation

(A) WT macrophages were treated with super-low dose LPS (50pg/mL) in the presence or absence of tunicamycin (5µg/mL) for the indicated times. The levels of p62 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of p62 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

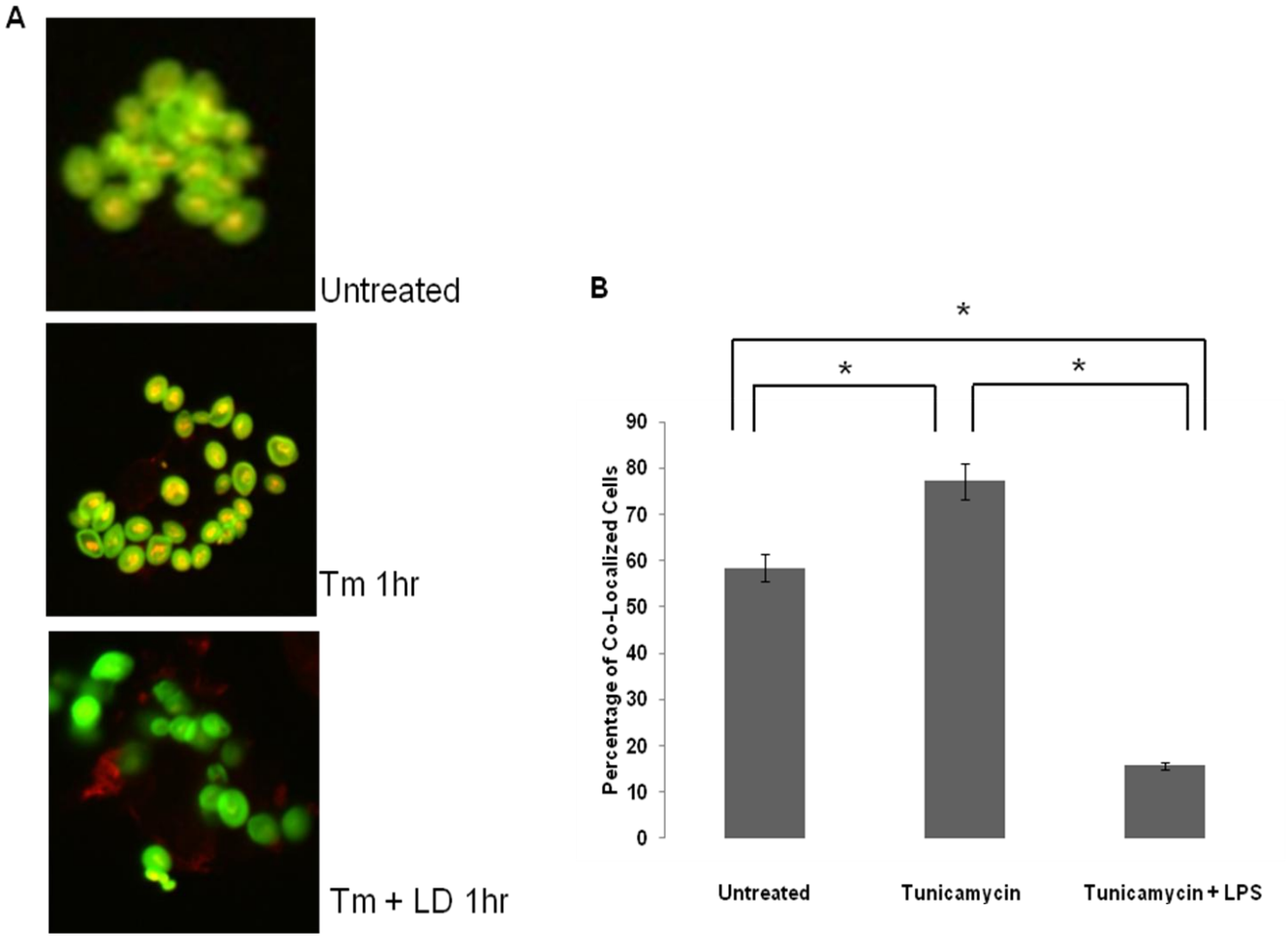

Figure 24: Super-low dose LPS prevents tunicamycin-induced endosomal-lysosomal fusion (A) WT macrophages were treated with super-low dose LPS (50pg/mL) in the presence or absence of tunicamycin (5µg/mL) for 1hr followed by 1hr incubation with dextran-alexa fluor 546. Cells were challenged with zymosan-FITC bioparticles for 1hr and then fixed with 4% PFA. Fluorescence images were obtained with a laser-scanning confocal microscope Zeiss LSM510 to observe lysosome (red)–endosome (green) fusion. (B) Graph represents quantification of endosomal-lysosomal fusion. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

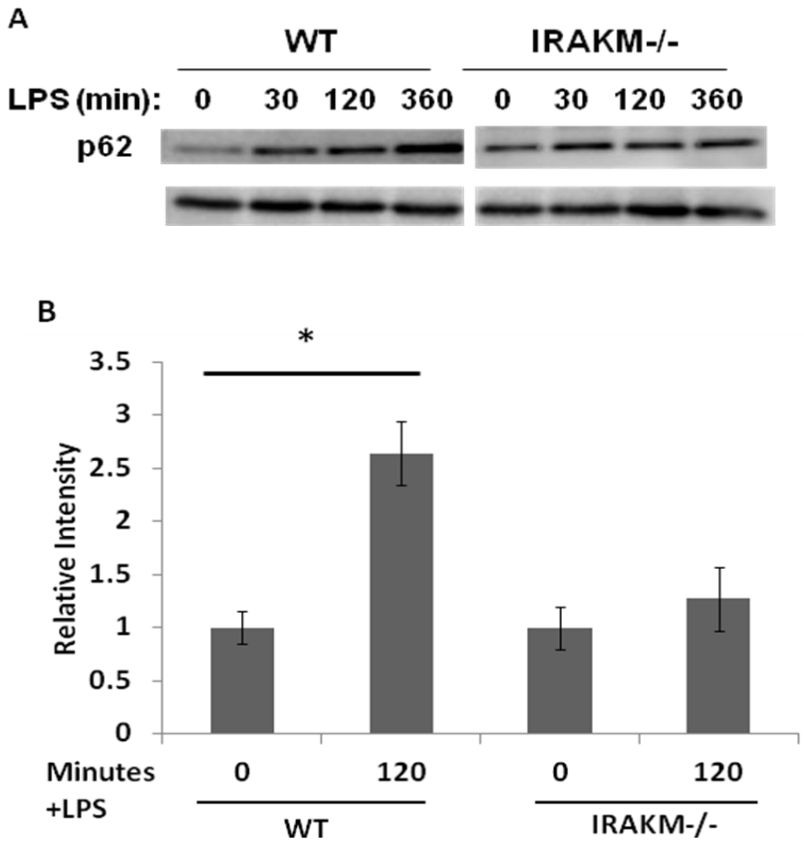

Figure 25: p62 accumulates at the basal level in IRAK-M deficient macrophages

(A) WT and IRAK-M deficient macrophages were treated with 50pg/mL LPS for the indicated times. The levels of p62 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of p62 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

