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Inhibitory Effects Of Shiitake-derived Exosome-like Nanoparticles On NLRP3 Inflammasome Activation

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INHIBITORY EFFECTS OF SHIITAKE-DERIVED EXOSOME-LIKE NANOPARTICLES ON NLRP3 INFLAMMASOME ACTIVATION

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The NLRP3 inflammasome is a critical mediator of inflammation and consists of the sensor NOD-like receptor family, pyrin domain containing 3 (NLRP3), the adaptor apoptotic speck protein containing a caspase recruitment domain (ASC), and the effector caspase-1. Dysregulated or excessive activation of the NLRP3 inflammasome contributes to pathogenesis of diverse inflammatory diseases such as Type 2 diabetes and atherosclerosis. Therefore, the NLRP3 inflammasome is a promising therapeutic target for treating these diseases. Extracellular vesicles (EVs) are membrane-enclosed tiny particles released by almost any type of cells and they are involved in the intercellular communication by transferring their cargos including RNAs, proteins, and lipids to target cells. Based on their sizes, origins, and biological functions, EVs are mainly classified into exosomes, microvesicles and apoptotic bodies. Recently, exosome-like nanoparticles (ELNs) have been isolated from some edible plants, and their beneficial effects on diseases have also been reported. Given that mushrooms have been utilized as medicines for long time in some countries and they have anti-cancer, anti-inflammation and anti-microbial properties, we isolated ELNs from six mushrooms and tested their effects on the NLRP3 inflammasome activation. Among these six mushroom-derived ELNs, shiitake-derived ELNs (S-ELNs) significantly suppressed NLRP3 inflammasome activation and therefore were chosen for further study. S-ELNs were nanoparticles that contained RNAs, proteins,

and lipids. RNA-labeled S-ELNs or lipid-labeled S-ELNs were taken up by macrophages in a dose-dependent manner. S-ELNs had a broad inhibitory effect on the NLRP3 inflammasome activated by FFA, alum, nigericin, and ATP. S-ELNs inhibited assembly of the NLRP3 inflammasome. Importantly, we further identified lipids in S-ELNs lipids as active biomolecules that play an important role in suppressing NLRP3 inflammasome activation. Taken together, our findings suggested that S-ELNs is a promising agent that suppresses NLRP3 inflammasome activation.

DEDICATION

This work is dedicated to

My dear father, Yongxiang Lu, and my dear mother Fengju, Wang for their support.

All my dear friends for their help and encouragement

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CHAPTER 1. LITERATURE REVIEW

Inflammation is important for eradication of pathogens and beneficial for the wound and tissue healing (1,2). There are two types of inflammation: acute inflammation and chronic inflammation (3). The clinical features of acute inflammation are pain, heat, redness and swelling (4,5). At the cellular and molecular levels, acute inflammation is characterized by increased permeability of the vascular, blood flow, leukocytic infiltration, and the release of inflammatory mediators (3,6). When the pathogens or dangerous signals are not eradicated completely, chronic inflammation occurs and lasts for weeks, months or even years (1). Chronic inflammation is mainly characterized by the development of immune responses at the infection site, which contributes to the pathogenesis of chronic diseases, including cardiovascular diseases, type 2 diabetes and renal diseases (6,7). Both acute and chronic inflammatory are involved in the recruitment of immune cells and the accumulation of inflammatory mediators, but the severity of chronic inflammation is subtle compared with acute inflammation (6).

In response to infection or cellular stress, the innate immune system plays an important role in detecting and eliminating dangerous signals, including microorganisms or endogenous damage (9,8). Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), recognize these dangerous signals by sensing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), leading to the recruitment of innate immune cells and the upregulation of proinflammatory cytokines (8). Inflammasomes, a subgroup of PRRs, are multiple protein complexes that are critically engaged in the immune responses to eradicate microorganisms and tissue repair (9). Among the inflammasomes, the NLRP3

inflammasome is most extensively and widely studied because it recognizes many DAMPs and PAMPs, and thus engaged in the chronic inflammation in many diseases (10).

