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INFLAMMASOME INDEPENDENT LEUKOTRIENE-B₄
PRODUCTION DRIVES CRYSTALLINE SILICA-INDUCED
STERILE INFLAMMATION

By

Bindu Hegde

A Dissertation Submitted to the Faculty of the School of Medicine of the University of
Louisville

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy in Microbiology and Immunology

Department of Microbiology and Immunology

University of Louisville

Louisville, Kentucky

May, 2018

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PRODUCTION DRIVES CRYSTALLINE SILICA-INDUCED
STERILE INFLAMMATION

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ABSTRACT

INFLAMMASOME INDEPENDENT LTB₄ PRODUCTION DRIVES CRYSTALLINE SILICA-INDUCED STERILE INFLAMMATION

Bindu Hegde

Ma & GE 2018

Silicosis is an irreversible lung inflammatory disease caused by chronic exposure to crystalline silica (CS) and is one of the most prevalent occupational diseases worldwide. Lipid chemoattractant Leukotriene B₄ (LTB₄) plays an important role in neutrophilic inflammation that drives silicosis and promotes lung cancer. Previous studies in our laboratory have demonstrated that CS-induced neutrophilic inflammation and lung tumor burden in K-ras^{LA1} mice is abrogated in the LTB₄-receptor deficient mice. Another pathway whose importance is well studied in the progression of silicosis is the Nalp3 inflammasome pathway. Studies have shown inflammasome- dependent IL-1 β to be important for the development of CS-induced pulmonary fibrosis.

In this study, we examined the cellular mechanisms involved in CS-induced inflammatory pathways. We demonstrate that phagocytosis of CS particles is essential for the production of LTB₄ and IL-1 β in macrophages, mast cells and neutrophils. CS uptake induced rapid formation of lipid bodies in the cytoplasm independent of inflammasome activation. The appearance of these lipid bodies correlated with LTB₄ production in mouse bone marrow-derived macrophages. LTB₄ synthesis enzymes 5-

LO, FLAP and LTA₄H, co-localized within the lipid bodies suggesting that they are not merely storage vesicles but the sites of CS-induced LTB₄ production. We coined the term “lipidosome” to define the functional unit of LTB₄ synthesis in these lipid bodies. Our studies with bafilomycin-A1 and NLRP3 deficient mice, confirmed that LTB₄ synthesis in the lipidosome is independent of inflammasome activation. siRNA knockdown and confocal microscopy studies revealed that lipidosome is closely associated with phagosome and their formation appeared to be seamlessly linked to the phagosome maturation pathway. GTPases Rab5c, Rab40c along with JNK1 are essential for lipidosome formation and LTB₄ production. Additionally, activation of JNK pathway is necessary for both LTB₄ and IL-1 β production. BI-78D3, a JNK inhibitor, completely abrogated CS-induced neutrophilic inflammation in an *in-vivo* air pouch model. In conclusion, these results highlight an inflammasome independent and JNK activation dependent lipidosome pathway as a major regulator of LTB₄ synthesis and CS-induced sterile inflammation.

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CHAPTER I

BACKGROUND

Silica Exposure

Silica or silicon dioxide is one of the most abundant elements on earth. Silica, also known as quartz or cristobalite exists in both crystalline and amorphous forms [1]. Occupational exposure to silica is a serious public health concern with millions of laborers working in mining, drilling, quarrying industries and construction sites continuously exposed to breathable silica particles [2-4].

Reports suggest that nearly 33 million workers in China and India, and around 2 million people in the U.S are occupationally exposed to crystalline silica (CS) [5, 6]. In developing countries like China, India and Brazil, it remains a major problem especially in the low and middle-income groups. With the growth in industry and the lack of effective measures to control the dust, silicosis cases continue to rise in China [7].

Pneumoconiosis¹ is the most prevalent occupational disease in China and accounts for 90% of all work related illnesses. In 2013 alone, 23,152 new cases of pneumoconiosis were diagnosed, 35% of those were confirmed to be silicosis (8,095 cases) [8].

In developed countries like the United States, silicosis was a major problem during the depression era. There have been several reports of silica epidemics in the past. The most significant one occurred in 1930-31 during the construction of Gauley Bridge tunnel in West Virginia, where more than 400 of the estimated 2000 workers died of silicosis and almost all the survivors developed silicosis [9]. More recently, in 1996, 60 of the 1072 workers in an automotive factory developed silicosis. In the last few decades, deaths due to silicosis appear to have declined due to awareness on the adverse effects of silica and improved workplace protection. Recently, the Occupational Safety and Health Administration (OSHA) revised the permissible limits of silica exposure at construction sites from 250µg/cc to 50µg/cc over an 8h period [10, 11]. These new guidelines can have a huge impact on the 2.3 million workers employed in construction industries. When in effect, this policy could save 600 lives and prevent 900 new cases of silicosis every year [10, 11].

Pneumoconiosis in coal miners is known as the “black lung” disease. The Coal Act of 1969 created the National Institute for Occupational Safety and Health (NIOSH)

¹ *Pneumoconiosis- Inflammation of the lungs caused by inhalation of coal dust, asbestos and*

for surveillance of coal dust exposure. The incidence of pneumoconiosis and progressive massive fibrosis (PMF) dropped to a historic low during 1990-99 with only of 31 cases reported. Although the overall incidence may appear to have decreased, this could be due a cluster of incidents undetected in the surveillance program, which was recently brought to attention by NIOSH. A higher number of miners are now seeking radiographs to obtain more information about their health, which has led to resurgence in the PMF cases (Fig. 1).

Figure 1. Prevalence of progressive massive fibrosis among underground-working coal miners from 1974–2015 in Kentucky, Virginia, and West Virginia [12].

Pathophysiology of development of silicosis

Silicosis is a chronic and irreversible inflammatory disease of the lungs caused due to silica exposure [13]. Silica particles less than 10 μ m in size are highly pathogenic

as they can escape the mucociliary defense mechanism and reach the distal parts of the lungs [2, 14]. Silicosis is a progressive, debilitating and often fatal lung disease. Exposure to silica can lead to other diseases such as lung fibrosis[15], chronic bronchitis[16], emphysema, increased susceptibility to tuberculosis [17], renal disorders[17] scleroderma [18], autoimmune diseases[18], and lung cancer [19-21].

Silicosis can be classified as chronic, accelerated, or acute based on the onset and rapidity of disease progression [22]. Chronic silicosis usually develops after 10-30 years of exposure to low concentrations of silica. This is the most common type. Acute and accelerated silicosis can take a few weeks to less than 10 years to develop and is caused by high doses of silica exposure. Silicosis is diagnosed with a combination of history of silica exposure, radiography, and CT scan [22, 23]. Pathologically, small round opacities (non-fibrogenic) are classified as simple silicosis whereas large opacities >1 cm size are classified as pneumoconiosis. Simple silicosis may be mostly asymptomatic, except for the presence of cough, and shows minor decrease in lung vital capacity. Pneumoconiosis is associated with chronic cough, dyspnea, pulmonary edema and progressive massive fibrosis. In severe cases, upper lobules of the lungs show dense nodules with central collagenous deposition or lipoproteinaceous material filling the alveolar spaces (silicoproteinosis) [13, 14, 22-24]. Fig 2 shows the histopathology of lungs of chronic silicosis patient.

Figure 2. Section of lungs of a chronic silicosis patient, showing central dense nodule with concentric whorls collagen fibers surrounded by a peripheral zone of silica dust-laden macrophages [25].

Evidence suggests that uncontrolled inflammation triggered by crystalline silica is responsible for the development of silicosis. Lung epithelial cells, fibroblasts, mast cells, and alveolar macrophages release an array of inflammatory mediators such as cytokines, neutrophil chemokines and reactive oxygen species (ROS) that contribute to the pathogenesis of the disease [26]. The mechanisms involved in the initiation of inflammation and its potential role in the pathogenesis of the disease are described here.

Sterile inflammation

Inflammatory cells like neutrophils and macrophages are the first line of defense against invasive pathogens. These phagocytes engulf the pathogens and release pro-inflammatory mediators that are responsible for the recruitment and activation of the adaptive immune system. Other than the pathogens, a myriad of substances like crystals, minerals, toxins, chemicals, antigens and mechanical trauma can trigger inflammation [27, 28]. Inflammation triggered by non-infectious agents is known as “**sterile inflammation**”. Both infectious and sterile inflammation have similar responses including the recruitment of neutrophils and macrophages, the production of inflammatory mediators and the induction of adaptive immune responses. The effector mechanisms adopted by these phagocytes to get rid of the pathogens are production of ROS, and degradation by proteases and acidic enzymes in the lysosomes [28]. The immune system is effective in clearing the infection rapidly; therefore, the tissue damage caused by these effector mechanisms is minimal. While these mechanisms might be useful in dealing with pathogens, they have the potential to cause more harm than good in case of sterile particulates. In the case of sterile inflammation, this could lead to chronic inflammation and significant tissue damage if the particles are not cleared. Due to this, sterile inflammation is the underlying cause for many inflammatory diseases [28]. Table 1 shows a list of sterile particles that can trigger inflammation along with the associated inflammatory disease.

Sterile particles	Condition	Affected area
Endogenous particles		
Monosodium urate crystals (MSU)	Gout	Joints
Calcium pyrophosphate crystals	Acute arthritis	Joints
β -amyloid	Alzheimer's disease	Brain
Cholesterol crystals	Atherosclerosis	Arteries
Exogenous particles		
Asbestos	Asbestosis	Lung
Silica	Silicosis	Lung

Table1. Inflammatory conditions caused by sterile particles

Once inhaled, silica particles persist in the system for a lifetime. Immune cells ingest the particles in an attempt to “digest” them. However, even the acid enzymes in the lysosomes are unable break it down. Eventually the phagocytes undergo apoptosis and the silica particles are released back into the system triggering a continuous cycle of phagocytosis and inflammation [29-31]. Unlike pathogen-induced cell death, which is helpful in containing the inflammation, sterile particle-induced cell death can induce an acute inflammatory response. Therefore, apoptotic cells and their debris themselves can play an important part in promoting the pathogenesis of the disease [32, 33]. Chronic

inflammation is followed by a reparative phase marked by increased proliferation of mesenchymal cells and neovascularization [26]. In order to repair the injured tissue, collagens, fibronectins, and growth factors are released. Certain cytokines released by the inflammatory cells, such as IL-1 β , TGF β and TNF- α are also fibrinogenic [34-36]. All these factors perpetuated by chronic inflammatory and reparative phases contribute to the formation of dense nodules that is responsible for respiratory failure in the lungs (Fig. 3).

Figure 3. Pathogenesis of silicosis: phagocytes ingest inhaled silica dust, deposited in the alveoli. Phagocytes undergo apoptosis releasing silica particles, several pro-inflammatory and fibrogenic factors that result in fibrous tissue formation. The process repeats resulting in formation of dense nodules and scarring of lung tissue [37].

Cellular mediators of silicosis

Alveolar macrophages, mast cells, and lung epithelial cells orchestrate the initial responses to silica in lung tissue. Production of neutrophil chemokines by these cells result in an **acute neutrophilic inflammatory response**, which is a hallmark of silica induced sterile inflammation [38]. Activation of the NLRP3 inflammasome and

inflammation triggered by necrotic cells contribute to a sustained neutrophilic inflammation in the lungs. The contribution of different cell types to the pathogenesis of the disease are discussed below.

Macrophages

Macrophages are one of the “first responders” at the site of silica deposition in lungs. Macrophages have specialized receptors known as scavenger receptors (SR) that recognize ligands, including bacteria and opsonized particles [1, 39]. SRs that are mainly associated with binding Crystalline Silica (CS) are SR-AI, SR-AII and MARCO [40-43]. Alveolar macrophages are present in large number and they amplify the signal by secreting several mediators. IL-1 β is one of the key mediators of sterile inflammation and several studies have demonstrated that persistent production of IL-1 β is a driving factor for silicosis [44-47]. IL-1 β is responsible for the upregulation of adhesion molecules necessary for recruitment of neutrophils and monocytes [48, 49], and is required for the production of several pro-inflammatory mediators [50].

IL-1 β and inflammasome pathway

A plethora of endogenous and exogenous agents is capable of activating the NLRP3 inflammasome pathway² [51]. Particulates such as low-density lipoprotein (LDL) cholesterol, MSU, amyloid- β peptides and crystalline silica capable of activating the inflammasome complex [52]. Inflammasome sensors can also detect various intracellular bacteria, viruses and intracellular protozoa [53, 54].

IL-1 β production is regulated by two separate mechanisms. First, activation of toll-like receptors (TLR) triggers the signaling cascade to increase the production of pro-IL-1 β via the transcription factor NF κ -B. The second step involves the activation of the pattern recognition receptor (PRR) NALP3, which results in the production of active IL-1 β from pro-IL-1 β [27]. NALP3 is a multi-ligand sensing receptor that can recognize a variety of “danger signals”. These danger signals can be pathogen-associated molecular patterns (PAMPs) present on pathogens (eg. LPS on the bacterial cell wall) or damage-associated molecular patterns (DAMPs) present on nuclear proteins or endogenous substances. Uptake of crystalline silica leads to phagolysosomal destabilization and activation of NALP3 [55]. NALP3 binds to the adaptor protein ASC and pro-caspase-1 to form the **inflammasome complex**. The assembly of inflammasome complex leads to the spontaneous activation of caspase-1, which cleaves the pre-formed pro-IL1 β to its active

² *Inflammasome activation is not exclusive to macrophages. It can occur in various myeloid and epithelial cells.*

form (Fig. 4). Similarly, inflammasome pathway can lead to the production of active IL-18 from its inactive form [27, 56, 57].