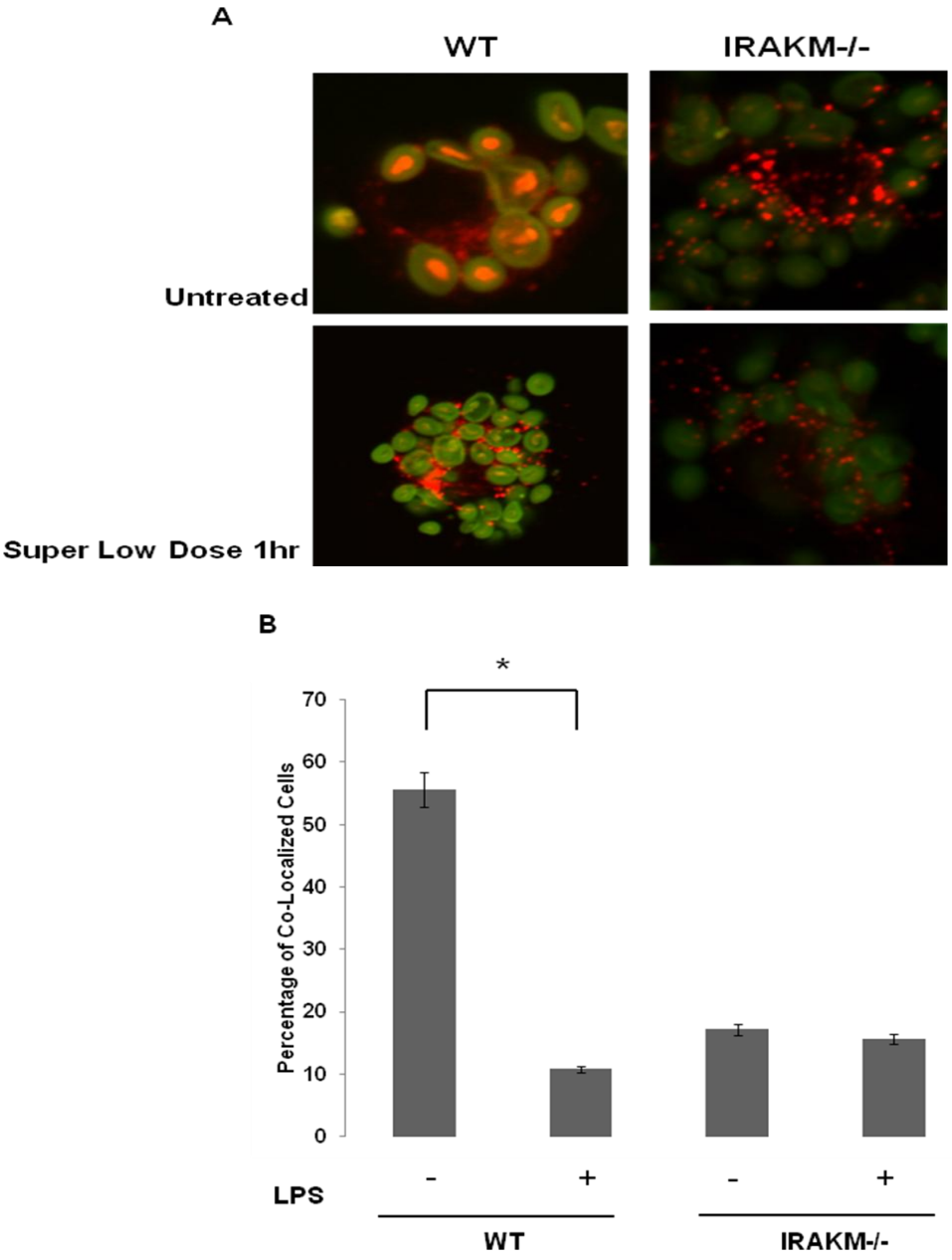

Figure 26: Inhibition of endosome-lysosome fusion irrespective of super-low dose LPS in IRAK-M deficient macrophages

(A) WT and IRAK-M deficient macrophages were treated with super-low dose LPS (50pg/mL) for 1hr followed by 1hr incubation with dextran-alexa fluor 546. Cells were challenged with zymosan-FITC bioparticles for 1hr and then fixed with 4% PFA. Fluorescence images were obtained with a laser-scanning confocal microscope Zeiss LSM510 to observe lysosome (red)–endosome (green) fusion. (B) Graph represents quantification of endosomal-lysosomal fusion. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

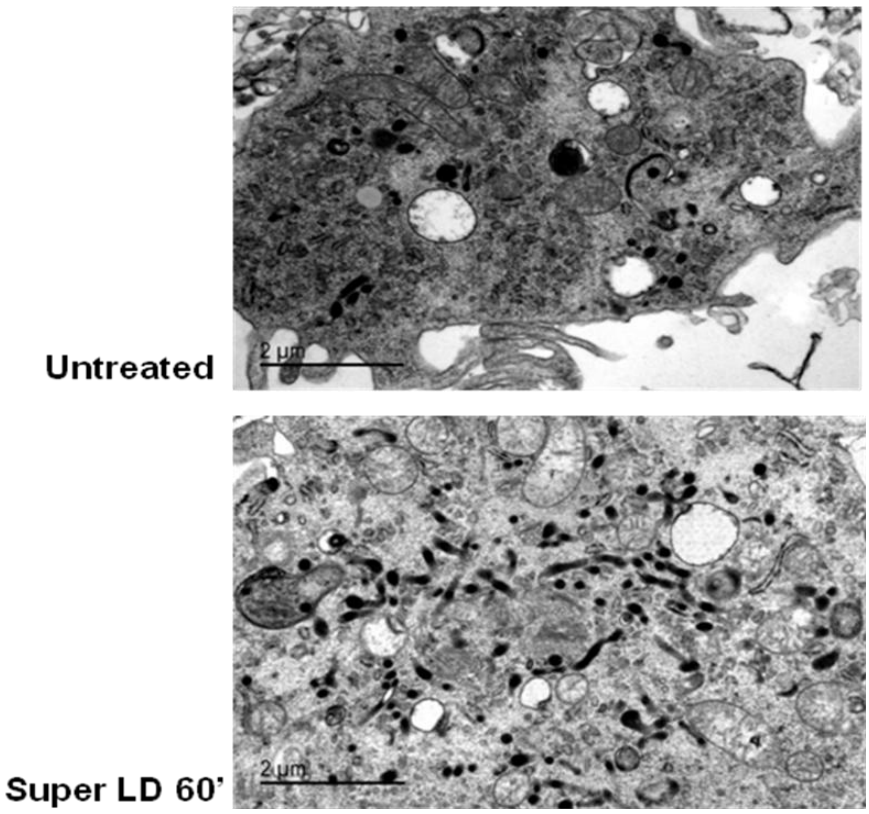

Figure 27: Lysosomal accumulation in IRAK-M deficient macrophages

IRAK-M deficient macrophages were treated with super-low dose LPS (50pg/mL) for 1hr. Cells were prepared and visualized under a JEOL JEM 1400 transmission electron microscope. Lysosomes were identified as small dark bodies. All data are representative of three independent experiments.