The NLRP3 inflammasome consists of the sensor NOD-like receptor family, pyrin domain containing 3 (NLRP3), the adaptor apoptotic speck protein containing a caspase recruitment domain (ASC), and the effector caspase-1 (Casp1) (10,11). It can be activated by various stimuli, including molecules introduced during both pathogen infection and endogenous damage (10,11). Activation of the NLRP3 inflammasome results in autocleavage of Casp1 to produce Casp1 p10 and p20, which cleaves pro-interleukin (IL)-1β and pro-IL-18 into active interleukin-1β (IL-1β) and IL-18 (10-12). IL-1β is one of the most important proinflammatory cytokines that plays a vital role in inflammatory responses (12), and engaged in various chronic inflammatory diseases, including type 2 diabetes and atherosclerosis (13,14).

Under normal physiological condition, activation of the NLRP3 inflammasome contributes to the microorganism elimination and tissue repair (9). However, dysregulated or excessive activation of the NLRP3 inflammasome promotes progress of diverse diseases (10,11). Increasing evidence has suggested that the NLRP3 inflammasome is a promising therapeutic target in diverse chronic inflammatory diseases (15). *Nlrp3*$^{-/-}$ obese mice have lower glucose levels in blood and higher insulin sensitivity, compared with *Nlrp3*$^{+/+}$ obese mice (16,17). The inhibitor MCC950 that specifically targets NLRP3 inflammasome alleviated the severity of experimental autoimmune encephalomyelitis (18).

Extracellular vesicles (EVs) are membrane-enclosed tiny particles released by

almost any type of cells and they are involved in the intercellular communication by transferring their cargos including RNAs, proteins, and lipids to target cells (19,20). EVs are mainly classified into three different categories based on their sizes, origins, markers or biological functions: exosomes (30-100 nm), microvesicles (50-2000 nm) and apoptotic bodies (500-5000 nm) (21). Exosomes are released from endosomal membrane through invagination. Exosomes are released from many mammal cells through exocytic fusion of multivesicular bodies with cell surface and they have been isolated from urine, plasma, saliva and milk (20, 25-28). Microvesicles are produced from plasma membrane through budding (22). Apoptotic bodies are usually larger than exosomes and microvesicles. They are produced during apoptosis progresses (21). However, the strict standard for EV's nomenclature or classification has not been well clarified and established. Importantly, EVs play a significant role in both physiological and pathological processes (23). Therefore, they begin to serve as biomarkers or therapeutic targets in many diseases (23). In the physiological process, EVs secret from donor cells and deliver their cargos to recipient cells, thereby maintaining homeostasis such as tissue repair and immune surveillance (24,25). On the other hand, EVs contribute to the pathological process of some diseases such as Alzheimer disease and cancer (26,27).

Recently, exosome-like nanoparticles (ELNs) have been isolated from some edible plants, and their beneficial effects on diverse diseases have also been reported (28-30). ELNs from plants share some similarity with mammal-derived exosomes: they are both sphere nanoparticles carrying cargos of RNAs, proteins and lipids. The exosomes from mammalian cells, mammalian tissues, or cow milk are often authenticated by immunoblot analysis of exosome-specific surface markers (31-33), but such specific surface markers of

ELNs from plants have not been established. Therefore, the nanoparticles isolated from plants are usually called exosome-like nanoparticles (ELNs) (34,35).

Dietary exosomes or ELNs can be taken up by cells and subsequently have biological effects on them (34,36-38). Dietary exosomes like milk exosomes were taken up by mammalian cells through endocytosis (36). It was suggested that glycoproteins on the surface of exosomes are essential for the uptake of milk exosomes (37). The membrane-enclosed structure of dietary exosomes or ELNs provides protection for their cargos such as RNAs and proteins from degradative condition of the gastrointestinal tract (39). One study showed that RNA cargos from milk exosomes were distributed to brain and kidneys in mice, which is distinctive from the distribution of the intact milk exosomes (40). Depletion of dietary milk exosomes could affect cognitive ability in mice, suggesting the important functions of milk exosomes in animals (38). Grape-derived ELNs were taken up by mouse intestinal stem cells, and promoted their proliferation, and therefore protect mice against DSS-induced colitis (30). All these findings suggest that dietary exosomes or ELNs have biological functions in the consumers.