Figure 4. A schematic of inflammasome pathway. Priming of macrophages with low levels of IL-1 β following CS exposure results in activation of NF- κ B and production of pro-IL-1 β . CS uptake leads to destabilization of phagolysosomal membrane and leakage of its contents. This results in assembly of a multi-protein inflammasome complex consisting of NALP3, ASC and caspase-1. Active caspase-1 in the complex can cleave preformed pro-IL-1 β to its active IL-1 β [57].

Activation of the NALP3 inflammasome/IL-1 β driven inflammation plays an important role in the pathogenesis of other crystal-mediated diseases namely, MSU induced gout and cholesterol crystal induced atherosclerosis [58]. Additionally, macrophages can also produce IL-1 α , IL-33, and neutrophil chemokines, such as Leukotriene B4 (LTB₄) and CXC chemokines, in response to sterile inflammation [59]. IL-33 is produced at high levels by endothelial cells in response to silica [60]. Both IL-1 α

and IL-33 are active cytokines that initiate necrosis and are important for recruitment of neutrophils [61, 62].

M1/M2 macrophages and fibrosis

Macrophages display characteristics of heterogeneity and plasticity. In response to different stimuli, they can differentiate into pro-inflammatory, cytotoxic M1 or anti-inflammatory, cell proliferation and tissue repair-promoting M2 phenotypes. Differentiation of macrophages into the M2 phenotype promotes a microenvironment that supports fibrosis in several disease conditions [63, 64]. However, in the case of silicosis, the role of M2 macrophages is controversial. Several studies have shown that alveolar macrophages express M2 markers such as YM1, FIZZ1, Arginase 1 (Arg1), Nitric Oxide Synthase 2 (NOS2) and SOCS3 in response to silica [65, 66] [67]. Although, *Mission et.al.* have shown that M2 markers are expressed in the early stages (at day3), they are not persistently in silica- treated mice [65]. Some argue that M2 macrophages help in uptake of extracellular matrix components and resolution of fibrosis [68]. *Cruz et. al.* showed that treatment with Dasatinib reduces lung inflammation and fibrosis by inducing macrophage polarization towards M2 phenotype [69].

Mast cells

Mast cells are a specific subset derived from hematopoietic progenitor cells present in all organs and abundant in skin and mucosal tissues [70]. Mast cells are

known for mediating allergic reactions by crosslinking their surface receptors to IgE leading to degranulation and release of histamines, cytokines, arachidonic acid metabolites, and proteolytic enzymes causing inflammatory reactions [70]. Mast cells have been long known to play a role in the onset and progression of various inflammatory diseases such as asthma, allergic diseases, neuroinflammatory diseases (Alzheimer's disease, Parkinson's disease, and multiple sclerosis), and also arthritis [71].

Histological examination of lungs with silicosis has revealed an increase in the number of mast cells in silicosis patients [72]. Mast cells produce inflammatory mediators and cytokines such as mast cell tryptase, fibroblast growth factor (bFGF), interferon -gamma (IFN- γ), and transforming growth factor -beta (TGF- β), which play an important role in fibrinogenesis. The presence of mast cell produced bFGF in silicotic nodules in lung sections of patients suffering from silicosis has been demonstrated [73]. Mast cell deficient mice B6.Cg-Kit^{W-sh} do not develop silica-induced inflammation or fibrosis [74, 75]. These evidences suggest that mast cells are crucial for the development for silicosis. However, the exact mechanism through which mast cells mediate this effect is not clear. Previous studies in our laboratory showed that mast cells are a major source of LTB₄ in in-vitro experiments with CS [76]. Although small in numbers, because of their ability to produce massive amounts of LTB₄ in response to silica (10 x more than macrophages), they appear to be critical for neutrophil recruitment and promotion of CS-induced inflammation.

Neutrophils

Neutrophils are the most abundant leukocyte sub-population in the circulation. Neutrophils are short-lived cells and produced at a rate of 10^{11} cells per day in the bone marrow [77]. Neutrophils play a fundamental role in the acute phase of inflammation, and are capable of activating the adaptive immune system [78]. Neutrophils are not only powerful phagocytes, they can release large amounts of superoxide and reactive oxygen species [79]. They also have another unique mechanism of killing microbes by releasing a web of chromatin loaded with proteases, known as neutrophil extracellular traps (NETs), to trap and kill the microbes [80-82]. When the inflammatory response fails to return to a homeostasis, it could lead to chronic inflammation.

Due to the possible damaging side effects of their effector mechanisms, neutrophils are the driving factor of several inflammatory diseases. Neutrophils and NETs are found in the atherosclerotic plaques at all stages of the disease [83]. In fact, neutrophil count can predict cardiovascular events in both mice and humans [84]. Neutrophil infiltration is also observed in mouse models of Alzheimer's in the β -amyloid plaques [85]. Ng's group described neutrophil recruitment and migration pattern to the site of injury in the case of sterile inflammation using a laser injury model [86]. Similarly, neutrophils have been found to play an important role in the pathogenesis of other sterile inflammatory diseases such as gout caused by MSU, arthritis, and silicosis. Several reports, including the studies done in our laboratory, have indicated an increase

in neutrophil influx in silicosis [69, 87-89]. Neutrophil chemokines, leukotriene B₄ (LTB₄) and IL-1 β are critical for the development for silicosis [76] .

Leukotriene B₄- synthesis and role in inflammatory diseases

Arachidonic acid (AA) derivatives or eicosanoids are synthesized *de novo* in the cells in response to bacterial agents, inflammatory mediators, allergens and various stress factors. Eicosanoids are one of the first mediators released in response to silica. Three different pathways can metabolize AA: cyclooxygenase (COX), lipoxygenase (LOX) and epoxigenase (EPOX) pathways. Cyclooxygenase pathway results in the production of prostaglandins, procyclins and thromboxanes [90]. Prostaglandins are powerful vasodialators and regulate smooth muscle contraction, whereas thromboxanes are vasoconstrictors [90].

AA, a precursor for eicosanoid synthesis, is not freely available in the cell. Ca²⁺ is important for activation and translocation of the enzymes to intracellular membranes [91]. Upon activation, cytosolic phospholipase 2 (c-PLA₂) translocates to the Golgi apparatus, the endoplasmic reticulum, and the nuclear envelope. Active c-PLA₂ catalyzes the release AA from the membrane. AA is metabolized by 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP) to biologically active Leukotrienes (LTs). Cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) and LTB₄ are the end products of lipoxygenase pathway (Fig. 5). Cysteinyl leukotrienes (CysLT) mediate contraction of smooth muscle and immediate hypersensitivity reactions [92]. LTB₄ has been implicated

as a major mediator of inflammation through induction of leukocyte and lymphocyte activation. LTB_4 is a potent neutrophil chemoattractant and exerts its effects by acting on G-protein coupled receptors BLT1 and BLT2 [93]. BLT1 is the high affinity receptor and is expressed on immune cells namely neutrophils, macrophages, eosinophils and T cells [94, 95]. BLT2 is the low affinity receptor and is more ubiquitously expressed [94, 95].

Figure 5. A schematic of Leukotriene B₄ biosynthesis. LTB₄ is synthesized on the nuclear membrane by the sequential action of cPLA₂, 5LO-FLAP and LTA₄H enzymes in the lipoxygenase pathway [96].

Site of LT synthesis

Synthesis of LTs is a highly regulated process and requires interaction of at least four proteins on the phospholipid membrane. Nuclear membrane and endoplasmic reticulum is widely considered as the site of LT synthesis [97-99]. However, there is now an evolving understanding of compartmentalization of enzymes required for LT synthesis in other locations. Patricia Bozza was the first to propose that depending on the stimulatory conditions, the enzymes required for LT synthesis could be localized to a cytoplasmic site. From the studies done in her laboratory and others, it is now well-established that LTs could be synthesized in cytoplasmic lipid bodies [100-102]. cPLA₂ was found to localize within the lipid bodies [103, 104]. Additionally, electron microscope studies with radiolabelled AA provided evidence for the presence of active cPLA₂ in these organelles [104]. LT synthesis in lipid bodies was observed in different cell types under varying pathological conditions. Eicosanoid-containing lipid bodies were shown to be found in increased numbers in cancer [105], hypoxia [106], and infections [107, 108]. Although the enzymatic pathway of LT synthesis is well known, knowledge on the mechanisms of LT synthesis in lipid bodies is very limited.

Eicosanoids in inflammatory diseases and cancer

CysLTs and LTB₄ play a central role in the pathophysiology of asthma. Elevated levels of CysLTs have been detected in the breath condensates, bronchoalveolar lavage fluids, and sputum of asthma patients. CysLT receptor antagonists (e.g., montelukast, zafirlukast, pranlukast) and 5-LO inhibitors (e.g., zileuton) have shown therapeutic efficacy in patients with asthma [109]. LTB₄ plays a critical role in the pathogenesis of various other inflammatory diseases such as rheumatoid arthritis [110], atherosclerosis [111], and autoimmune uveitis [112]. The importance of LTB₄ pathway in the development of arthritis is demonstrated in 5-LO, LTA₄ hydrolase, and BLT1 knockout mice that do not develop arthritis [113].

Eicosanoids are implicated not just in inflammatory diseases but also in cancer. Studies have shown an upregulation in both COX2 and 5-LOX in colon, esophagus, breast, and pancreatic cancer [114]. A few studies have also reported polymorphisms in the ALOX-5 gene to be associated with colorectal and ovarian cancer risk [115-117]. Further evidence in support of the role of eicosanoids in cancer comes from epidemiological studies showing reduced incidence of solid tumors with the use of non-steroidal anti-inflammatory drugs (NSAID). The role of neutrophils and LTB₄ in silicosis-associated lung cancer is discussed further in the next section.

Current treatment for silicosis

Silicosis leads to decreased pulmonary function and increased susceptibility to various lung infections like tuberculosis. There is a lack of effective therapeutic interventions for silicosis. Most treatment options aim at alleviating pain and discomfort associated with the disease. Management of silicosis is similar to other chronic lung diseases, with the use of bronchodilators and corticosteroids to reduce the secondary symptoms like COPD, fibrosis, and inflammation [13, 118, 119]. However, there are no treatment options available to halt or reverse the progression of the disease. Lung transplantation is the only viable option in many cases [120].

Using protective measures remains the best way to avoid the disease. Respirable dust must be within legal limits at work places. Regular check-ups every 1-3 years is recommended for workers exposed to silica dust to diagnose the disease at an early stage. Once diagnosed, all exposure must be avoided to prevent the progression of the disease [23].

IL-1 β has been a potential target for sterile inflammatory disorders including silicosis. IL-R antagonist anakinra, was highly effective in patients with gout who failed to respond to previous therapies. However, blockade of IL-1 β only moderately reduced lung inflammation and fibrosis in mice and failed to show any promising therapeutic benefit in human clinical trials [35].

Another relatively recent approach for the treatment of silicosis is cell-based therapies. Local infusion of bone marrow derived mononuclear cells resulted in reduced inflammation, fibrosis and apoptosis, and improved lung functions in an experimental silicosis model. However, the beneficial effects lasted only 60 days [121]. Phase I clinical trials with autologous transplantation of bone marrow derived mononuclear and mesenchymal cells in silicosis patients showed improved lung function and no adverse effects after one year [122, 123]. Despite these efforts, there is a lack of effective therapeutic strategy for silicosis. Therefore, understanding the mechanisms of silica – induced inflammation to identify new therapeutic approaches becomes vital.

Silicosis and Lung Cancer

Lung cancer is a major cause of cancer related deaths worldwide. It is the second most common type of cancer in men and women (prostate and breast cancer being the most common type in men and women, respectively). More people die of lung cancer each year than prostate, breast, and colon cancer combined [124, 125]. Cigarette smoking is by far the leading cause for lung cancer. Exposure to radon, asbestos, and silica are some of the other causes for lung cancer. Exposure to asbestos leads to a type of cancer known as mesothelioma. Asbestos was widely used for insulation purpose in homes and industries. The use of asbestos has now been greatly restricted by the government after

recognizing the serious ill effects of asbestos [14]. Although the relative risk of lung cancer due to silica exposure is lower than smoking or asbestos exposure, it is significant nevertheless. According to OSHA, there is a 6% risk of getting lung cancer in workers exposed to a working lifetime (45 years) of silica [3]. Connection between CS exposure and lung cancer has been controversial. There are studies linking CS exposure to lung cancer, but they have been inconsistent. Long latent periods for cancer development, exposure to other carcinogens like cigarette smoke, genetic susceptibility, and poor nutrition have made it difficult to establish a cause–effect relationship [126]. Nevertheless, a 2016 meta-analysis supported the carcinogenic role of silica at higher exposures [127]. Another report suggested that silica exposure is at a higher risk of lung cancer among smokers. After careful assessment of studies with low confounding factors, the International Agency for Research on Cancer (IARC) classified CS as a Group I human carcinogen in 1997 [14, 124].

Inflammation is a promoter of various cancers including colorectal cancer, ovarian, pancreatic, and lung cancer. Several types of cancer arise at the site of inflammation or infection. In fact, inflammation is now considered a hallmark of cancer and a key component of the tumor microenvironment [128-130]. A tumor microenvironment consisting of immune cells, stromal cells and fibroblasts foster cancer survival and are indispensable players in tumorigenesis. The innate immune system is an integral part of the tumor microenvironment as well as the invasion, migration and

metastasis processes [131]. Cytokines, chemokines, transcription factors and infiltrating leukocytes can all contribute to the progression of cancer [131]. Although it is clear that inflammation alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, mediators and growth factors can promote cancer growth. Inflammation being key player in cancer development is also demonstrated by regression of tumors or a reduced risk of colon cancer development by COX2 inhibitors, NF- κ B inhibitors, aspirin, and other NSAIDs.