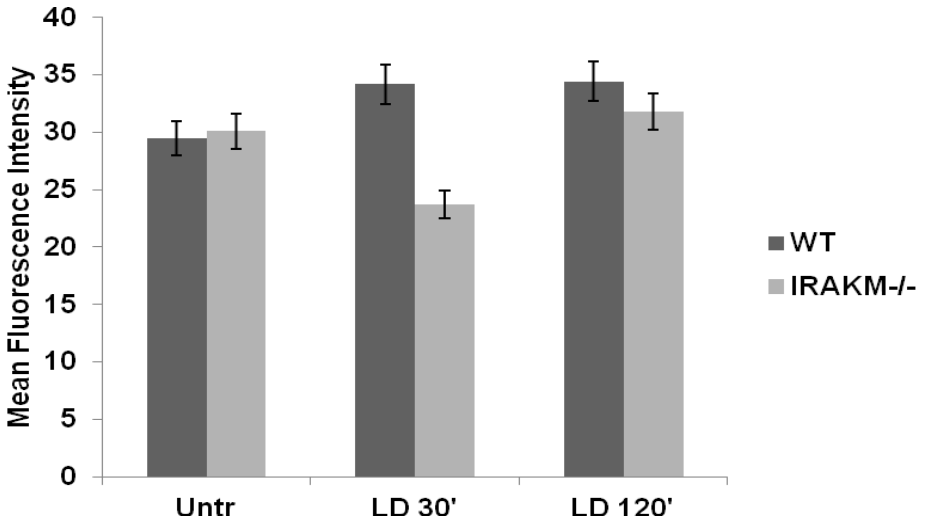

Figure 28: Super-low dose LPS fails to induce endosomal acidification in WT and IRAK-M deficient macrophages

WT and IRAK-M deficient macrophages were treated with super-low dose (50pg/mL) LPS for the indicated times. Cells were harvested and incubated in media containing 1 mg/ml fluorescein and tetramethylrhodamine double-labelled dextran (70 kDa) for 10min at 37℃. Cells were extensively washed and analyzed using a FACScanto flow cytometer. An increase in fluorescence intensity indicated a decrease in endosomal pH. All data are representative of three independent experiments and represented as mean value ± SD.

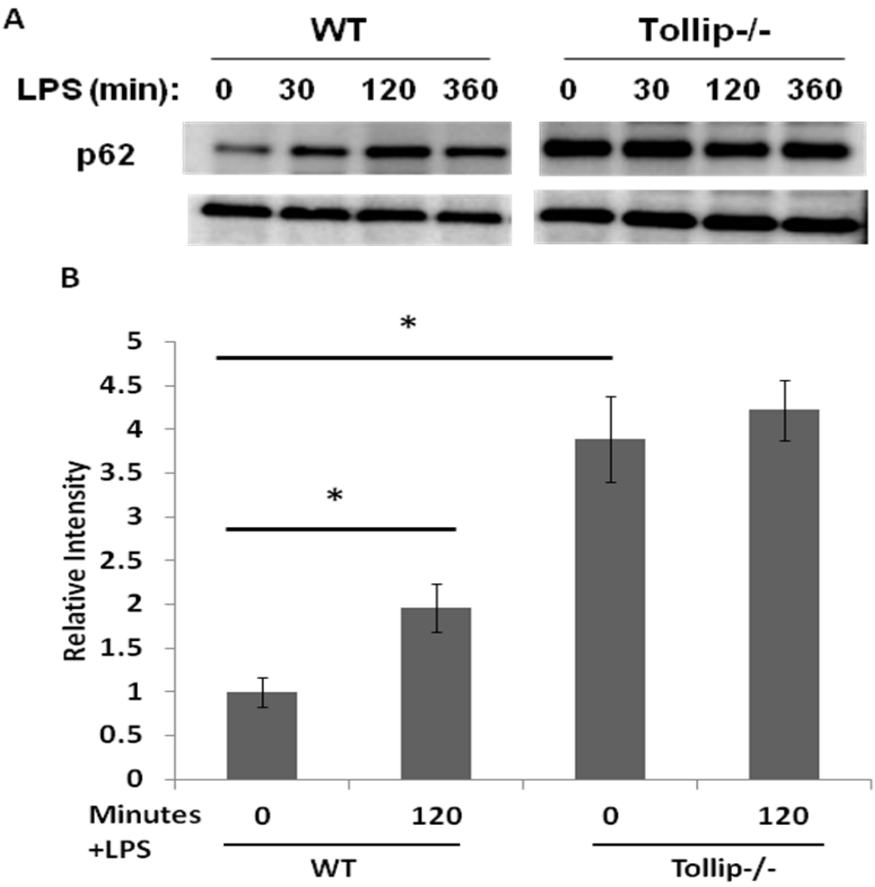

Figure 29: p62 accumulates at the basal level in Tollip deficient macrophages

(A) WT and Tollip deficient macrophages were treated with 50pg/mL LPS for the indicated times. The levels of p62 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of p62 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