Mushrooms, which belong to Basidiomycetes and Ascomycetes, have been considered as one of the most nutritional foods because they are enriched in carbohydrates, proteins, unsaturated fatty acids, and vitamins (41). Moreover, some of mushrooms have been widely used for the treatment of many diseases in certain countries because of their medical properties including anti-inflammatory, anti-cancer, antioxidant, anti-microbial and anti-diabetes properties (42-47). Increasing evidence has suggested that mushrooms have therapeutic potential for the prevention or treatment of diseases including cancers, diabetes and atherosclerosis (48-50). Mushrooms play an important role in inhibiting

tumor growth, reducing blood glucose, enhancing immunity, and protecting against infection (49,51-53). A variety of bioactive compounds, including polysaccharides, flavonoids, phenolic compounds, terpenoids and lectins, have been identified in mushrooms and they are suggested to account for the medical functions of mushrooms (43).

The regulatory roles of diet in the NLRP3 inflammasome activity has been reported. Saturated fatty acids (SFAs) including palmitate and stearate activate the NLRP3 inflammasome and thus induce the release of IL-1β (54). However, unsaturated fatty acids (UFAs) including oleate linoleate, and omega-3 fatty acids suppress activation of the NLRP3 inflammasome, therefore reducing IL-1β secretion (55,56). Like UFAs, a variety of natural dietary compounds including polyphenols and flavonoids inhibit activation of the NRLP3 inflammasome (57,58). Hence, these studies suggest an alternative strategy of using natural dietary compounds or adjusting our dietary patterns for the treatment of chronic inflammatory diseases. Here, the NLRP3 inflammasome, the mechanism of NLRP3 inflammasome activation, inflammasome-related inflammatory diseases, extracellular vesicles in disease, functions of dietary exosomes or ELNs, effects of dietary molecules on activation of the NLRP3 inflammasome, and medical functions of mushrooms are discussed.

1.1 NLRP3 inflammasome

NLRP3 is one member of the nucleotide-binding domain and leucine-rich repeat containing gene family (NLR) family (59). NLR family members usually serve as sensors for recognition of pathogens and other dangerous signals. There are 34 NLR family members in mice and 22 NLR family members in humans (60). The NLRs contain a N-

terminal effector region, a centrally located nucleotide-binding-and-oligomerization (NOD) domain (or NACHT domains) and C-terminal leucine-rich repeats (LRRs) (60). The N-terminal effector region mediates downstream interactions between proteins; The centrally located NOD domain participates in oligomerization; The C-terminal LRRs are involved in stimuli recognition (60). The NLRP3 inflammasome is a multiple protein complex, which contains the sensor NLRP3, the adaptor ASC, and the effector caspase-1. ASC participates in assembly of the NLRP3 inflammasome and contains caspase recruitment domain (CARD) domain which is responsible for recruiting caspase-1 (61). Upon activation of the NLRP3 inflammasome, ASC is recruited by NLRP3, and subsequently forms specks in the perinuclear area of the cells. Therefore, ASC specks could be used as an indicator of inflammasome assembly (62-64). ASC further recruits the effector caspase-1 to form the NLRP3 inflammasome complex. Activation of the NLRP3 inflammasome causes the autocleavage of caspase-1 to produce the proteolytic active caspase-1 p10 and p20, which cleaves pro-IL-1β and pro-IL18 into active IL-1β and IL-18 (65,66) (Figure 1). In addition, active caspase-1 also induces pyroptosis, a specific type of inflammatory cell death that release DAMPs (67). Both cytokine release and pyroptosis are essential contributors to the inflammasome-mediated inflammatory process.