The main function of neutrophils is to defend the host from organisms and aid in wound healing. Once the neutrophils encounter the microorganisms they release a variety of cytotoxic substances, including proteases, that can damage the surrounding host tissue [132]. The role of neutrophils in cancer biology is increasingly becoming clear. Neutrophils make up a significant number of immune cells infiltrating the tumor microenvironment. High neutrophil numbers do not always correlate with increase in host defense. Tumor-associated neutrophils (TAN) have a distinct phenotype compared to blood neutrophils. They can be either anti-tumorigenic (N1) or pro-tumorigenic (N2) [132-136]. A high neutrophil count has been associated with a favorable prognosis in gastric tumors. On the contrary, neutrophilia has been considered a poor prognosis in many cancers, including colorectal cancer, non-small cell lung cancer, bronchoalveolar and renal carcinoma [135, 136]. In the case of mesothelioma, a higher neutrophil-lymphocyte ratio (NLR) is considered a poor prognosis and is now used as a predictive

marker for malignant mesothelioma [137-139]. Several studies have noted an increase in neutrophil population in silicosis [69, 87-89]. However, there is a lack of experimental mouse models studying silica-induced lung carcinogenesis. *Satpathy et. al.* from our laboratory developed a mouse model for examining the role of CS in promoting lung tumorigenesis [76]. While the neutrophilic inflammation was found to be predominant at the tumor sites, the precise role of neutrophils in accelerating tumor growth in these models remains to be determined.

Role of LTB₄-dependent neutrophilic inflammation in silica-induced lung cancer

The role of LTB₄ in several sterile inflammatory diseases is well established as discussed previously. Additionally, elevated levels of LTB₄ were detected in plasma, urine, and breath condensates of patients with silicosis [140]. Although, inflammatory conditions in selected organs are known to increase the risk of cancer, the role of LTB₄ in silica-induced lung cancer was not clearly understood. In our laboratory, we investigated the role of LTB₄-BLT1 pathway in CS-promoted lung tumor growth [76]. In K-ras^{LA1} mice that spontaneously develop lung cancer, CS exposure led to an increase in lung tumor burden. In BLT1^{-/-} K-ras^{LA1} mice that were exposed to CS, there was a significant attenuation of lung tumor burden in these mice. Histological examinations of lung

sections of BLT1^{+/+} K-ras^{LA1} mice exposed to CS showed neutrophil aggregates. CS-exposed BLT1^{-/-} K-ras^{LA1} showed reduced overall inflammation. Further analysis of bronchoalveolar lavage fluid (BALFs) and immunohistochemical sections revealed that CS-exposed BLT1^{-/-} K-ras^{LA1} had significantly lower neutrophil numbers compared to CS-exposed BLT1^{+/+} K-ras^{LA1}. Real-time PCR of RNA isolated from lungs indicate an elevated level of neutrophil active chemokines and cytokines. Ex-vivo studies in primary macrophages, mast cells, and neutrophils derived from mice produced large amounts of LTB₄ on stimulation with CS. Mouse macrophages also secreted IL-1 β and CXC CC neutrophil active chemokines. The importance of LTB₄-BLT1 axis in promoting was confirmed using two other murine models: an air pouch model- a simple inflammation model to investigate sterile inflammation -and an orthotopic tumor model.

Collectively the data suggests that neutrophil inflammation drives CS-induced lung cancer. More importantly, the LTB₄-BLT1 pathway is crucial for promotion of CS-induced lung cancer. Absence of LTB₄-BLT1 pathway led to a significant abrogation of CS-induced lung tumor promotion, further confirming the importance of LTB₄ in the pathogenesis of silicosis. This knowledge opens up various therapeutic possibilities for the treatment of silicosis and silicosis-associated lung cancer.

Goals of my study

Our previous studies have established LTB₄ as an important mediator in CS-induced sterile inflammation and lung tumor promotion (Fig. 6). CS-exposure results in the production of IL-1 β through the activation of inflammasome pathway, and LTB₄. While the inflammasome pathway is well studied, the key events leading up to LTB₄ synthesis is not clearly understood. In this dissertation, I set out to understand the mechanisms involved in CS-induced LTB₄ production. The goal of this study is to delineate the signaling pathways leading to LTB₄ and IL-1 β production. In addition, I wanted to understand how these pathways are integrated to mediate an overall inflammatory response. Lastly, I wanted to test the efficacy of select inhibitors of LTB₄ pathway in *in-vivo* models of CS-induced sterile inflammation.

Figure 6. Overview of CS-induced tumor promotion: Activation of resident macrophages, mast cells, and lung epithelial cells in response to CS, results in the production of LTB₄, IL-1 β and CXC and CC chemokines. This results in the recruitment of neutrophils to the lungs. Neutrophils themselves can secrete LTB₄, resulting in an amplification of the loop. Neutrophil inflammation is responsible for CS-induced promotion of lung tumor.

AIM I: To investigate whether CS-induced LTB₄ production is dependent on inflammasome pathway or IL-1 β production. I used specific phagocytosis inhibitors to determine whether uptake of CS is necessary for LTB₄ synthesis. I also used inflammasome inhibitors, BMDMs from inflammasome, and 5LO KO mice to determine how the two pathways are regulated. I used bone marrow-derived macrophages to study the interaction between LTB₄ and IL-1 β pathway. To address cell type-specificity, I performed experiments in primary mast cells, bone marrow-derived neutrophils and human neutrophils.

AIM II: To identify the source of LTB₄ production. Using confocal microscopy, various staining techniques, and siRNA knockdowns, I examined the molecular mediators required for lipid body formation and activation. I used bone marrow-derived macrophages for labeling cellular compartments and proteins. I used RAW264.7 cells in siRNA knockdown studies.

AIM III: To evaluate the role of JNK pathway in CS-induced sterile inflammation. Using *in-vitro* CS stimulations assays and an *in-vivo* airpouch model, I evaluated the role

of JNK activation in liposome activation and LTB₄ production. *In-vitro* experiments were performed in bone marrow-derived macrophages, mast cells, and neutrophils and human neutrophils.

CHAPTER II

MATERIALS AND METHODS

Reagents

Crystalline Silica (MIN-U-SIL-5; average particle diameter 1.7 μ m) was obtained from US Silica Co., WV, and was baked at 200°C overnight to make it endotoxin free. Zinc Oxide (ZnO) nanoparticles 10-30nm in size (US Research Nanomaterials, Inc., Houston, TX). Monosodium urate (MSU) crystals (tlrl-msu; Invivogen, San Diego, CA), mCherry expressing E. coli was described earlier in (16) and fluorescent red latex beads (L3280; Sigma-Aldrich, St. Louis, Missouri) were used as controls in phagocytosis experiments. Calcium ionophore A23187 was purchased from Sigma-Aldrich. The following pharmacological inhibitors were used in the study: Zileuton, Cytochalasin D, SB202190, and BI-78D3 (all from Sigma-Aldrich), Bafilomycin-A1 (Santa Cruz, Dallas, TX) and AG-126 (Cayman Chemicals, Ann Arbor, MI).

Mice, cell lines and primary cells

C57BL/6, 5-LO-deficient $Alox5^{-/-}$ and NALP3-deficient $Nlrp3^{-/-}$ mice were purchased from Jackson laboratories and bred at the University of Louisville. 6-8 weeks age old mice were used in ex-vivo and in-vivo experiments. All mice were cared for in accordance with the institutional and National Institutes of Health (NIH) guidelines. The University of Louisville Institutional Animal Care and Use Committee (IACUC) approved all the procedures.

Bone marrow derived macrophages

Mice (6–8-week-old) of indicated genotypes were euthanized by cervical dislocation. The hind legs were dissected and the bone marrow cells were flushed out. The bone marrow cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 50 mM β -mercaptoethanol supplemented with 50 ng/ml recombinant mouse macrophage colony-stimulating factor (BioLegend; San Diego, CA). The cells were plated at a density of 1 million cells per 100-mm tissue culture dishes containing 10 ml of medium. After 3 days, the medium was replaced by 10 ml of fresh growth medium. The culture were maintained for another 3 days before the experiments. The purity of the cells (>99%) was confirmed using flow cytometry by surface staining for F4/80 and CD11b.

Bone marrow derived mast cells

6–8-week-old mice of indicated genotypes were euthanized by cervical dislocation. The hind legs were dissected and the bone marrow cells were flushed out. The bone marrow cells were plated at density of 1 million cells in T-75-cm² flask containing 15 ml of DMEM medium containing 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 50 mM β -mercaptoethanol supplemented with 12.5 ng/ml recombinant mouse stem cell factor (R&D, 455 MC) and 10 ng/ml recombinant mouse IL-3 (R&D, 403-ML). The non-adherent cells were transferred after 48 h into fresh flasks. The cells were transferred into a new flask weekly with media containing freshly supplemented cytokines. The homogenous population of mast cells was visible after 4 weeks of culture and propagated further for 4 weeks. The purity of primary mast cells was confirmed to by flow cytometry using CD117 and Fc ϵ R1 cell surface markers [76].

RAW 264.7 macrophages

RAW 264.7 cells (ATCC, TIB-71) were maintained in DMEM containing 10% FBS, 100 units/ml Penicillin, 100 μ g/ml Streptomycin, 2mM L-Glutamine and 50 μ M β -mercaptoethanol.

Neutrophils

Human neutrophils were isolated from healthy donors using the plasma-Percoll gradient as previously described (15). The recruitment of healthy donors and all the procedures involved in blood draws were in accordance with the guidelines approved by the University of Louisville Institutional Review Board. Microscopy evaluation of isolated cells determined to be 90-95 % neutrophils and >97% of cells were viable by Trypan blue exclusion. Following Percoll gradient, a second purification step was performed to obtain 99.9% purity using EasySep Neutrophil Enrichment Kit (StemCell Technologies; Vancouver, Canada).

In-vitro CS stimulation assays

BMDMs were plated at a density of 0.3 million cells per well in a 12 well culture dish in 1 ml or 0.1 million cells per well in a 96 well culture dish in 200 μ l of DMEM containing 10% FBS and allowed to attach overnight. BMDMs were primed with 10 ng/ml of LPS (LPS-EK; InvivoGen, San Diego, CA) for 3 hours. The media was changed to 1% FBS containing media to achieve a final volume of 400 μ l in 12 well plate or 200 μ l in a 96 well plate. The cells were then pre-treated with the pharmacological inhibitors at the indicated concentrations for 1 hour prior to stimulation with 100 μ g/cm² of CS for 3-6 hours.

RAW 264.7 cells were plated at a density of 0.1 million cells in 100 μ l in a 96 well plate in 10% FBS containing media. They were primed for 3 hours with 10 ng/ml LPS. The media was changed to 1% FBS containing media. The cells were pre-treated with the compounds for an hour before stimulation with 100 μ g/cm² CS for 3 hours.

Mast cells were plated at a density of 0.1 million cells per well in a 12 well culture dish in 400 μ l of serum-free media. Mast cells required no LPS priming. The cells were pre-treated with the inhibitors for an hour before stimulation with 100 μ g/cm² CS for 3 hours.

Neutrophils plated at 0.1 million cells per well in a 96 well plate with 100 μ l of serum free media, were primed with 50 ng/ml LPS and stimulated with 100 μ g/cm² CS for 3 hours after pre-treatment with the inhibitors for an hour.

ELISA

LTB₄ and IL-1 β in the supernatants of cell culture and air pouch lavage fluid were measured using LTB₄ EIA Kit (Cayman Chemicals) and Mouse IL-1 β ELISA MAX Standard Kit (Biolegend) respectively, using manufacturer's instructions. Absorbance was measured using a BioTek (Winooski, VT) microplate reader at 405nm and 450nm for LTB₄ and IL-1 β respectively.

qRT-PCR

Cells were lysed using TRIzol reagent and total RNA was isolated using an RNeasy Mini Kit (Qiagen) using manufacturer's protocol. RNA samples were treated with DNase (Qiagen) to remove any trace of DNA from the samples before reverse transcription with TaqMan reverse transcription reagents (Applied Biosystems) using random hexamer primers. Quantitative PCR was performed using 'power SYBR-green master mix' (Applied Biosystems). Expression of the target genes was normalized to GAPDH and the relative fold changes relative to the PBS (vehicle)-treated were calculated using the delta CT method. Data were representative of triplicate cell cultures. GAPDH, CXCL1, CXCL2, CXCL3, CXCL5, CCL2, CCL3, CCL4 and CCL5 primers obtained from Real Time Primers.

MTT assay

RAW264.7 macrophages were pre-treated with the pharmacological inhibitors for an hour, followed by CS treatment. 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide (MTT) was added to the cells post CS-treatment. After 2h, absorbance was measured at 562 nm using a BioTek reader.

Immunofluorescence

1 x 10⁶ BMDMs or RAW 264.7 cells were plated in a 35 mm cover-glass bottom dish (World precision Instruments, Sarasota, FL) in DMEM with 10% FBS for attachment overnight. The media was changed to DMEM with 1% FBS and the cells were primed with LPS as described above. The cells were stimulated with 35 µg/cm² CS and processed for different staining protocols for microscopy as described below. The confocal images were captured using Nikon A1R confocal microscope at 60 x magnification with appropriate lasers as indicated. A minimum of five fields were captured for each sample. Confocal reflection microscopy combined with fluorescence microscopy was used to visualize internalized CS particles. Reflection was captured by allowing the laser light to directly pass to the detector channel.

BODIPY staining

After 3 hours of treatment with 35 µg/cm² CS, cells were washed with Hanks-buffered salt solution without calcium chloride and magnesium chloride (HBSS^{-/-}) and incubated with 1 µM BODIPY: 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-sindacene (ThermoFisher Scientific, Waltham, MA) for 1 hour at 37 °C to stain the lipid bodies. Cells were washed again with HBSS^{-/-} and fixed with 4% paraformaldehyde for 15 min. Subsequently, cells were permeabilized with 0.1% saponin and stained

with nuclear stain DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; ThermoFisher Scientific) (17).