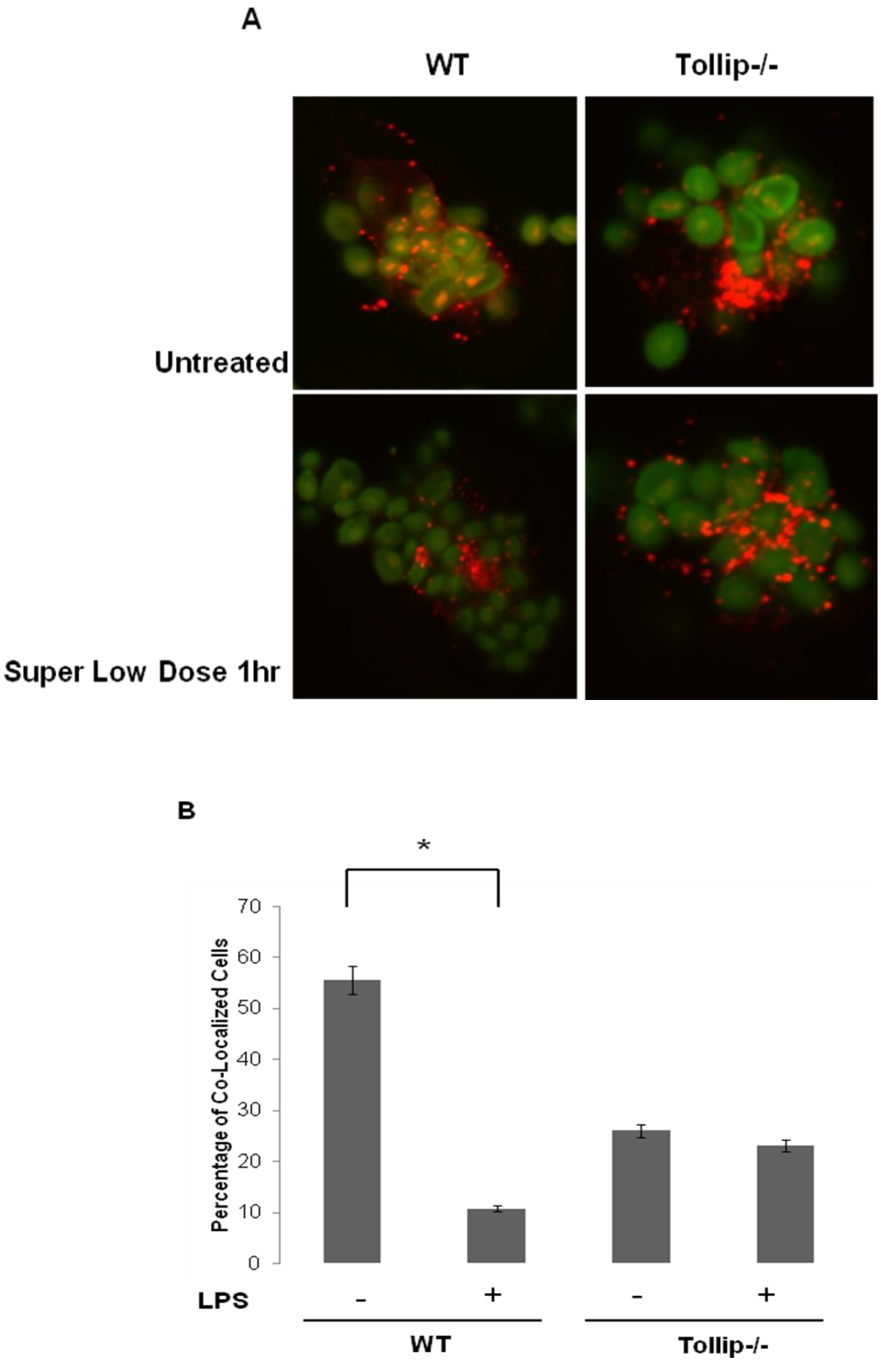

Figure 30: Inhibition of endosome-lysosome fusion irrespective of super-low dose LPS in Tollip deficient macrophages

(A) WT and Tollip deficient macrophages were treated with super-low dose LPS (50pg/mL) for 1hr followed by 1hr incubation with dextran-alexa fluor 546. Cells were challenged with zymosan-FITC bioparticles for 1hr and then fixed with 4% PFA. Fluorescence images were obtained with a laser-scanning confocal microscope Zeiss LSM510 to observe lysosome (red)–endosome (green) fusion. (B) Graph represents quantification of endosomal-lysosomal fusion. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

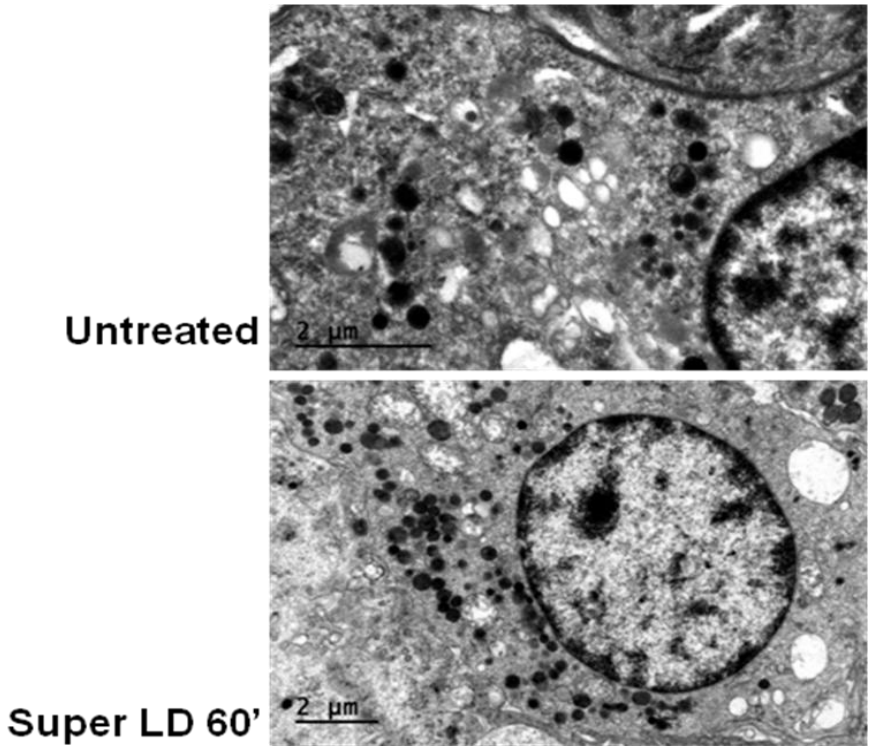

Figure 31: Accumulation of lysosomes and autophagic bodies in Tollip deficient macrophages

Tollip deficient macrophages were treated with super-low dose LPS (50pg/mL) for 1hr. Cells were prepared and visualized under a JEOL JEM 1400 transmission electron microscope. Lysosomes were identified as small dark bodies while autophagic bodies are represented by white double membrane vesicles. All data are representative of three

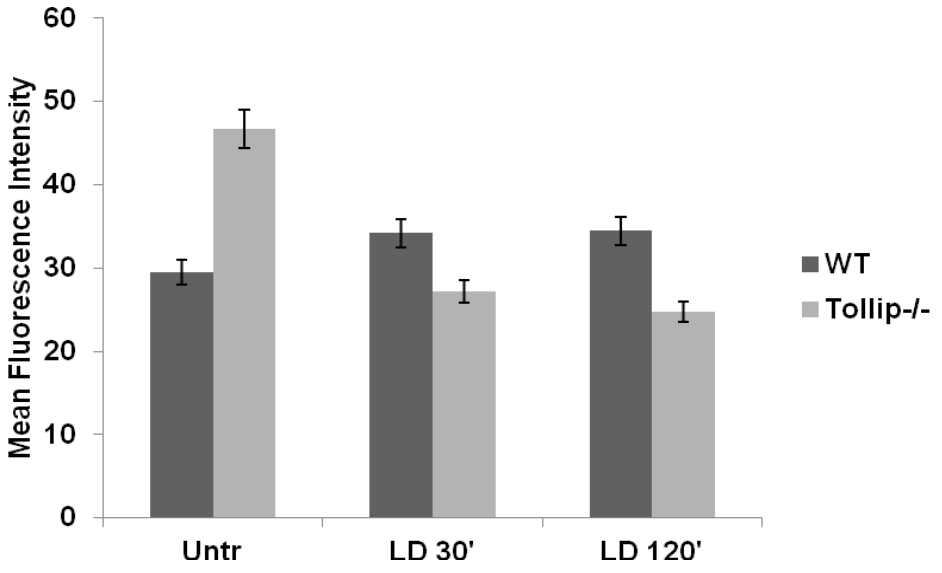

Figure 32: Super-low dose LPS fails to induce endosomal acidification in WT and Tollip deficient macrophages

WT and Tollip deficient macrophages were treated with super-low dose (50pg/mL) LPS for the indicated times. Cells were harvested and incubated in media containing 1 mg/ml fluorescein and tetramethylrhodamine double-labelled dextran (70 kDa) for 10min at 37°C. Cells were extensively washed and analyzed using a FACScanto flow cytometer. An increase in fluorescence intensity indicated a decrease in endosomal pH. All data are representative of three independent experiments and represented as mean value ± SD.