1.2 Activation of the NLRP3 inflammasome

Activation of the NLRP3 inflammasome requires two signals (68). The first signal, such as exogenous pathogens or endogenous molecules, induces *Nlrp3* and *Il1b* gene expression through toll-like receptor (TLR) / nuclear factor (NF)- κB pathway. The second signal triggers the assembly of inflammasome and activation of caspase-1. The second

signal could be PAMPs and DAMPs, such as adenosine triphosphate (ATP), pore-forming toxins, and viral RNA (61) (Figure 1). Intracellular K+ efflux, reactive oxygen species (ROS) production, lysosomal destabilization and rupture have been suggested as upstream events of NLRP3 inflammasome activation (9,69).

1.2.1 Intracellular K+ efflux

The release of extracellular ATP triggered by cellular injury or necrosis stimulates K+ efflux via the purinergic P2X7 receptor and thus activates the NLRP3 inflammasome (70). The efflux of K+ induced by pore-forming toxin nigericin is necessary for activation of the NLRP3 inflammasome (71). The low intracellular levels of K+ induced by ATP and nigericin promote the release of IL-1β (72), while inhibition of K+ efflux suppresses the release of IL-1β (73), which suggests the indispensable role of K+ efflux in ATP and nigericin-mediated NLRP3 inflammasome activation. However, Intracellular K+ depletion alone is not sufficient for promoting IL-1β release (74). Furthermore, particulate matter activates NLRP3 inflammasome by triggering the cell membrane permeable to K+ (71). The release of IL-1β induced by imiquimod is not blocked by manipulating K+ efflux. Thus, K+ efflux might not be an upstream event for NLRP3 inflammasome activation induced by imiquimod (75).

1.2.2 Reactive oxygen species (ROS) production

ROS production was suggested to serve as upstream of NLRP3 inflammasome activation. ROS production induced by ATP stimulates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, resulting in activation of the NLRP3 inflammasome and secretion of IL-1β (76). When ROS inhibitors *N*-acetyl-l-cysteine or (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate were used, the secretion of IL-1β was decreased,

implicating that ROS production is crucial for the NLRP3 inflammasome activation (77). Furthermore, when RNA interference (RNAi) was used to downregulate ROS detoxifying proteins, the secretion of IL-1β was increased (77). NAPDH oxidases (NOXs) are involved in generation of ROS in cells (78). Interestingly, cells deficient in NOX activity exhibited the normal amounts of IL-1β secretion, but treatment of ROS scavengers in cells inhibited the secretion of IL-1β, suggesting ROS is required for activation of the NLRP3 inflammasome, and NOXs are not the only source for ROS production during inflammasome activation (79). Consistently, another ROS inhibitor diphenyliodonium also inhibits NLRP3 inflammasome activation (80).

1.2.3 Lysosomal destabilization and rupture

Particulate matter, also called particle pollution, is solid and liquid droplets suspended in air (81). A range of particulate matter (less than 1% of total particle mass) such as silica, asbestos and alum activate the NLRP3 inflammasome (81-83). The phagocytosis of particulate matter causes lysosomal swelling and rupture, release of lysosomal contents into cytoplasm, and subsequent NRLP3 inflammasome activation (82). The inhibition of phagocytosis impaired NRLP3 inflammasome activation in response to particulate matter (82). The H+ ATPase inhibitor, which blocked lysosomal acidification, attenuated NLRP3 inflammasome activation (82). The inhibition of lysosomal protease also reduced activation of the NLRP3 inflammasome (82). These studies indicated that lysosomal destabilization is required for the particulate matter-induced NLRP3 inflammasome activation.

1.3 Role of NLRP3 inflammasome activation in diseases

The NLRP3 inflammasome plays an important role in inflammatory responses

and is incriminated in the pathogenesis of many metabolic diseases, including type 2 diabetes, atherosclerosis and liver disease.