Acridine orange staining

BMDMs were loaded with 5 µg/ml acridine orange (Sigma- Aldrich) for 30 min post CS treatment and washed with HBSS^{-/-} before imaging.

Phagosome staining

BMDMs were loaded with 40 µg/ml pHrodo Red Dextran (ThermoFisher Scientific) together with 1 µM BODIPY and DAPI for 1 hour at 37°C, after 3 hours of CS treatment. The cells were washed thoroughly with HBSS^{-/-} before analyzing by confocal microscopy.

Antibody staining

The cells were fixed with 4% paraformaldehyde for 15 min post CS treatment. The cells were permeabilized with 0.1% saponin and blocked with 5% BSA for 1 hour. Subsequently, the cells were incubated with either anti-Caspase-1, anti-Rab-40c, anti-FLAP (sc-56036, sc-514826, sc-28815; Santa Cruz), anti-5-LO (160402; Cayman Chemicals) or anti-LTA₄H (ab196607) antibodies at 1:500 dilution overnight at 40°C. The cells were washed three times with 1x PBS and incubated with the appropriate secondary antibody (Alexa Fluor 594 goat anti-mouse IgG A-11032 or Alexa Fluor 594

goat anti-rabbit IgG A-11012; ThermoFisher Scientific) at 1:500 dilution for 1 hour. The cells were washed and stained with DAPI. Additionally, for phagocytosis experiments, Alexa Fluor 488 or 594-Cholera toxin Subunit B (ThermoFisher Scientific) was used at the last step along with DAPI. The cells were washed again before analyzing by confocal microscopy.

For time-lapse studies, LPS-primed BMDMs were loaded with 1 μ M BODIPY or Active Caspase-1 stain (ab219935; abcam) along with nuclear stain Hoechst. After an hour, cells were stimulated with 35 μ g/cm² CS for different times, as indicated. The cells were washed with HBSS^{-/-} before analyzing by confocal microscopy.

Air pouch experiment

5 ml of sterile air was injected subcutaneously into the back of 6-8 weeks old mice to generate an air pouch. An additional 3 mL was injected into the pouch 3 days later to maintain the air pouch. 3 days later, the animals were either injected with 25 mg/kg BI-78D3 or vehicle into the air pouch. After an hour, 1 mg of CS in 1 ml PBS was injected into the air pouch. The control animals received PBS. After 6 hours, the air pouch was rinsed by injecting 3 ml of cold PBS and the lavage fluid was collected.

Flow Cytometry

Cells from the air pouch lavage were stained with CD45, CD11b-APC, Ly6G-FITC, F480-PerCP, SiglecF-PE fluorochrome labeled antibodies purchased from BD Biosciences or Biolegend, following standard protocols. Cells labeled with isotype matched antibodies were used as controls. Flow cytometry data were acquired on FACS Calibur or FACS Canto (BD Biosciences) and analyzed using Flowjo software (Tree Star).

Cytospin

The air pouch lavage fluid cells were spun down Shandon Cytospin centrifuge (Shandon Lipshaw) followed by staining with Hema-3 reagents (ThermoFisher Scientific) according to the manufacturer's recommendations.

siRNA

For siRNA transfection, 20 μ l of 168 nM Silencer siRNA (Life Technologies) diluted in Opti-MEM (Life Technologies) was mixed with 10 μ l of 0.03% (v/v) Lipofectamine RNAiMax/Opti-MEM (Life Technologies) as described by the manufacturer. 30 μ l of the siRNA-lipofectamine mixture was added to each well of a white flat-bottom 96-well plate (Greiner). After 10 min at room temperature, 1×10^4 RAW264.7 macrophages suspended in 80 μ l of DMEM+10% FBS were added. Cells were incubated for 48 h at 37°C with 5%

CO₂. 48h later, the cells were re-suspended in DMEM+1% FBS medium, primed with 10ng/ml of LPS for 3 h and stimulated with 100 µg/cm² CS. After 6h, the supernatant was analyzed for LTB₄ production. A total of 110 genes were screened using three distinct siRNAs for each gene. 5-LO siRNA and scrambled siRNAs served as positive and negative controls, respectively.

Statistical Analysis

All *in-vitro* experiments were repeated at least three times. Represented data are a mean ± S.E of triplicate cultures. All data were analyzed with GraphPad Prism4 Software (San Diego, CA). Statistical significance was determined using unpaired Student's *t*-test.

CHAPTER III

LTB₄ PRODUCTION IS INDEPENDENT OF INFLAMMASOME ACTIVATION

Introduction:

Uptake of CS is essential for the production of various pro-inflammatory mediators including IL-1 β [45, 55, 56]. Internalized particles go through distinct intracellular compartments before they are degraded in the lysosomes (or attempted to be degraded, in the case of silica). The contents in the early endocytic vesicles are delivered to Early Endosomes (EE) in the peripheral cytoplasm. EEs receive cargo from both Clathrin and Caveolin-mediated pathways [141]. The components to be recycled are sorted out and transported back to the surface via recycling endosomes. Endocytosed materials are then delivered to Late Endosomes (LE), which are large vesicles found in the perinuclear region. pH is an important factor for the maturation of the endosomes. Vacuolar proton pumps (V-ATPases) are responsible for progressively reducing the pH: 6.2 in the early endosomes, 5.5 in the late endosomes and 4.5 in the lysosomes. Finally,

LEs fuse with the lysosomes where its contents are digested by the lysosomal hydrolases [141].

Uptake of crystals and particulates like alum and silica go through the phagosomal maturation pathway in the same way a pathogen would. The production of ROS and lysosomal proteases in response to the surface ligands result in degradation of pathogens, whereas in case of silica, it results in phagosomal membrane destabilization and release of cathepsin proteases into the cytosol. This in turn triggers NLRP3 inflammasome and caspase-1 activation. Uptake of silica induces a caspase -1 dependent cell death known as Pyroptosis³ [142-144]. David Knecht's group studied the timeline of events after silica uptake demonstrated that phagosomal leakage occurs 30-120 min after silica uptake and cell death is triggered 3-6 hours post-uptake [29]. The production of several CS-induced pro-inflammatory mediators including IL-1 β , bFGF, HMGB1, CXCL1,2,3,5 are directly linked to the activation of inflammasome pathway [50]. However, the sequence of events leading to the synthesis of LTB₄ in response to CS and its relationship to the inflammasome pathway is not clearly understood.

Here, I sequentially evaluated the requirement of each step leading to the inflammasome activation in the synthesis of LTB₄. First, I evaluated the requirement of phagocytosis for LTB₄ production by using several pharmacological inhibitors that

³ Pyroptosis does not require apoptotic caspases 3, 6 or 8. Moreover, loss of mitochondrial integrity and cytochrome c release is not seen in pyroptosis.

inhibit phagocytosis: Cytochalasin D (Cyt D) - an F-actin polymerase inhibitor; Chlorpromazine (CPZ) – a clathrin-dependent endocytosis inhibitor; and Genistein – a caveolin mediated endocytosis inhibitor. Next, I used Bafilomycin-A1 (Baf-A1), a macrolide antibiotic isolated from *Streptomyces* species that inhibits the vacuolar H⁺ ATPase (V-ATPase), to evaluate the requirement of phagolysosome fusion for LTB₄ production. Baf-A1 prevents fusion of phagosome and lysosome by inhibiting the acidification of phagosomes [141, 145]. Finally, I used macrophages derived from 5LO and NLRP3 KO mice to determine whether inflammasome activation or IL-1 β is required for LTB₄ production.

Results:

Phagocytosis is required for CS-induced LTB₄ and IL-1 β production. To examine the requirement of phagocytosis for CS-induced LTB₄ production, LPS-primed BMDMs were stimulated with CS in the presence of phagocytosis inhibitors. CytD (10 μ M), CPZ (10 μ g/mL) and Genistein (100 μ M), all blocked the production of IL-1 β and LTB₄ in BMDM (Fig. 7a-b). To determine whether uptake was necessary in other cell types that produce LTB₄ in response to CS, mouse mast cells, mouse neutrophils and human neutrophils were stimulated with CS in the presence of phagocytosis inhibitors. Phagocytosis inhibitors completely blocked CS-induced LTB₄ production in all these cell types (Fig. 7c). Mast cells and neutrophils did not make any detectable levels of IL-1 β .

Reflective microscopy in combination with fluorescence staining techniques was used to visualize CS particles inside the cell. In the presence of CytD, the cells did not take-up CS particles (Fig. 7d). Together, these results indicate that phagocytosis of CS particles is necessary for LTB₄ production.

Inhibition of phagolysosome formation enhances LTB₄ production: The production of several CS-induced pro-inflammatory mediators including IL-1 β , bFGF, and HMGB1 are directly linked to the activation of inflammasome pathway. Bafilomycin-A1 (Baf-A1), an ATPase inhibitor prevents fusion of phagosome and lysosome by inhibiting vacuolar acidification. To investigate whether phagolysosome formation is necessary for

LTB₄ production, LPS-primed BMDMs were treated with varying doses of Baf-A1 prior to CS stimulation. Baf-A1 had opposite effects on LTB₄ and IL-1 β production. As expected, Baf-A1 blocked IL-1 β production (Fig. 8a) [55]. In contrast, Baf-A1 enhanced CS-induced LTB₄ production in BMDM (Fig. 8b). It is important to note that Baf-A1 alone did not cause LTB₄ production. A similar trend in RAW 264.7 cells, mouse mast cells, mouse neutrophils and human neutrophils was observed (Fig. 8c). Approximately three-fold increase in LTB₄ production over CS control was observed at 5 μ M, 0.5 μ M and 1 μ M in BMDM, RAW 264.7 cells and human neutrophils, respectively.

The effect of Baf-A1 on lysosomal acidification was measured using acridine orange staining. Acridine orange emits green fluorescence in nuclear and cytoplasmic compartments, whereas acidic lysosomes or phagolysosomes are stained orange-red. CS-treated cells show enlarged lysosomal compartments. Acridine orange staining in the lysosomal compartments was completely lost in Baf-A1 treated cells, in the presence of CS (Fig. 8d). Together, these results indicate that phagolysosomal fusion is not required for LTB₄ production.

LTB₄ production is independent of inflammasome activation and IL-1 β production:

To evaluate whether LTB₄ production is independent of inflammasome activation, I used BMDMs from 5LO^{-/-} and NLRP3^{-/-} mice. LPS-primed BMDMs derived from the knockouts were stimulated with CS for 6 h and the supernatants were assayed for LTB₄

and IL-1 β production using ELISA. BMDMs from 5LO^{-/-} mice were deficient in CS-induced LTB₄ production but produced IL-1 β . Similarly, BMDMs derived from NLRP3^{-/-} mice made LTB₄ in response to CS but did not make IL-1 β . Collectively, these results indicate that CS-induced LTB₄ production is completely independent of inflammasome activation (Fig.9).

CS-induced lipid body formation is inflammasome independent: To visualize the divergence in CS-induced LTB₄ and IL-1 β pathways, I used BODIPY and caspase-1 staining. LPS-primed BMDMs with/without pre-treatment with pharmacological inhibitors were stimulated with CS for 3 h, followed by staining with BODIPY, a yeast extract that stains lipid bodies in the cell. An increase in accumulation of lipid bodies was observed in the cytoplasm of CS- stimulated cells. In the presence of CytD, lipid bodies were absent, whereas in the presence of Baf-A1, there was a significant increase in the formation of lipid bodies in cells (Fig. 10a, upper panel). Confocal microscopy images are quantified in terms of fluorescence intensity of lipid bodies and the number of lipid bodies per cell. Inflammasome activation under similar conditions was measured by staining for active caspase-1 as described in *Materials and Methods*. CS-exposed cells showed caspase-1 activation, whereas the cells that were pre-treated with CytD or Baf-A1 prior to CS-stimulation showed no caspase 1 staining (Fig. 10b). Formation of lipid bodies and inflammasome pathway are regulated independent of each other as observed in BODIPY and caspase-1 staining of CS-stimulated BMDMs from NLRP3^{-/-} and 5-LO^{-/-}

mice. (Fig. 13). Since the formation of lipid bodies correlated with LTB₄ production, we speculated that these could be sites of CS-induced LTB₄ synthesis.

Kinetics of lipid body formation and inflammasome activation: To examine the kinetics of lipid body formation and inflammasome activation, LTB₄ and IL-1 β production in the supernatants were measured at time points ranging from 1 hour to 6 hours (Fig. 11c). Cells loaded with BODIPY and active caspase-1 stain were stimulated with CS and live images were captured at various time points (Fig. 11b). Although the accumulation of both LTB₄ and IL-1 β in the supernatant appears to follow a similar time course, live staining data reveals that lipid body formation occurs as early as 5 min, whereas the earliest caspase -1 activation was observed at 30 min after CS exposure. These results indicate that lipid body formation precedes inflammasome activation.

A23187 induced LTB₄ but not lipid body formation

To determine whether a calcium ionophore that induces LTB₄ production activates lipid body formation, I stimulated LPS-primed BMDMs with A23187. Stimulation with A23187 resulted in huge amounts of LTB₄ secretion within 5 min of exposure but did not show any lipid body formation (Fig. 12). These results indicate that phagocytosis of CS triggered a unique pathway that was different from the conventional LTB₄ production.