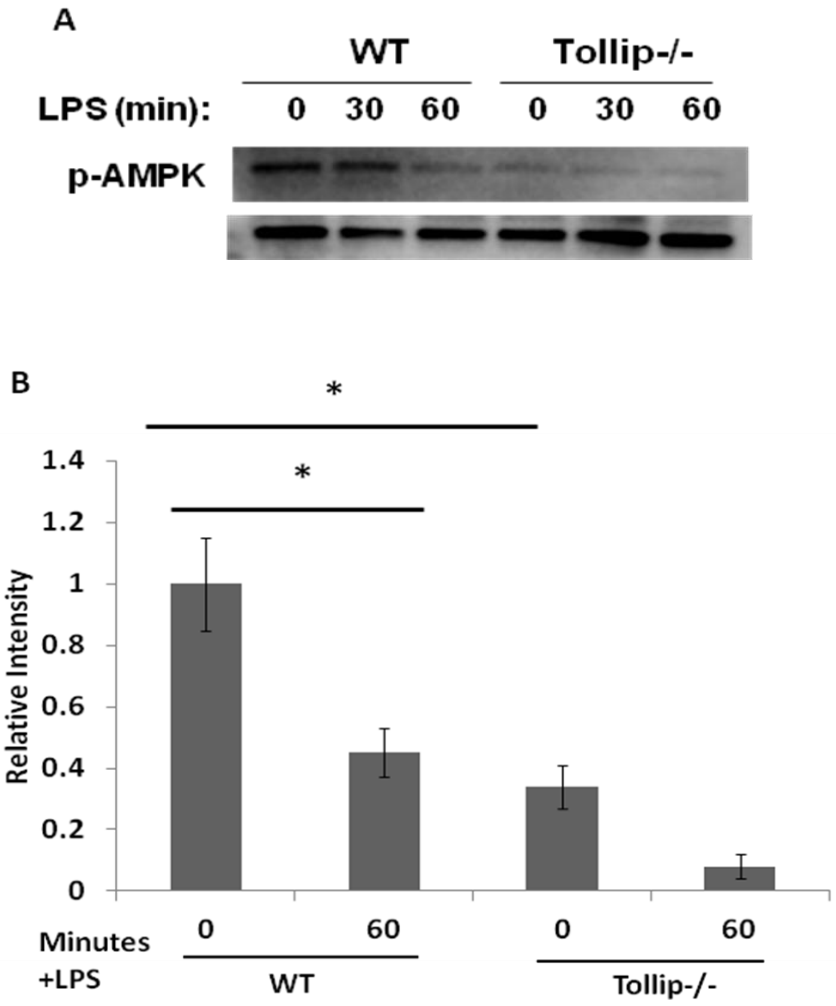

Figure 33: Super-low dose LPS fails to activate AMPK in a Tollip dependent manner

(A) WT and Tollip deficient macrophages were treated with 50pg/mL LPS for the indicated times. The phosphorylation levels of AMPK were determined by western blot. The same blots were probed with total AMPK as a loading control. (B) Band intensities of phosphorylated AMPK levels were quantified and normalized relative to total AMPK content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

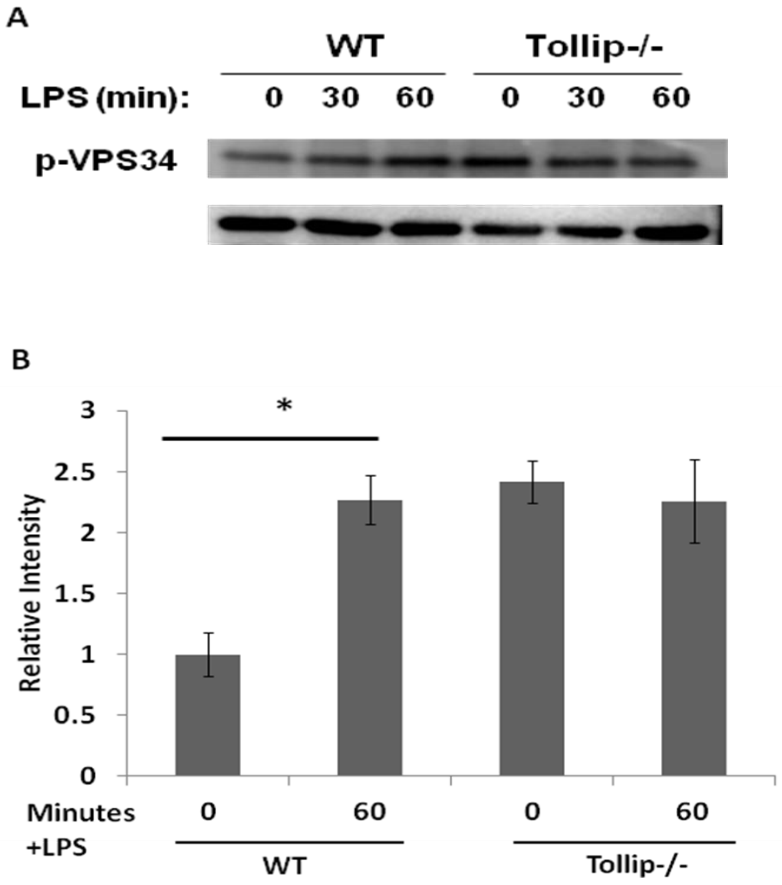

Figure 34: Super-low dose LPS induces VPS34 phosphorylation which is constitutive in Tollip deficient macrophages

(A) WT and Tollip deficient macrophages were treated with 50pg/mL LPS for the indicated times. The phosphorylation levels of VPS34 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of phosphorylated VPS34 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