1.3.1 Type 2 Diabetes

The NLRP3 inflammasome contributes to the progression of type 2 diabetes (84). IL-1β, one downstream of NLRP3 inflammasome activation, induces the inflammation in pancreatic islets, which leads to the destruction of β-cells, and therefore reduces insulin production (85). Also, IL-1β leads to insulin resistance and affects the glucose uptake (86). Increased macrophage infiltration has also been found in the pancreatic islets of patients with type 2 diabetes (87). Knockout of NLRP3 in mice protected pancreatic β-cells from damage and thus these mice had increased insulin levels in the blood (17). The antidiabetic drug glyburide was shown to inhibit NLRP3 inflammasome activation (88,89). TXNIP inhibitor, which inhibited the NLRP3 inflammasome activation, enhanced glucose tolerance and insulin sensitivity in mice (16).

1.3.2 Atherosclerosis

Atherosclerosis has been considered as an inflammatory disease and is characterized by the accumulation of lipids, cholesterol, and other substances in the artery wall (90). Cholesterol metabolism is tightly linked with inflammation in atherosclerosis (91). Cholesterol crystals are accumulated in atherosclerotic lesions and trigger activation of the NLRP3 inflammasome and thus lead to IL-1β release from macrophages in the lesions (92). The level of IL-1β is correlated with the severity of atherosclerosis (92). IL-1β deficiency decreased the severity of atherosclerosis in apolipoprotein E (ApoE) knockout mice (93). Similarly, knockdown of the *Nlrp3* gene in ApoE knockout mice showed lower levels of proinflammatory cytokines, less

macrophage infiltration in the atherosclerotic lesions, and overall less severity of atherosclerosis induced by hyperhomocysteinemia (94). Silencing the *Nlrp3* gene or using caspase-1 inhibitor blocked homocysterine-induced NLRP3 inflammasome activation in macrophages (94). In addition, when RNAi was used to silence the sensor NLRP3, cholesterol crystal-induced IL-1β secretion were decreased, suggesting the important role of NLRP3 inflammasome in cholesterol crystal-induced inflammation (95).

1.3.3 Liver disease

IL-1 β secreted by macrophages (Kupffer cells) in liver is key driven for alcohol-induced liver inflammation, steatosis, and damage, because IL-1 receptor antagonist significantly attenuates this alcoholic hepatitis (96). The hepatocytes damaged by alcohol release DAMPs, which activate the NLRP3 inflammasome in Kupffer cells (97). When a mutant form of *Nlrp3* gene resulting in a hyperactive NLRP3 was introduced in mice, these knock-in mice show severe inflammation, fibrosis in liver and remarkable hepatocyte pyroptotic cell death (98). Sulforaphane, which inhibits activation of the NLRP3 inflammasome through AMP-activated protein kinase-autophagy axis, attenuates hepatic steatosis in high fat diet-treated mice (99). Therefore, strategies that inhibit IL1-β and NLRP3 inflammasome activation could have high therapeutic potential to treat liver disease.

1.4 Extracellular vesicles

Extracellular vesicles (EVs) are phospholipid bilayer-enclosed tiny particles released from eukaryotic and prokaryotic cells (19,20). Almost all the cell types, such as T cell, dendritic cells, tumor cells, stem cells and endothelial cells, release EVs (23).

EVs have been found in urine, saliva, blood plasma and breast milk (100-103). Based on their sizes, origins, surface markers, and biological functions, EVs are mainly classified into exosomes, microvesicles and apoptotic bodies (20-22,104). Ultracentrifugation, immunoaffinity and chromatography are typical approaches to isolate EVs and subtypes of EVs can be isolated using different approaches (105). EVs are recognized to transfer biological information between cells by carrying cargos of RNAs, proteins and lipids (106). Importantly, EVs play a key role in both physiological and pathological processes (19, 107).