Discussion:

Silica can be internalized by receptor mediated phagocytosis and endocytosis depending on physical and chemical properties of the particles such as size, shape or surface charge. Several studies have highlighted the importance of scavenger receptors SR-AI, SR-AII and MARCO in internalization of silica [146]. However, scavenger receptor-knockout studies have revealed that they are important but not essential for CS uptake [147, 148]. These studies suggest that there could be other mechanisms by which silica can be taken up, such as clathrin and caveolin mediated endocytosis. In this study, blocking uptake of CS using CytD, Genistein, and CPZ, abrogated IL-1 β and LTB₄ production (Fig 7). These results suggest that CS particles could possibly be taken up by more than one mechanism. Particles of size < 200 nm are internalized by clathrin-coated pits, whereas slightly larger particles up to 500 nm are taken up via caveolin mediated endocytosis [149, 150]. Phagocytosis typically involves uptake of larger particles (>250nm). Since the CS particles size used in this study ranged from 500-5000 nm in diameter, it is likely to be taken up by more than one ways.

Inflammatory pathways triggered by phagocytosis of CS particles appears closely linked to the phagosome maturation pathway with different mediators such as LTB₄, IL-1 β and CXC chemokines produced at different stages. Treatment of cells with bafilomycin-A1 prevents phagolysosome formation [151]. Although this is a key step in IL-1 β production [55], based on the results presented here, it appears to be downstream

of LTB₄ synthesis (Fig 8). Dissociation of inflammasome activation that requires phagosome fusion with lysosome clearly places the lipid body formation upstream of this phagosome maturation event (Fig 10). Kinetics study showing lipid body formation as early as 5 min after CS exposure as opposed to 30 min for caspase-1 activation (Fig. 11) also supports the hypothesis that lipid body formation might precede inflammasome activation.

The complete independence of LTB₄ and IL-1 β pathways is further supported by the use of primary cells from NLRP3^{-/-} and 5-LO^{-/-} mice (Fig 9). BODIPY and caspase-1 staining in BMDMs from 5LO and NLRP3 KO mice showed that lipid bodies are formed independent of inflammasome activation (Fig 13). The appearance of the lipid bodies correlated with the LTB₄ production under similar conditions allowing us to speculate that they may be a source of CS-induced LTB₄ production. Interestingly, A23187, a calcium ionophore, induced LTB₄ production instantly but did not show any appearance of lipid bodies, suggesting that CS-induced LTB₄ synthesis in the lipid bodies could be a novel phagocytosis-dependent pathway. Together, these data provide evidence that inhibition of phagocytosis inhibits both LTB₄ and IL-1 β producing pathways, whereas Baf-A1 enhances LTB₄ production while blocking the inflammasome pathway. Our preliminary data with lipid bodies suggest that they could be sites of LTB₄ production.

Figure 7. Phagocytosis is required for the production of CS-induced LTB₄ and IL-1 β production. Bone marrow-derived macrophages (BMDM) from WT mice were LPS-primed and stimulated with 100 μ g/cm² CS for 6h in the presence or absence (control) of phagocytosis inhibitors Cytocholasin D, Chlorpromazine and Genestein. ELISA was done to assess (a) LTB₄ levels (b) IL-1 β levels in the cell culture supernatants. LTB₄ production in (c) mouse bone marrow-derived mast cells, mouse bone marrow-derived neutrophils and human neutrophils upon stimulation with 100 μ g/cm² CS in the presence phagocytosis inhibitors was measured using ELISA. Data represented is from one of the three experiments. Data are expressed as mean $\pm$ SE. **p< 0.01, ***p< 0.001 non-parametric t-test. (d) CS uptake in the presence and absence of CytD was analyzed in LPS-primed BMDM, 2h post stimulation with 100 μ g/cm² C'S. Cell membrane was stained with fluorescent cholera toxin (red), nucleus with DAPI (blue) and CS particles were observed using reflective microscopy.

Figure 8. Inhibition of phagolysosome formation enhances LTB₄ production.

BMDMs derived from WT mice were LPS-primed and stimulated with 100µg/cm² CS for 6h in the presence or absence (control) of varying doses of Bafilomycin-A1. ELISA was done to assess (a) IL-1β and (b) LTB₄ levels in the cell culture supernatants. LTB₄ production in (c) LPS-primed RAW264.7 cells, mouse and human neutrophils upon stimulation with 100µg/cm² CS in the presence of Baf-A1. 5-LO inhibitor Zileuton was used as a positive control in these experiments. Data represented is from one of the three experiments. Data are expressed as mean ± SE. **p< 0.01, ***p< 0.001 non-parametric t test. (e) LPS-primed BMDMs were stimulated with 35µg/cm² CS for 3h in the presence or absence of Baf-A1. Cells were stained with acridine orange (red) for 30 min after CS treatment and analyzed by confocal microscopy.

Figure 9: LTB₄ production is independent of inflammasome activation and IL-1 β production: LPS-primed BMDMs from WT, 5-LO^{-/-} and NLRP3^{-/-} were stimulated with 100 μ g/cm² CS for 6h. ELISA was done to assess (a) LTB₄ levels and (b) IL-1 β levels in the cell culture supernatants. Data are expressed as mean $\pm$ SE. **p < 0.01, ***p < 0.001. ns = not significant, non-parametric t-test. Representative experiment of n=2.

a

DAPI (Nucleus), BODIPY (Lipid body)

b

DAPI (Nucleus), Cholera Toxin (Membrane), Caspase-1 (Inflammasome)

Figure 10. CS induces formation of lipid bodies. LPS-primed BMDMs were stimulated with $35\mu\text{g}/\text{cm}^2$ CS for 3h in the presence or absence (control) of CytD or Baf-A1 (a) The cells were loaded with BODIPY (green) for 60 min. Subsequently, cells were fixed, permeabilized (saponin 0.01%) and stained for nucleus using DAPI (blue) and analyzed by confocal microscopy to visualize lipid bodies (upper panel). Lipid bodies were quantified based on the intensity of BODIPY in cell (assessed in at least 20 cells/sample) and number of lipid bodies/cell (counted in at least 25 cells/sample). Representative experiment of n=5. (b) The cells were fixed, permeabilized and stained for: Caspase-1 (red), cell membrane using Cholera toxin (green) and nucleus using DAPI (blue), post CS-treatment to visualize inflammasome activation (lower panel).

a

b

Figure 11. Kinetics of lipid body and inflammasome activation. LPS-primed BMDMs (0.3×10^6 cells in 400 μ l media) were stimulated with 100 μ g/cm² CS. (a) LTB₄ and IL-1 β levels were measured in the supernatants collected at the indicated time points from 1 hour through 6 hours. Data are expressed as mean $\pm$ SE. **p < 0.01, ***p < 0.001 non-parametric t-test. (b) LPS-primed BMDMs were loaded with BODIPY or active caspase-1 stain along with nuclear Hoechst stain, stimulated with 35 μ g/cm² CS and live cell images were captured at indicated time points to observe lipid body and inflammasome activation. Images shown are representative of one of the three experiments.

Figure 12. A23187 induces LTB₄ production but not lipid body activation. LPS-primed BMDMs were stimulated with A23187 (10 μ M) and LTB₄ was measured in the supernatants. Representative experiment of n=3. Data are expressed as mean $\pm$ SE. **p<0.01, ***p<0.001 non-parametric t-test. In parallel cultures, the cells were stained with BODIPY at 5, 10, 15 and 30 min after A23187 (10 μ M) addition and confocal images

were obtained. Representative Images from one of the three independent experiments are shown.

Figure 13. CS-induced lipid body formation is inflammasome independent. LPS-primed BMDMs from $5LO^{-/-}$ and $NLRP3^{-/-}$ were stimulated with $35 \mu\text{g}/\text{cm}^2$ CS for 3 h. They were stained with: BODIPY (green) and DAPI (blue) to visualize lipid body formation (upper panels). The cells were also stained for Caspase-1 (red), cholera toxin (green) and DAPI (blue) to visualize inflammasome activation (lower panels). Images shown are representative from one of the three experiments.

CHAPTER IV

CHARACTERIZATION OF LIPIDOSOME

Introduction:

Lipid bodies are highly regulated cytoplasmic organelles that are found in yeast, plant and eukaryotic cells [152]. They consist of a hydrophobic lipid core mostly consisting of triacylglycerol and cholesterol lined by a single layer of phospholipid [153-155]. They are called by various names such as adiposomes, oil bodies (plants), lipid droplets, etc. [156-158]. In mammalian cells, they are predominantly seen in adipocytes as fat storing organelles that release energy during starvation [159, 160]. Recent studies have revealed that they are not just storage organelles but are induced in response to various stimuli. Leukocytes, including macrophages, neutrophils, mast cells, eosinophils, and basophils, can also induce cytoplasmic lipid bodies in response to various stimuli [161]. Naturally, their dysregulation is associated with various disease conditions like inflammation, cancer, diabetes, liver diseases and stress responses [162-166]. For example, macrophages that have densely packed lipid bodies, called foam cells, are associated with atherosclerosis [102, 167].

Biogenesis of lipid bodies

Lipid body biogenesis involves formation of a monolayer bound organelle from a bilayer membrane [168]. Lipid bodies mostly arise from endoplasmic reticulum; however, they can also be derived from plasma membrane, endosomes, and mitochondria [169, 170]. Several studies have shown lipid bodies to be closely associated with the phagosomes, although the physiological relevance of this is not known [171]. The mechanisms of budding of lipid droplets from the membrane is not well understood. Additionally, the proteins required for the formation of lipid bodies have also not been identified. In mammalian tissues, lipids are stored in lung, brain, and mammary glands in cytoplasmic lipid bodies. ADRP is highly expressed in all these tissues [172]. Adipocytes and steroidogenic cells in the adrenal cortex, ovarian follicles, and testis also store lipids in lipid bodies. The composition and formation of lipid bodies in the later is distinct from those present in mammary glands and brain. These possess perilipins in addition to ADRP [172]. Lipid bodies can be triggered in leukocytes in response to certain stimuli. Enzymes required for lipid synthesis, and proteins involved in membrane trafficking like specific Rabs and SNARES may be associated with lipid bodies, although the composition of lipid bodies may depend on its stimulatory conditions [173-175]. Lipid bodies are usually encompassed of three main proteins collectively known as the PAT proteins: Perilipins, ADRP (Adipose differentiation related protein) and TIP47 [102, 170, 176]. Proteomics, subcellular fractionation, and Western blotting techniques have

revealed various other proteins to be localized within the lipid bodies. Two other proteins S3-12 and OXPAT are other coat proteins later discovered to be important [177, 178]. *Kimmel et. al* described a new nomenclature for these coat proteins as follows: perilipin 1 for perilipin; perilipin 2 for ADRP; perilipin 3 for TIP47; perilipin 4 for S3-12, and perilipin 5 for OXPAT [179]. While perilipins 1 and 2 constitutively associated with lipid bodies, perilipins 3, 4, and 5 are exchangeable between cytosol and lipid body [180]. *Skinner et. al* have found perilipin 3 to be important the formation of lipid bodies from ER [181]. Attempts to characterize the signaling pathways required for biogenesis of lipid bodies have revealed that it is specific to the stimuli [152, 182, 183]. PAF induced lipid bodies involved increase in intracellular cAMP, which activates protein kinase A, which then activates perilipins which probably regulate lipid body formation [152, 182, 183]. Nascent lipid bodies are of the size range 50-100 nm in diameter, whereas mature cytoplasmic lipid bodies have diameters in the range of 250-500 nm [184, 185]. Larger lipid bodies can arise from coalescence of smaller lipid bodies to form a single, larger lipid body [168].

Here, I characterize CS-induced lipid bodies that are activated upon phagocytosis of CS, independent of the inflammasome pathway. Using several staining techniques and RNAi approach, I sought to answer several important questions such as:

Are lipid bodies merely a storage vesicle or site of LTB₄ synthesis? Are the enzymes required for LTB₄ synthesis such as 5-LO, FLAP, and LTA₄H be localized to the lipid bodies?

Do other crystals similar to crystalline silica, inert particles, or infectious agents activate lipid bodies? Do they all correlate with LTB₄ synthesis?

At what stage in the phagosome maturation pathway are lipid bodies formed? What are the molecules necessary for the formation of LTB₄ associated lipid bodies?

Results:

Lipid bodies are the primary site for CS-induced LTB₄ production: To investigate whether the observed lipid bodies are in fact the source of CS-induced LTB₄ production, LPS-primed BMDMs were stained for 5-LO, FLAP and LTA₄H, using specific antibodies, along with BODIPY, 3h after CS stimulation. The enzymes required for LTB₄ synthesis: 5-LO, FLAP, and LTA₄H co-localized with the lipid bodies, confirming our hypothesis that they are not merely a storage vesicle but are the primary sites of CS-induced LTB₄ production (Fig. 14).

Lipid bodies are closely associated with phagosomes: CS-induced LTB₄ production in the lipid bodies appears to be seamlessly linked to the phagocytosis pathway. In order to spatially localize lipid bodies, the LPS-primed BMDMs were loaded with endosome stain pHrodo dextran along with BODIPY 3h after CS treatment. A z-section and quantification using the intensity plot shows the close association of the two organelles (Fig. 15), providing a proof of connection between phagosome pathway and lipid bodies formation.

LTB₄ production and lipid body formation with other phagocytic particles: Next, we wanted to further explore the correlation between phagocytosis and lipid body formation. We used an array of phagocytic particles: Monosodium Urate (MSU) crystals, Zinc oxide nanoparticles, inert latex beads, and mCherry labeled *E.Coli* particles in this experiment. LPS-primed BMDMs stimulated with CS, MSU, *E.Coli* particles induced LTB₄

production and lipid body formation (Fig. 16). Whereas, phagocytosis of ZnO nanoparticles or inert latex beads did not induce LTB₄ production or lipid body formation. In agreement with previous studies, CS, MSU and latex beads were also capable of activating the inflammasome pathway and IL-1 β production (Fig. 16).