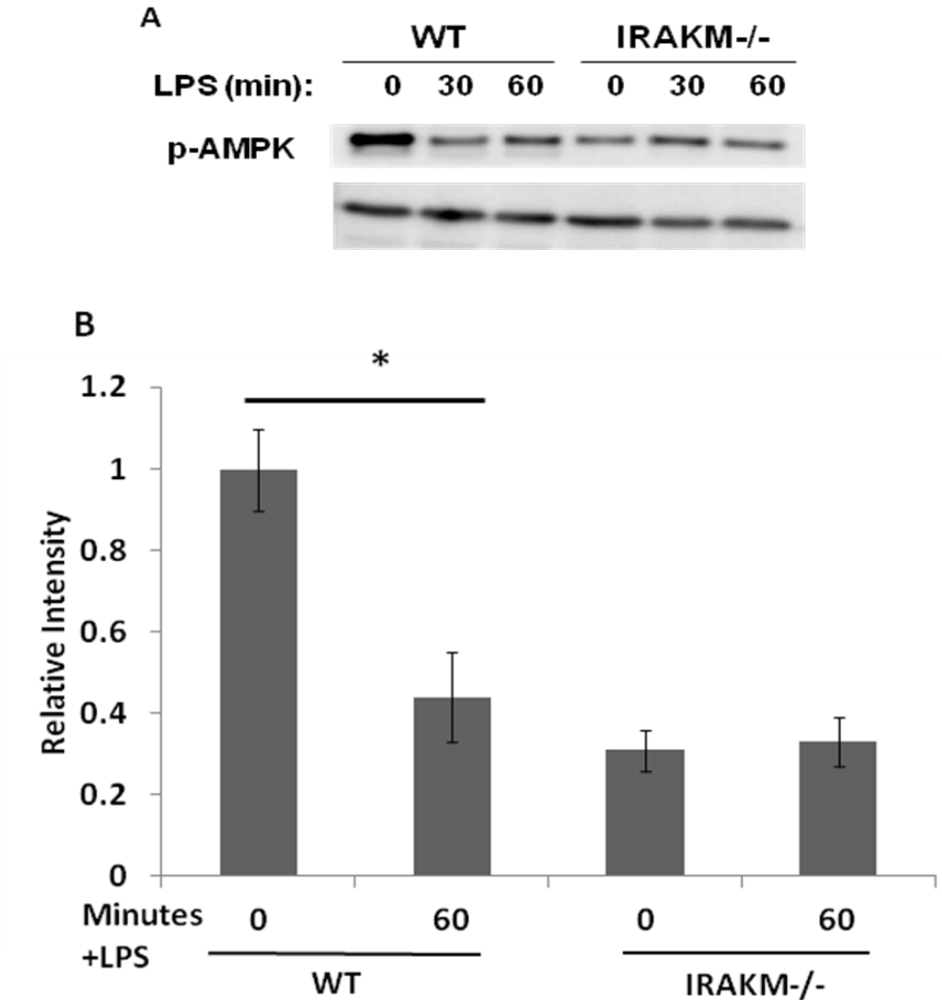

Figure 35: Super-low dose LPS fails to activate AMPK in an IRAK-M dependent manner
(A) WT and IRAK-M deficient macrophages were treated with 50pg/mL LPS for the indicated times. The phosphorylation levels of AMPK were determined by western blot. The same blots were probed with total AMPK as a loading control. (B) Band intensities of phosphorylated AMPK at the basal levels were quantified and normalized relative to total AMPK content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

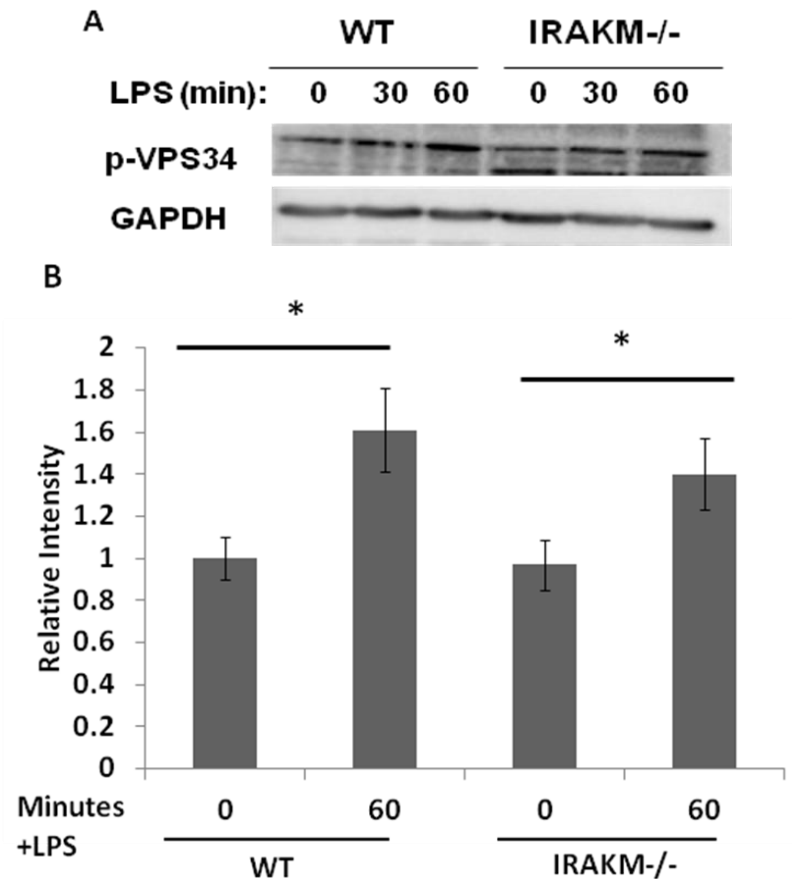

Figure 36: Super-low dose LPS induces VPS34 phosphorylation in WT and IRAK-M deficient macrophages

(A) WT and IRAK-M deficient macrophages were treated with 50pg/mL LPS for the indicated times. The phosphorylation levels of VPS34 were determined by western blot. The same blots were probed with GAPDH as a loading control. (B) Band intensities of phosphorylated VPS34 levels were quantified and normalized relative to GAPDH content. All data are representative of three independent experiments and represented as mean value ± SD. *P<0.05.

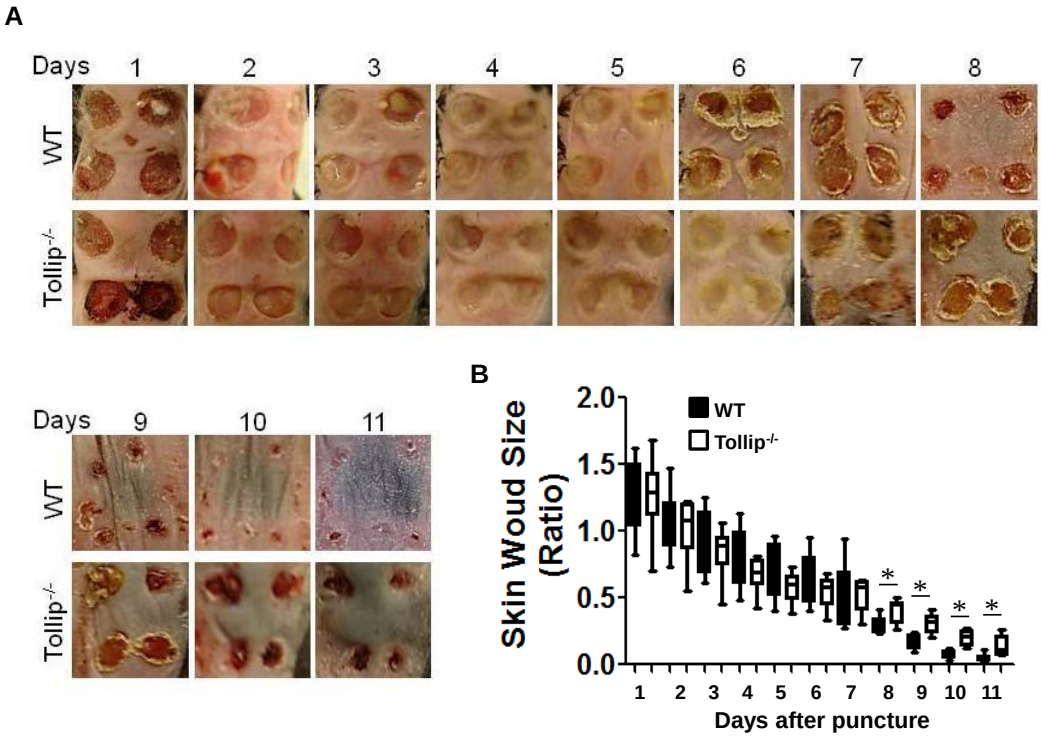

Figure 37: The influence of Tollip-absence on murine dermal wound healing.

Four excisional full-thickness wounds were created on each mouse dorsum. (A) Representative pictures of wound healings for mice from each group are shown. (B) The quantitative analysis of cumulative results. Each group of figures is representative of 8 mice at each time point. Wound sizes at any given time point after wounding were expressed as ratio of initial (day 0) wound area for Tollip$^{-/-}$ and WT mice. Results are expressed as the mean ± SE. n = 8 mice per group. *$P < 0.05$.

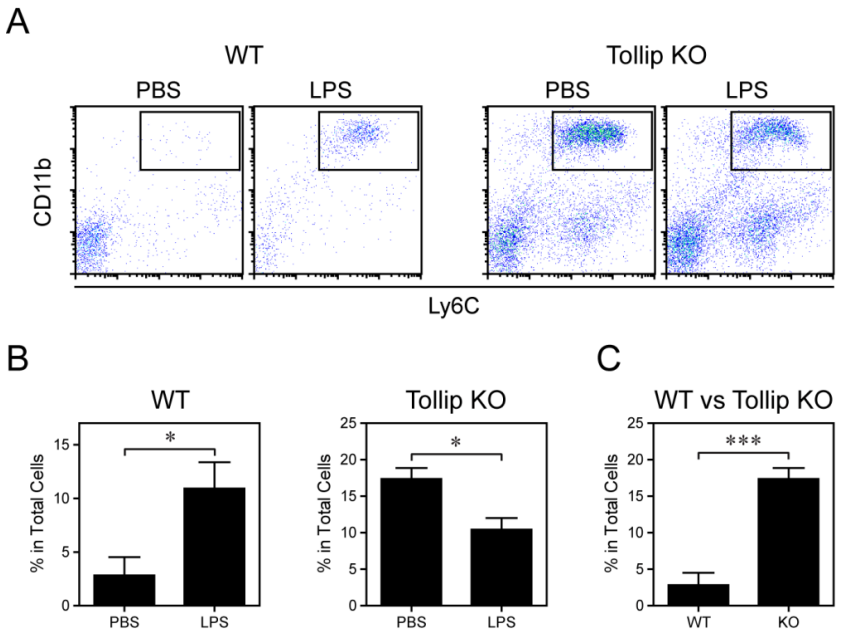

Figure 38: Increased level of inflammatory macrophages in the skin of Tollip deficient mice (A) Wound tissue was harvested from WT and Tollip-deficient mice and single cell populations were extracted and labeled with pro-inflammatory markers. Cells were first gated in Ly6G- population, and then analyzed for CD11b+/Ly6C+ inflammatory macrophages using a FACScanto flow cytometer. (B) Graph which demonstrates the frequency of Ly6G-/CD11b+/Ly6C+ inflammatory macrophages within the total skin cells. (C) Graph which shows the comparison between WT and Tollip-deficient mice after treatment with PBS. n = 8 mice per group $*P < 0.05$.

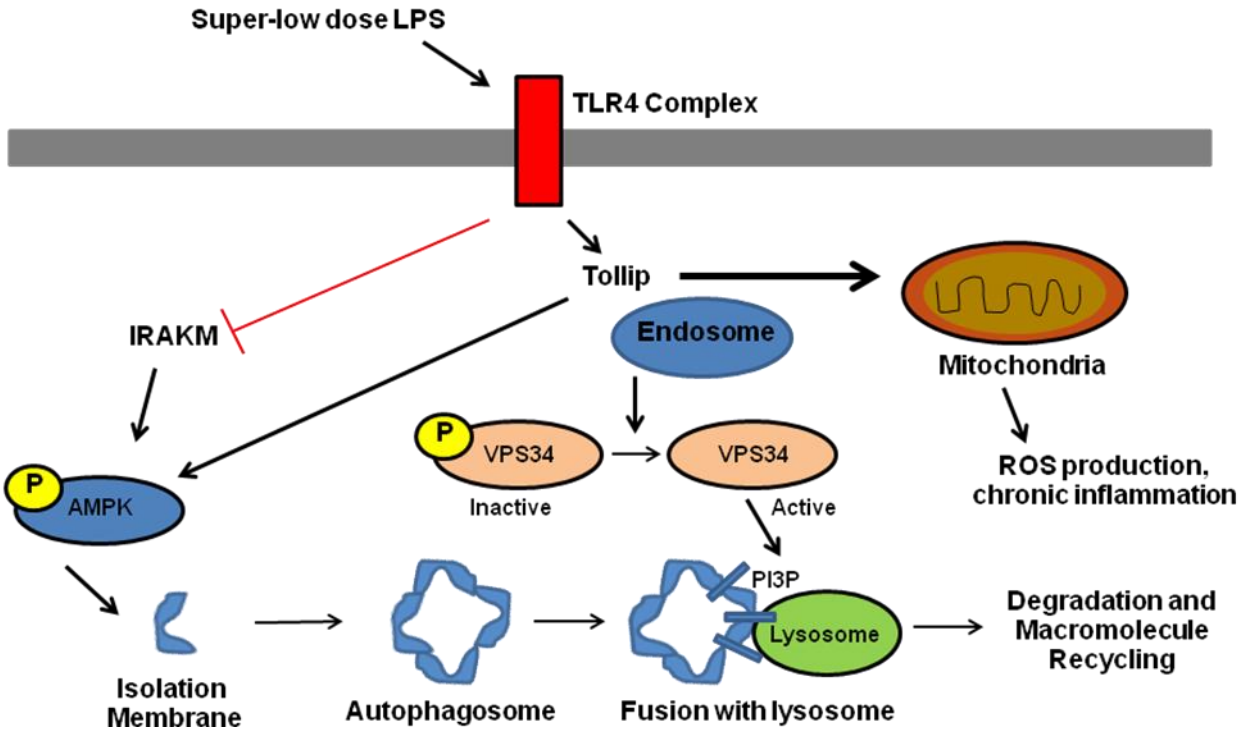

Figure 39: Super-low dose LPS hinders endosome-lysosome fusion promoting cellular stress and low-grade inflammation

Schematic representation of the novel mechanism utilized by super-low dose LPS to induce cellular stress and low-grade inflammation via the inhibition of endosome-lysosome fusion. Tollip facilitates the activation of AMPK and VPS34 which are crucial steps in the initation of autophagy and endosomal-lysosomal fusion. In addition, IRAK-M plays a role in the activation of AMPK and thus the initiation and formation of the autophagosome. Super-low dose LPS promotes non-resolving inflammation and endosome-lysosome fusion inhibition through the suppression of IRAK-M and the translocation of Tollip to the mitochondria where it contributes to ROS production and chronic inflammation.

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Chapter 4: Conclusions and Future Directions

4.1 Conclusions

Research into the innate immune response to the bacterial endotoxin LPS has been extensively studied. As a result of high doses of LPS inducing septic shock and sepsis, this mechanism has been extensively studied and well characterized. However, subclinical levels of LPS in circulation have been shown to lead to chronic inflammation through the mild but, persistent activation of pro-inflammatory mediators. With chronic inflammation being increasingly recognized as the underlying cause of numerous devastating inflammatory diseases including cancer, atherosclerosis, and Parkinson's disease, we must begin to understand the cellular and molecular mechanisms utilized by super-low dose LPS to propagate persistent low-grade inflammation. The following is a summary of my doctoral studies which I hope will contribute to furthering the understanding of low-dose endotoxemia and aid in the identification of potential therapeutic targets.