Molecular mediators of CS-induced lipidosome formation and LTB₄ production:

Since LTB₄ production in response to CS occurs after phagocytosis and before the fusion of phagosome with the lysosome, we sought to identify specific components in the phagosome maturation pathway by siRNA knockdown experiments to identify the key molecules necessary for lipidosome formation. In previous studies, screening 18,000 murine genes for interference with *Yersenia pestis* persistence in macrophages led to identification of ~300 genes involved in the phagocytosis and autophagy pathway to be important for *Y. pestis* survival in macrophages. Of these, 110 genes were selected to investigate their role in CS-induced LTB₄ production in RAW264.7 cells. RAW264.7 cells were incubated with the siRNA for 48h. The cells were then LPS-primed and stimulated with CS. After 6h, the LTB₄ levels were measured in the supernatant using ELISA (Fig. 18a). Inhibition of these proteins led to more than 50% inhibition of LTB₄ levels compared to the scrambled siRNA control. These included genes involved in phagocytosis such as clathrin, dynamin-2; GTPases Arf6, Rab1, Rab14, Rab40c; signaling kinases like PI3K, JNK1/2, PKA, PKC. In a second screen, we specifically examined proteins involved in lipid metabolism and adapters/ scaffold proteins and found

that 5-LO, cPLA2- α , Pld2 as well as Plin3, Hip1r, Akap1 were also critical in CS-induced LTB₄ production. Almost sixty other genes in our screen such as Caveolin-1, Rab7, Rab8a, Rab20 and Seipin reduced the LTB₄ levels by less than 50% relative to scrambled siRNA control (Fig. 17).

To further explore the contribution of some of these molecules in lipidosome formation, 7 genes (Table1) were selected. RAW264.7 cells were incubated with the siRNA for 48h. The cells were then LPS-primed and stimulated with CS. After 6h, the LTB₄ levels were measured in the supernatant using ELISA (Fig. 18a). RAW264.7 cells plated in confocal dishes were transfected with siRNA for 48 hours. The cells were then LPS-primed, stimulated with CS for 3 hours and stained with BODIPY and nucleus stain DAPI to analyze lipidosome formation. LTB₄ levels were measured in supernatants collected from cells plated in parallel after 6 hours of CS exposure. Knockdown of early endosome marker Rab5c, components of JNK pathway (JNK1, JIP1/2) and Rab40c inhibited LTB₄ production whereas knockdown of ASC and Rab18 did not. Complete abrogation of lipid body formation was observed in the presence of Rab5c, JNK1 and Rab40c siRNAs (Fig 18). In concurrence with previous results, knockdown of inflammasome activation with LAMP1 and ASC enhanced lipidosome formation. To further validate whether Rab40c is an integral part of the lipidosome structure, LPS-primed BMDMs were stained with Rab40c along with BODIPY 3h after CS stimulation. Fig. 19 shows co-localization of Rab40c with lipidosome.

Discussion:

Leukotriene biosynthesis was suggested to occur exclusively on the nuclear membrane after the translocation of 5-lipoxygenase (5-LO) into the nucleus [99, 186, 187]. However, recent studies have demonstrated that leukotriene synthesis can also take place in cytosolic lipid bodies [100, 161, 188, 189]. Diverse physiological functions including lipid homeostasis, testosterone production and eicosanoid generation were attributed to lipid bodies that are variably named as lipid droplets, adiposomes, lipid bodies etc. [171, 190, 191]. However, not all of these are associated with LTB₄ synthesis. Localization of eicosanoid synthesis enzymes within lipid bodies have confirmed that they could be sites of leukotriene synthesis depending on the cell type and the state of activation [101, 102, 152, 192, 193].

In our study the appearance of CS-induced lipid bodies in the cytoplasm correlated with the LTB₄ production under similar conditions. Additionally, antibody staining of 5-LO, FLAP and LTA₄H revealed that it is exclusively localized within the lipid bodies (Fig. 14), showing that they were the site of CS-induced LTB₄ production. Here, we coined the term “**lipidosome**” to distinguish LTB₄ synthesizing lipid bodies from other similar but unrelated cytoplasmic organelles of lipid storage. Lipidosome is not only the physical form but also a functional complex that allows the coupling of phagocytosis to LTB₄ production. The lipidosome can compartmentalize the enzymatic machinery necessary for leukotriene production.

Simultaneous staining with pHrodo dextran and BODIPY showed non-overlapping but close association of phagosome and lipidosome, providing evidence that it is an early event initiated by phagocytosis (Fig. 15). Interestingly, phagocytosis of Urate crystals and *E.Coli*, which induced LTB₄ production in a similar fashion, also triggered lipidosome activation. Whereas phagocytosis of other particles such as ZnO nano particles or non-toxic latex beads did not induce LTB₄ production or the appearance of lipidosome (Fig. 16). Calcium ionophore A23187 induced LTB₄ production independent of lipid body formation, suggesting that CS-induced lipidosome is a distinct phagocytosis-dependent pathway that is different from the conventional synthesis on nuclear membrane. Phagocytosis-induced LTB₄ synthesis appears to be a specific process, as uptake of ZnO and latex beads did not induce lipid body formation. The mechanisms that trigger the synthesis of LTB₄ in lipid bodies needs further understanding. The differences in the kinetics of appearance, composition and functional significance of lipidosome activation during the uptake different materials also needs further investigation.

siRNA knockdown studies allowed further examination of the phagosome maturation pathway to lipidosome activation. Clearly, knockdown of large number of genes involved in phagocytosis pathways reduced the levels of CS-induced LTB₄ synthesis (Fig. 17). Caveolin-1, which is a structural protein commonly found on plasma membrane is often associated with the lipid bodies [194]. In our study knockdown of

Caveolin-1 resulted in inhibition of LTB₄ production. Consistent with the results using Baf-A1 and inflammasome KO, knockdown of Rab7a, LAMP1 and ASC did not affect LTB₄ production or lipidosome formation (Fig. 18). In agreement with our inhibitor studies and known roles of certain Rab proteins [195], knockdown of Rab5c and JNK pathway completely abrogated LTB₄ production and lipidosome formation. Proteomic studies of lipid bodies in adipocytes have shown high concentration of Rab18 and Rab40c along with ADRP [196, 197]. Rab18 is one of the better-characterized proteins associated with lipid bodies. Although its function is not entirely understood, the studies indicate that Rab18 could have a role in biogenesis of ER-derived lipid bodies [197, 198]. Surprisingly Rab18 was not found to be essential for the formation CS-induced lipidosome or LTB₄ synthesis (Fig. 18). However, Rab40c was clearly required for lipidosome formation. Antibody staining with Rab40c also revealed that it is co-localised with BODIPY, suggesting that it is an important structural component of the lipidosome (Fig. 19). Further studies are needed to determine the biochemical composition and activation mechanisms of the CS-induced lipidosome.

Figure 14. CS-induced LTB₄ production occurs in lipid bodies: LPS-primed BMDMs were stimulated with 35μg/cm² CS for 3h. The cells were stained for 5LO, FLAP, or LTA₄H (red) using specific antibodies and BODIPY (green) as described in the *Materials and Methods*. Images shown are representative from one of three independent experiments.

Figure 15. Lipid bodies are closely associated with phagosomes: LPS-primed BMDMs were loaded with pHrodo red dextran (red) and BODIPY (green) post CS stimulation. A slice of a z-stack showing appearance of lipid bodies and phagosomes. The intensity plot shows the proximity of non-overlapping green and red signals. The intensity plot shown is representative of ten separate measurements.

Figure 16. LTB₄ production and lipid body formation with other phagocytic particles. LPS-primed BMDMs were stimulated with 100 µg/cm² CS, 100 µg/ml and 200 µg/ml MSU, 25 µg/ml and 50 µg/ml zinc oxide nano particles, fluorescent red latex beads and mCherry labeled *E.Coli* particles. (a) The supernatant was collected after 6 h and LTB₄ was measured using ELISA. (b) The cells were stained with BODIPY (green), DAPI (blue), cholera toxin (red) and particles (white). Representative experiment of n=2. Data are expressed as mean ± SE. *p< 0.05, **p< 0.01, ***p< 0.001 non-parametric t-test.

Figure 17. Molecular mediators of CS-induced LTB₄ production. RAW 264.7 cells were incubated with various siRNA as indicated for 48h. These cells were primed with LPS and stimulated with CS (100μg/cm²) for 6h. After 6h, LTB₄ in culture supernatants were measured. Data represent average inhibition of LTB₄ production by at least two siRNA for each gene and are representative of at least two independent experiment.

Table 2. Select siRNAs tested for their effects on lipidosome formation and LTB₄ production

Sl.No	Gene Symbol	Full Gene Name	Gene ID
1	Alox5	Arachidonate 5-lipoxygenase	11689
2	Rab5c	RAB5C, member RAS oncogene family	19345
3	Pycard	ASC, inflammasome	66824
4	Rab40c	Rab40c, member RAS oncogene family	224624
5	Rab18	RAB18, member RAS oncogene family	19330
6	Mapk8ip	mitogen activated protein kinase 8 interacting protein 1	19099
7	Mapk8	mitogen activated protein kinase 8	26419

Figure 18. Molecular mediators of CS-induced lipidosome formation. RAW 264.7 cells were incubated with siRNAs as indicated, for 48h. Subsequently the cells were LPS-primed and stimulated with $100 \mu\text{g}/\text{cm}^2$ CS. LTB_4 production after 6h of CS-stimulation was measured using ELISA. The cells were stained for lipid bodies with BODIPY (green) and nucleus with DAPI (blue) 3h after CS stimulation. Representative experiment of $n=3$. Data are expressed as mean $\pm$ SE. ** $p < 0.01$, *** $p < 0.001$ non-parametric t-test.

Figure 19. Rab40c is a structural component of lipidosome. After CS treatment, LPS-primed RAW 264.7 cells were stained for lipidosome (BODIPY, green). The cells were then fixed, permeabilized (saponin 0.01%) and stained for Rab40c (red) and DAPI (blue). Representative images from one of the three experiments are shown. A minimum of five fields were captured for each sample in every experiment.

CHAPTER V

JNK ACTIVATION IS ESSENTIAL FOR CS-INDUCED LTB₄ AND IL-1 β PRODUCTION

Introduction:

Silica can cause a spike in the intracellular calcium levels, implicating that there is a possibility of intracellular signaling [199]. ERK1/2, JNK, and p38 MAP Kinases have roles in various signaling pathways activated by CS [199-201]. Silica nanoparticles have shown an upregulation of MEK-ERK1/2 signaling pathway, whereas p38 and JNK pathways were unaffected [202]. Conversely, silica was found to inhibit phosphorylation of ERK1/2 and p38 MAPK expression in dermal fibroblasts that resulted in increased type-1 collagen and decrease in collagen-degrading enzyme MMP-1, both contributing to increased fibrosis [203]. In another study with crystalline silica, Src tyrosine kinase was found to be important for CS-induced NF- κ B activation [204]. Studies have also shown that Cox-2 expression is regulated both at the transcriptional and post-transcriptional level by members of the MAPK family [200, 205, 206]. Although MAP Kinases are

crucial in various signaling pathways activated by CS, their role in activation of CS-induced LTB₄ and IL-1 β pathways is not well defined.

In chapters III and IV, we established that CS-induced LTB₄ and IL-1 β production are independently regulated. Understanding the role of signaling kinases in CS-induced lipidosome and inflammasome pathways would help us define these mechanisms better. This understanding might also provide useful insights into therapeutic strategies for silicosis or silica induced lung cancer.

Here, I investigate the role of MAPKs in CS-induced LTB₄ and IL-1 β production using specific pharmacological inhibitors. I used: AG126 that selectively inhibits the phosphorylation of ERK1 and ERK2, a potent p38 inhibitor SB202190, and BI-78D3, a competitive JNK inhibitor that inhibits binding of JNK to its interacting protein J1P1. Our preliminary results revealed that JNK pathway is important for both CS-induced LTB₄ and IL-1 β production. The role of JNK in CS-induced lipidosome formation and LTB₄ production was further explored. JNK inhibitor, BI-78D3 was tested in an in-vivo air pouch model for CS-induced sterile inflammation.

Results:

Activation of JNK pathway is essential for CS-induced LTB₄ production: To investigate the involvement of MAP Kinases in CS induced LTB₄ production, LPS-primed BMDMs were pre-treated with specific MAPK inhibitors prior to CS exposure. p38 inhibitor SB202190 (10 μ M) moderately inhibited LTB₄ but not IL-1 β production. MEK inhibitor AG-126 had no effect on production of CS-induced LTB₄ or IL-1 β . JNK inhibitor BI-78D3 (10 μ M) significantly inhibited production of both LTB₄ and IL-1 β in BMDM (Fig. 20a-b). The inhibitory effect of BI-78D3 was confirmed in mast cells, mouse and human neutrophils (Fig. 20c). Real-time PCR analysis revealed that BI-78D3 significantly lowered the expression on CXCL1, 2, 3 and 5 neutrophil active chemokines (Fig. 21a). MTT assay revealed that treatment with BI-78D3 did not increase the cytotoxicity of BMDMs over the CS control (Fig. 21b). The JNK inhibitor also blocked Bafilomycin induced increase in LTB₄ production in BMDM (data not shown). Together, these results indicate that JNK activation is crucial for CS-induced LTB₄ and IL-1 β production.

Activation of JNK pathway is essential for lipidosome formation: To determine the requirement of JNK pathway in activation of lipidosome and inflammasome pathways, we used BODIPY and caspase-1 staining. LPS-primed BMDMs with/without pre-treatment with pharmacological inhibitors were stimulated with CS for 3 h, followed by staining with BODIPY. Inflammasome activation under similar conditions was measured

by staining for active caspase-1. In the presence of BI-78D3, lipidosome and inflammasome were completely absent (Fig. 22). These results indicate that activation of lipidosome and inflammasome require JNK activation.