In Chapter 2, we observed that super-low dose LPS selectively induces cellular stress and necroptosis in an IRAK-1 dependent manner. Necroptosis represents caspase-independent programmed cell death and is gaining attention as a type of cell death which contributes to the inflammatory response by resulting in the release of various DAMPs (damage-associated molecular patterns). Super low-dose LPS causes the rapid and sustained activation of RIP3, the kinase responsible for initiating necroptosis. Necroptotic initiation leads to the activation of PGAM5 which dephosphorylates Drp1, the master regulator of mitochondrial fission, resulting in its activation. We observed that super-low dose LPS induces the dephosphorylation and activation of Drp1. In addition, super-low dose LPS degrades Mfn1, a protein involved in maintaining proper mitochondrial fusion

and antagonizing the function of Drp1. Collectively these represent mechanisms utilized by super-low dose LPS to induce mitochondrial fission and necroptosis in macrophages thus contributing to the propagation of non-resolving inflammation observed in low-grade endotoxemia. These phenotypes were abolished in IRAK-1 deficient cells highlighting its novel role in the initiation of necroptosis induced by super-low dose LPS. With research indicating that selective inhibition of necroptosis may constitute a viable treatment for chronic inflammatory diseases, IRAK-1 may represent a potential target in the intervention of persistent low-grade inflammation induced by super-low dose LPS.

In Chapter 3, we observed that super-low dose LPS potentiates cellular stress by hindering endosomal-lysosomal fusion and suppressing autophagy completion via the modulation of AMPK and VPS34. Autophagy constitutes a cell survival mechanism utilized by cells to survive in suboptimal conditions including nutrient deprivation. Cytoplasmic contents are sequestered in autophagosomes which ultimately fuse with lysosomes allowing for the degradation of cellular components and recycling of macromolecules necessary to maintain homeostasis. Perturbations in autophagy have been linked to cell vulnerability to stress, the promotion of pro-inflammatory immune responses, and the pathogenesis of chronic inflammatory diseases. This novel pathway utilized by super-low dose LPS represents another mechanism to induce persistent low-grade inflammation and cellular stress by interfering with endosome-lysosome fusion. We observed that Tollip and IRAK-M play critical roles in mediating this process. In addition, Tollip deficient cells displayed delayed wound healing and increased release of pro-inflammatory mediators furthering highlighting its significant role in reconciling the innate immune response to super-low dose LPS. Collectively, this study provides another

novel mechanism utilized by super-low dose to propogate non-resolving inflammation in an IRAK-M and Tollip dependent manner.

Collectively, these studies identify novel pathways utilized by super-low dose LPS to induce persistent inflammation which contributes the pathogenesis of a variety of devastating and debilitating diseases (Fig 41).

4.2 Future Directions

In the future, it will be important to explore possible interventions in these novel pathways. With the persistent cellular stress induced by super-low dose LPS, JNK and GSK3β inhibitors may represent effective treatments for preventing or reversing the chronic low-grade inflammation and disease progression observed in vivo and in vitro. Additionally, as we begin to understand the role of super-low dose LPS induced chronic inflammation in these novels pathways, it will be essential to identify molecular and cellular players which converge with the activation of the TLR4 pathway and could provide therapeutic targets to disrupt the propagation of non-resolving inflammation. We would like to pay particular attention to phosphatases, which are known to be involved in mediating the innate immune response to LPS and could promote chronic inflammation.

4.3 Final Remarks

Taken together, this work identifies novel cellular and molecular mechanisms utilized by super-low dose LPS to induce chronic inflammation. Beginning to recognize and clarify the selective and preferential pathways used to sustain the pro-inflammatory immune response observed in low-grade endotoxemia will allow us to identify potential therapeutic targets for the treatment and prevention and/or reversal of numerous chronic

inflammatory diseases. As we continue to uncover the mechanisms at the foundation of persistent low-grade inflammation, future studies focusing on intervention in these novel pathways are warranted.

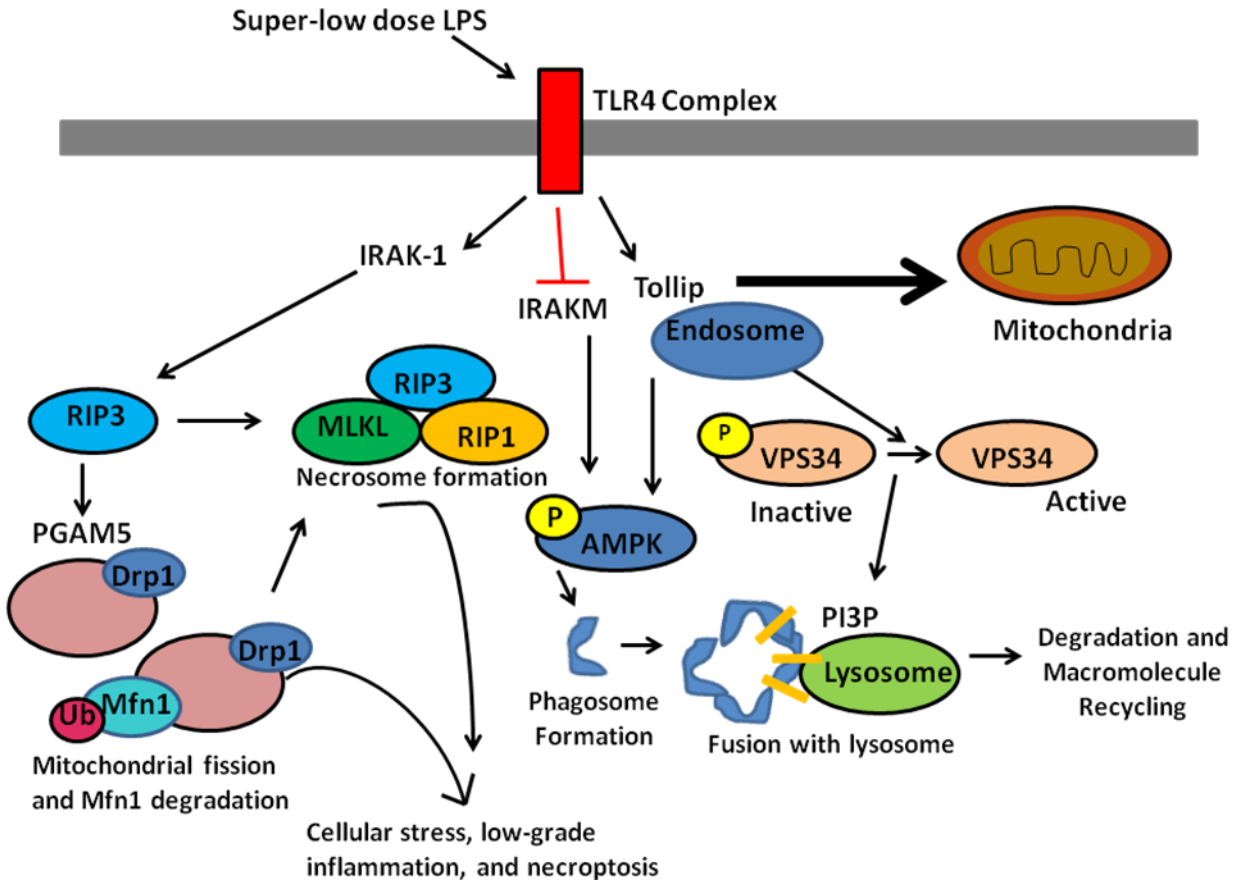

Figure 40: Novel mechanisms utilized by super-low dose LPS to promote chronic inflammation

Schematic representation of the novel mechanisms identified in this dissertation which are utilized by super-low dose LPS to induce inflammation. Chronic inflammation underlies the progression of various debilitating inflammatory diseases and disorders and by understanding mechanisms such as these we can begin to identify targets for prevention and treatment.