JNK signaling is required for CS-induced neutrophilic inflammation: Given that JNK inhibitor, BI-78D3, completely blocked the production of neutrophil chemokines LTB₄, IL-1 β and CXC chemokines in macrophages, mast cells and neutrophils in our *in-vitro* studies, I wanted to test the efficacy of this compound in an *in-vivo* model. The air pouch model was used to study the effect of BI-78D3 on CS induced inflammation (Fig. 23). The air pouch formed provides a localized environment to study infiltration of immune cells in response to an inflammatory irritant [207, 208]. This is also a convenient model to assess the anti-inflammatory efficacy of a test compound. Sterile air was injected into the back of the mouse and the air pouch was maintained for a week. BI-78D3 (10 mg/kg) was injected into the air pouch an hour prior to CS injection (2 mg/mouse) into the air pouch. The air pouch was lavaged 6h after CS administration with 3 ml of cold PBS. Analysis of cellular infiltrates upon CS administration using cytospin and flow cytometry revealed that CS induced recruitment of leukocytes, mainly neutrophils and macrophages into the air pouch (Fig. 24a, b). Pre-treatment with BI-78D3 resulted in significant reduction in leukocytes especially neutrophils into the air pouch. These results indicate that BI-78D3 was effective in reducing CS-induced neutrophilic inflammation *in-vivo* by inhibiting the production of neutrophil chemokines in the air

pouch.

Discussion:

Neutrophil mediated inflammation is the driving factor for progression of silicosis and CS-induced lung cancer. Activation of neutrophil chemoattractants LTB₄ and IL-1 β production is a hallmark of sterile inflammation [45, 55, 56]. Studies with ASC and NLRP3 KO mice showed decreased pulmonary inflammation and fibrosis after CS installation [2, 209]. Therefore, IL-1R antagonists and inflammasome inhibitors have been tested in various mouse models and in clinical trials for silicosis [45]. IL-1R antagonists showed moderate reduction in fibrosis and inflammation in some trials, however, its overall therapeutic benefit remains to be established. Similarly, although LTB₄ has been implicated in various sterile-inflammatory diseases, LTB₄ receptor antagonists and LTA₄H inhibitors have not seen much success clinically due to the complexities involved in the pathway [210]. Therefore targeting the production both IL-1 β and LTB₄ might be an effective strategy to control CS-induced neutrophilic inflammation.

Our *in-vitro* studies revealed that JNK activation was essential for CS-induced lipidosome formation and LTB₄ production (Fig. 20, 22). Blocking JNK activation also blocked CS-induced IL-1 β production (Fig. 20). Additionally, real-time data showed that the JNK inhibitor could lower the expression of neutrophil CXC chemokines CXCL2, 3

and 5 (Fig. 21). Therefore, targeting LTB₄, IL-1 β , and CXC chemokines production through JNK signaling could be an effective way of reducing CS-induced sterile inflammation. Hence, we tested the JNK inhibitor, BI-78D3 *In-vivo* and found that it blocked the recruitment of neutrophils into the air pouch and reduced CS-induced sterile inflammation (Fig. 24).

JNK pathway plays an important role in various cellular events and is implicated in diseases like diabetes, cancer, atherosclerosis and neurodegenerative diseases [211]. JNK activation has been associated with liver cancer [212, 213], glioma [214], leukemia [215], multiple myeloma [216] and lymphoma [217]. Studies have also found JNK signaling to play an important role in COPD associated mucus overproduction and tobacco smoke-induced lung tumor promotion [218, 219]. Strategies to target JNK signaling for cancer have resulted in the development of two broad categories of JNK inhibitors: ATP-competitive inhibitors and ATP-non-competitive inhibitors. The key issue with some of the commercially available JNK inhibitors targeting the ATP-binding site, is their potential “off target” effects as they display poor kinase specificity [220]. BI-78D3 disrupts the interaction between JNK and its scaffold protein JNK interacting protein J1P1. In *in-vitro* studies, BI-78D3 was found to inhibit the phosphorylation of its substrate c-Jun but not ATF2 (which is a substrate of both JNK and p38) [221]. For this reason, BI-78D3 specifically inhibited JNK and J1P1 without causing cytotoxicity [221]. BI-78D3 has been tested in various animal studies for other indications. BI-78D3 was

able to restore insulin sensitivity in a mouse model of type-2 diabetes [222]. BI-78D3 also blocked Con-A induced liver damage in mice [221].

One of the ways by which JNK could be inhibiting LTB₄ synthesis is by directly targeting cPLA₂ enzyme. Several reports suggest that JNK is required for cPLA₂ activation [223, 224]. Additionally, both p38 and JNK MAPK activation is also reported to be essential for lipid droplet biogenesis by activation of cPLA₂ enzyme [225, 226]. Our studies indicate that JNK activation is necessary for both LTB₄ and IL-1 β production. However, it is not clear from this study if an initial step required for activation of both the pathways is targeted by JNK inhibition. *Okada et. al* report that activation of TAK1-JNK pathway after lysosomal rupture is necessary for ASC oligomerization and complete activation of inflammasome [227]. Therefore, it is possible that JNK pathway could be regulating LTB₄ and IL-1 β production by two different mechanisms.

Our *In-vitro* data provides substantial evidence of the role of JNK pathway in CS-induced LTB₄, IL-1 β and CXC chemokine production. Our preliminary *in-vivo* data suggests that BI-78D3 can effectively reduce CS-induced sterile inflammation. However, its efficacy in reducing silicosis and CS-induced lung cancer needs further evaluation.

Figure 20. Activation of JNK pathway is essential for CS-induced LTB₄ and IL-1 β production. LPS-primed BMDMs were stimulated with 100 μ g/cm² CS for 6h in the presence or absence (control) of MAPK inhibitors: AG-126, SB-202190 and BI-78D3. Zileuton was used as a positive control for LTB₄ inhibition. ELISA was done to assess (a) LTB₄ levels and (b) IL-1 β levels in the BMDM cell culture supernatants. ELISA was done to assess LTB₄ levels in (c) mouse bone marrow-derived mast cells, mouse bone marrow-derived neutrophils and human neutrophils upon stimulation with 100 μ g/cm² CS in the presence MAPK inhibitors.

a

b

Figure 21. Effect of BI-78D3 on CXC chemokine expression and cytotoxicity. (a)

Quantitative real time PCR of neutrophil-active chemokines was performed in the presence and absence of BI-78D3 in LPS-primed BMDM, 6h post exposure to CS. **(b)** Cytotoxic effects of BI-78D3 was measured by addition of 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl-tetrazolium bromide (MTT) post CS-treatment. After 2h, absorbance was measured at 562 nm using a BioTek reader. Representative experiment of n=3. Data are expressed as mean $\pm$ SE.

Figure 22. Activation of JNK pathway is essential for lipidosome activation: LPS-primed BMDMs were stimulated with $35 \mu\text{g}/\text{cm}^2$ CS for 3h in the presence or absence (control) of BI-78D3. The cells were loaded with BODIPY (green) for 60 min. Subsequently, cells were fixed, permeabilized (saponin 0.01%) and stained for nucleus using DAPI (blue) and analyzed by confocal microscopy to visualize lipid bodies (upper panel). The cells were fixed, permeabilized and stained for: Caspase-1 (red), cell membrane using Cholera toxin (green) and nucleus using DAPI (blue), post CS-treatment to visualize inflammasome activation (lower panel).

Figure 23. Experimental set-up of air pouch model: Sterile air is injected subcutaneously to the back of the mouse to form an air pouch. The air pouch is maintained for a week by injecting air on day 3. BI-78D3 is injected directly into the air pouch 1 hour prior to CS injection. After 6 hours the air pouch is lavaged with cold PBS and the cells are analysed using cytopspin and flow cytometry.

a

b

Figure 24. JNK signaling is required for CS-induced neutrophilic inflammation.

Sterile air was injected subcutaneously to the back of the WT mice to form an air pouch. CS was injected into air pouch in the presence/absence of BI-78D3 and 6 hours later inflammation was assessed. **(a)** Leukocytes on cytopsin slides and **(b)** total leukocytes, neutrophils and macrophages as identified by flow cytometry in the air pouch lavage fluid. Data are expressed as mean $\pm$ SE. * $p < 0.05$, *** $p < 0.001$ non-parametric t-test. Data are representative of at least nine mice per group.

CHAPTER VI

CONCLUSION

Silicosis is an irreversible, progressive inflammatory disease caused by acute or chronic exposure to silica particles [13]. Despite preventive measures taken for decades, it continues to be a health concern worldwide [3]. Although the overall incidences have decreased substantially in the U.S over the last 30 years, silicosis cases that were earlier under-reported due to poor surveillance are now coming to light [12, 228].

Silica exposure increases the risk of tuberculosis and other mycobacterial, fungal, and bacterial lung infections, even without silicosis [17]. Silicosis could lead to autoimmune diseases such as scleroderma, sarcoidosis, rheumatoid arthritis and lung cancer [13]. So far, silicosis remains a preventable disease with no cure. Understanding the CS-induced pathways will provide key insights on the pathogenesis of silicosis and help in identifying the molecular targets that could halt its progression.

Neutrophil recruitment from blood to the extravascular sites of damage is a hallmark of sterile inflammation [38]. Among the molecular mediators, LTB₄ plays a

critical role. LTB₄ is unique as it can amplify the local cell death signal to enhance neutrophil recruitment. *Lammermann et. al.* demonstrated that initial chemotaxis is followed by a second phase of neutrophil swarming from distant sites in a sterile skin injury model [38]. This could be attributed to phagocytosis of dead cells that enhances autocrine LTB₄ production and signaling [38]. We find similarities in our study in the silicosis model where mast cell and macrophage- produced LTB₄ leads to a sustained neutrophilic inflammation perpetuated by dead cells. In this study, we demonstrated that LTB₄ production in these cells is closely linked to the phagocytosis maturation pathway. In fact, we found that several molecules like Dynamin, Clathrin and Rab GTPases Rab5 and Rab40 play a role in LTB₄ production.

Inflammasome is a well-studied pathway in pathogenesis of silicosis [45]. Here, we showed that CS-induced LTB₄ production is independent of the inflammasome pathway. We showed for the first time that CS-induced LTB₄ synthesis mainly occurs in cytoplasmic lipid bodies. Lipid bodies, lipid rafts, oil bodies etc. are described in various studies [157]; however, some are not linked to inflammation. Peter Weller and colleagues described eicosanoid synthesis in lipid bodies which are similar to the lipid bodies in our study. We coined the term “lipidosome” to describe the functional unit of LTB₄ synthesis that occurs in lipid bodies and to differentiate it from other lipid bodies not related to phagocytosis-dependent LTB₄ synthesis. Careful analysis of time-lapse images showed that lipidosome activation occurs prior to inflammasome activation.

We also demonstrated that JNK activation is essential for both lipidosome and inflammasome activation. JNK inhibitor BI-78D3 was effective in reducing CS-induced sterile inflammation in an air pouch model. Hence, targeting the JNK pathway may be good strategy for silicosis and silicosis associated lung cancer. Figure 25 summarizes the key findings of this dissertation in a diagram.

Overall, this study describes a unique phagocytosis and JNK activation-dependent pathway for CS-induced LTB₄ production in lipidosome. More importantly, it highlights the underappreciated role of LTB₄ in CS-induced sterile inflammation. Better understanding of the cellular mechanisms involving CS-induced LTB₄ synthesis may provide valuable insights into therapeutic interventions of silica-associated disease.

GRAPHICAL SUMMARY

Figure 25. Graphical Summary. Cells take up silica by phagocytosis. During phagosome maturation process distinct lipid bodies appear in the cytosol that are the primary source of CS-induced LTB₄ production. JNK activation is required for both LTB₄ and IL-1β production. Lipidosome is formed independent of inflammasome

activation. Enzymes required for LTB₄ production, Rab40c are essential structural components of CS-induced lipidosome.

FUTURE DIRECTIONS

This dissertation describes a novel mechanism for CS-induced LTB₄ production in cytoplasmic lipidosome that is JNK dependent and inflammasome independent. Although this study outlines the basic mechanism of CS-induced LTB₄ synthesis, it raises several important questions that are worth exploring.

Our studies here indicate that uptake of CS induces LTB₄ synthesis in cytoplasmic lipidosome. The fact that only specific phagocytic substances can trigger lipidosome indicates a potential signal that is recognized by the LTB₄ pathway. The “triggers” that induce assembly and activation of lipidosome are not known. The differences in the mechanisms of phagocytosis-dependent LTB₄ synthesis in lipidosome vs the known LTB₄ synthesis in the nuclear envelope is worth exploring.

Future studies could be aimed at understanding the difference between CS-induced lipidosome and the lipid bodies generated by other phagocytic particles such as MSU or microbial pathogens. We do not know if lipidosome is activated in other sterile inflammatory conditions such as gout, atherosclerosis or arthritis. If so what are the similarities and differences between CS-induced and other lipidosome. Do they also require JNK activation? Can they be targeted in a similar way? The relevance and

implications of lipidosome in other diseases where LTB₄ is an important mediator needs further understanding.

Time-lapse images revealed that lipidosome activation occurs as early as 5 min after CS exposure. These are dynamic structures that are constantly generated as long as CS persists in the system. However, we do not know how long these persist and whether their activation would cease once the stimulus is removed. Kinetics with *E.Coli* would reveal important information about the stability and dynamics of lipidosome.

Majumdar et. al describe that LTB₄ is packaged in multivesicular bodies and secreted as exosomes in neutrophils in response to fMLP [229]. In their study, they observed that these multivesicular bodies were tightly associated with the nuclear membrane. They also found 5-LO, LTA₄H and FLAP enzymes within the exosomes. They describe that this exosomal LTB₄ is able to regulate neutrophil chemotaxis in an autocrine and paracrine manner. Secretion of LTB₄ in exosomes, encapsulated in a larger multivesicular body could lead to establishment of a stable gradient and signal relay during neutrophil chemotaxis. Despite having a distinct mode of formation, lipidosome could have similar functions and implications. Given that lipidosomes are activated in response to CS exposure, these could possibly be used as a biomarker for silicosis.

This study highlights the role of JNK pathway in CS-induced LTB₄ and IL-1 β production. We demonstrated the importance of JNK activation in the production of these

mediators using inhibitor and siRNA knockdowns. However, the direct target of JNK in this pathway remains unknown. Future experiments could be aimed at understanding why JNK activation is crucial in CS-induced lipidosome and inflammasome pathways.

The importance of lipidosome pathway and its direct implications in the progression of silicosis needs to be established. Although BI-78D3 reduced CS-induced inflammation in an air pouch model, whether it can halt/slow the progression of silicosis remains to be answered. The efficacy of BI-78D3 on lung inflammation post CS-installation needs to be tested.

In our previous study, we have shown that BLT1 knockout mice have significantly reduced tumor burden after CS exposure. Our study here has contributed to the understanding that both lipidosome and inflammasome activation play a crucial role in CS-induced sterile inflammation. The importance of lipidosome pathway in CS-induced lung tumor promotion could be studied in BLT1-NLRP3 double knockouts or in JNK1 knockout mice. Based on our preliminary results here, we expect to see a decrease in CS-induced lung inflammation and promotion of lung tumor with the JNK inhibitor BI-78D3.⁴

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APPENDIX

ABBREVIATIONS

1. CS	Crystalline Silica
2. LTB ₄	Leukotriene B ₄
3. IL	Interleukin
4. 5-LO	5-Lipoxygenase
5. FLAP	5-Lipoxygenase Activating Protein
6. LTA ₄ H	Leukotriene A ₄ Hydrolase
7. NLRP3	NOD-Like Receptor family Pyrin domain containing 3
8. JNK	c-Jun N-terminal Kinases
9. OSHA	Occupational Safety and Health Administration
10. NIOSH	National Institute for Occupational Safety and Health
11. PMF	Progressive Massive Fibrosis
12. ROS	Reactive Oxygen Species
13. TGF β	Transforming Growth Factor beta
14. TNF α	Tumor Necrosis Factor alpha
15. SR	Scavenger Receptor
16. MARCO	Macrophage Receptor with Collagenous structure
17. LDL	Low-Density Lipoprotein
18. MSU	Mono-Sodium Urate crystals

19. TLR	Toll-Like Receptors
20. NF- κ B	Nuclear Factor kappa beta
21. PRR	Pattern Recognition Receptor
22. PAMP	Pathogen-Associated Molecular Pattern
23. DAMP	Damage-Associated Molecular Pattern
24. NOS	Nitric Oxide Synthase
25. bFGF	Basic Fibroblast Growth Factor
26. IFN	Interferon
27. NET	Neutrophil Extracellular Traps
28. c-pLA2	cytosolic Phospholipase 2
29. AA	Arachidonic Acid
30. COX	Cyclooxygenase
31. LOX	Lipoxygenase
32. EPOX	Epoxygenase
33. LT	Leukotrienes
34. CysLT	Cysteinyl Leukotrienes
35. NSAID	Non-Steroidal Anti-Inflammatory Drugs
36. COPD	Chronic Obstructive Pulmonary Disease
37. IARC	International Agency for Research on Cancer
38. TAN	Tumor-Associated Neutrophils
39. NLR	Neutrophil-Lymphocyte ratio

40. IACUC	Institutional Animal Care and Use Committee
41. WT	Wild Type
42. DMEM	Dulbeco's Modified Eagle Medium
43. FBS	Fetal Bovine Serum
44. LPS	Lipopolysaccharide
45. BMDM	Bone Marrow Derived Macrophages
46. BODIPY sindacene	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-
47. DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
48. BSA	Bovine Serum Albumin
49. PBS	Phosphate Buffered Saline
50. EE	Early Endosomes
51. LE	Late Endosomes
52. ADRP	Adipose differentiation related protein
53. CPZ	Chlorpromazine
54. MAPK	Mitogen-Activated Protein Kinase

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EDUCATION

Ph.D. in Microbiology and Immunology, University of Louisville, USA	2012-Present
MS in Medical Biotechnology, Manipal University, India	2006-2008
BS in Biotechnology, Manipal University, India	2003-2006

RESEARCH EXPERIENCE

University of Louisville, School of Medicine. (August 2012 to present)

PhD candidate, Microbiology and Immunology Department, Louisville, KY, USA.

“Leukotriene B4 Induced Sterile Inflammation by Crystalline Silica”

- Performed *ex-vivo* studies to understand the cellular mechanisms activated in crystalline silica-induced sterile inflammation
- Tested the efficacy of select inhibitors in silica-induced sterile inflammation model
- Trained and supervised summer trainees in basic cell culture and immunology techniques
- Studies led to the discovery of an inflammasome independent “lipidosome” pathway, that drives crystalline silica mediated LTB₄ production

Piramal Life Sciences Ltd. (August 2008 to June 2012)

Research Associate, High Throughput Screening, Mumbai, India

- Screened a library consisting of ~50,000 natural products and synthetic compounds for anti-inflammatory activity
- Performed extensive studies using physiologically relevant cells like hPBMCs and synovial cells to elucidate the mechanism of action of the potential lead anti-inflammatory agents

- Standardized cell based and enzymatic assays for screening of compounds on anti-inflammatory targets
- Gained experience in handling automated workstation (Tecan Freedom EVO), script designing and database management
- Trained and supervised new inductees in conducting experiments and in the use of various laboratory instruments
- Planned purchase of laboratory consumables and equipment
- Gained proficiency in working independently as well as in a team

-

Piramal Life Sciences Ltd. (October 2007 - June 2008)

Project Trainee, High Throughput Screening, Mumbai, India

“Transcriptional Analysis of Human Monocytes in Response to Specific Anti-inflammatory Agents”

- Screened a library of natural products for their anti-inflammatory activity by evaluating inhibition of TNF- α , IL-6 and IL-2 using ELISA
- Elucidated the molecular mechanism involved in the immunosuppressive effect of an active lead compound using qRT-PCR

-

Manipal Life Sciences Centre (February 2006- June 2006)

Project Trainee, Manipal Life Sciences Centre, Manipal, India

“Cytogenetic and molecular analysis of congenital cataract”

- Screened for mutations in the amplified region of the crystallin genes using PCR
- Carried out karyotyping of blood samples to detect chromosomal abnormalities in cataract patients
- Performed pedigree analysis using information about familial history of the patients to understand the pattern of inheritance of the disease
- Studies led to the discovery of a novel human mutation in the gamma-D crystallin gene responsible for juvenile autosomal dominant cataract

SPECIALISED SKILLS

Immunology Techniques <ul style="list-style-type: none">• ELISA• Multicolor flow cytometry• Immunohistochemistry• Confocal microscopy	Molecular Biology Techniques <ul style="list-style-type: none">• qRT-PCR• SDS-PAGE and Western blotting• Karyotyping and various banding techniques• Cloning and transfection	In-vivo Techniques <ul style="list-style-type: none">• Mouse colony maintenance• Experience with lung cancer model, orthotopic tumor model• Tumor measurements
Cell Biology Techniques <ul style="list-style-type: none">• Mammalian cell culture• Subset isolation of leukocytes from peripheral blood• Isolation of cells from synovial tissue• Cytotoxicity assays, Proliferation assays	Software tools <ul style="list-style-type: none">• Bioinformatics tools like BLAST• Primer designing• Graph-Pad Prism• FlowJo• Automated workstation (Tecan Freedom EVO) script designing	

PATENT APPLICATIONS

WO/2016/020724. Sphaeranthus indicus composition as IL-17 inhibitor and uses thereof:

The present invention relates to herbal composition comprising an extract of the flowering and fruiting heads of the plant *Sphaeranthus indicus* as interleukin-17 (IL-17) inhibitor. Further, the invention relates to the use of the said herbal composition for the treatment of disorders mediated by IL-17.

PUBLICATIONS

- **Hegde B**, Bodduluri SR, Satpathy SR, Alghsham RS, Jala VR, Uriarte SM, Chen D, Lawrenz MB, Haribabu B. Inflammasome independent leukotriene-B4 production drives crystalline silica induced sterile inflammation. J Immunol. 2018 Apr 2. pii: ji1701504. doi: 10.4049/jimmunol.1701504. [Epub ahead of print]

- Kulkarni-Almeida A, Shah M, Jadhav M, **Hegde B**, Trivedi J, Mishra PD, Mahajan GB, Dadarkar S, Gupte R, Dagia N. A semi-synthetic natural product blocks collagen induced arthritis by preferentially suppressing the production of IL-6. *Int Immunopharmacol*. 2016 Apr;33:63-9. doi: 10.1016/j.intimp.2016.01.001. Epub 2016 Feb 9. PubMed PMID: 26869203.
- Satpathy SR, Jala VR, Bodduluri SR, Krishnan E, **Hegde B**, Hoyle GW, Fraig M, Luster AD, Haribabu B. Crystalline silica-induced leukotriene B4-dependent inflammation promotes lung tumour growth. *Nat Commun*. 2015 Apr 29;6:7064. doi: 10.1038/ncomms8064. PubMed PMID: 25923988; PubMed Central PMCID: PMC4418220.
- Saklani A, **Hegde B**, Mishra P, Singh R, Mendon M, Chakrabarty D, Kamath DV, Lobo A, Mishra PD, Dagia NM, Padigar M, Kulkarni-Almeida AA. NF- κ B dependent anti-inflammatory activity of chlorojanerin isolated from *Saussurea heteromalla*. *Phytomedicine*. 2012 Aug 15;19(11):988-97. doi: 10.1016/j.phymed.2012.05.016. Epub 2012 Jul 3. PubMed PMID: 22762939.
- **Hegde B**, Vadnal P, Sanghavi J, Korde V, Kulkarni-Almeida AA, Dagia NM. Vitamin E is a MIF Inhibitor. *Biochem Biophys Res Commun*. 2012 Feb 10;418(2):384-9. doi: 10.1016/j.bbrc.2012.01.031. Epub 2012 Jan 18. PubMed PMID: 22281490.

MANUSCRIPTS UNDER PREPARATION

- Singh R, Chandrashekharappa S, Bodduluri SR, Baby BV, **Hegde B**, Kotla GN, Hiwale AA, Saiyed T, Patel P, Kumar MV, Langille MGI, Douglas GM, Dryden GW, Alatassi H, Zhang H, Haribabu B, Vemula PK, Jala VR. Microbial metabolite. Urolithin A enhances gut barrier function through AhR pathway. *Nat Commun*.
- Alghsham RS, Bodduluri SR, Satpathy SR, **Hegde B**, Jala VR, Sunkara M, Haribabu B. Activation of distinct inflammatory pathways by Zinc oxide Nanowires. *Journal of Particle and fiber toxicology*.
- Burlison JA, **Hedge B**, Jala VR, Haribabu B, Trent JO. The Discovery and Structure-Activity Relationship Studies of 3-(1-(2-Fluorobenzyl)-1H-indol-3-yl)-N-(pyridin-3-ylmethyl) propanamide, a BLT1 Agonist for the Treatment of Cancer.

POSTER PRESENTATIONS

- **Hegde B, Satpathy SR, et. al.** Inflammasome Independent LTB₄ Production Drives Crystalline Silica Induced Sterile Inflammation. AAI2017, Washington D.C., USA, May2017
- **Hegde B, Satpathy SR, et. al.** Inflammasome Independent LTB₄ Production Drives Crystalline Silica Induced Sterile Inflammation. Research! Louisville2016, Louisville, USA, September 2016
- **Hegde B, Satpathy SR, et. al.** Role of Phagocytosis Pathway in Crystalline Silica Induced LeukotrieneB4 Production. Research! Louisville2015, Louisville, USA, October 2015
- **Hegde B, Vadnal P, et. al.** Vitamin E is a Potent MIF Inhibitor. 5th Congress of the Federation of Immunological Societies of Asia-Oceania, Delhi, March 2012
- **Hegde B, Saklani A, et. al.** Chlorojanerin – an interesting natural compound from Sausurrea heteromalla with anti-inflammatory activity. SBCI Conference, IISc, Bangalore, India, December 2010
- **Hegde B, Dias C A, et. Al.** Th17 cell inhibition by lapatinib – implications for the treatment of inflammatory diseases. SBCI Conference, IISc, Bangalore, India, December 2010

CERTIFICATIONS

- Introduction to the Principles and Practice of Clinical Research (IPPCR) by NIH, April. 2016
- Scientific Writing by Stanford Online, Sept. 2015
- Health in Numbers: Quantitative Methods in Clinical & Public Health Research by HarvardX, Feb. 2013
- Fundamentals of Clinical Trials by HarvardX, Feb. 2013

AWARDS AND RECOGNITION

- 2017 AAI Trainee Poster Award (May 2017)
- GSC travel scholarship (April 2017)
- Served as a Graduate Student Ambassador for the School of Interdisciplinary and Graduate Studies, University of Louisville 2016-2017
- Integrated Program in Biomedical Sciences Fellowship – 2012-2014
- Award for dedication and excellence exhibited for scientific support at Piramal Life Sciences Ltd, Mumbai (Nov 2010)