1.4.1 Content of EVs

A variety of techniques have been used to characterize protein composition of EVs, including mass spectrometry-based proteomics, western blotting, enzymatic activity and serological reactivity (108,109). Some of proteins in EVs could serve as biomarkers for diseases detection or targets of immunotherapies (110). CD63, CD9, CD81, MHC I, MHC II, Gp96, Actinin-4, Tsg 101, heat-shock proteins 70 have been identified as common surface biomarkers of EVs and some of them are specific surface markers for some subgroups of EVs (111). In addition to the proteins, EVs are enriched with RNAs, including mRNAs, miRNAs, rRNAs and tRNA fragments (112-114). Most of the RNAs in EVs are very short and fragmented (115). RNAs delivered to the recipient cells are actually functional (116). miRNAs in dendritic cell-derived exosomes downregulate target genes in recipient dendritic cells (117). miR-155 encapsulated in exosomes was delivered to cardiac fibroblasts and inhibited proliferation of these cells (118). miR-135b in hypoxia-resistant multiple myeloma cell-derived exosomes increased endothelial tube formation (46). Like proteins, miRNAs in EVs can be used

as biomarkers for disease diagnosis or therapy because specific miRNAs could be aberrantly enriched in EVs under certain pathological conditions (119,120). Exosomal miR-1290 and miR-375 have been studied as promising biomarkers in diagnosis of castration-resistant prostate cancer (121). EVs display a specific lipid composition and organization (122). Usually glycosphingolipids, sphingomyelin, cholesterol and phosphatidylserine are highly enriched in most EVs, indicating an important role of these lipids in EVs biogenesis (123). These lipids contribute to the stability and structural rigidity of EVs (124,125). Phosphatidylserine on the surface of exosomes is thought to be an intercellular signaling, which can be recognized by Tim4 receptors on the macrophages (126).

1.4.2 Physiological and pathological role of EVs

EVs are engaged in a variety of physiological and pathological processes by mediating intercellular communication (127). Direct cell-cell communication is often achieved through membrane surface molecules (128). In contract, EVs mediate intercellular communication through their cargos of protein, RNAs, and lipids (106). After EVs are taken up by recipient cells, their cargos are released and thus mediate the functions of target cells (129). EVs are involved in stem cell maintenance, tissue repair, immune surveillance, and blood coagulation (130). EVs from embryonic stem cells deliver mRNA to recipient stem cells to increase their pluripotency (107). Microvesicles released from mesenchymal stem cells inhibited apoptosis and promoted proliferation of tubular epithelial cells, thereby protecting rats from acute kidney injury (131). Exosomes released from B lymphocytes induce T cell responses (25). EVs released from hepatocytes transferred RNAs to liver stellate-like cells and activate their proliferation

(132). EVs released from neurons represent a novel way for inter-neuronal communication, as specific neuron-derived EVs directly regulated signal transduction and protein expression in target neurons cells (133).

Exosomes released from dendritic cells regulate the inflammatory response by regulating inflammatory gene expression in recipient dendritic cells (134). Exosomes derived from FasL-expressing dendritic cells inhibit inflammation in a murine arthritis model (135). This study indicated that these specific exosomes may have the therapeutic potential to treat arthritis (135). LPS-stimulated macrophage-derived exosomes have an immunoprotective role, because an intraplantar injection of these exosomes reduced paw thickness and thermal hypersensitivity in a mouse model of inflammatory pain (136). Exosomes from body fluids of ovarian cancer patients have immunomodulatory capacities because they trigger the gene expression of NF-κB- and STAT3-mediated cytokines in monocytic precursor cells (137). Exosomes released from adipose-derived stem cells increased insulin sensitivity, reduced weight and attenuated hepatic steatosis in obese mice (138). Donor mouse-derived peripheral exosomes inhibited the inflammation of the allograft heart in recipient mice by inducing Treg cells, which potently suppress immune responses (139).

Because EVs transfer biological information between cells, it is possible that EVs also transfer disease-driven factors between cells to spread diseases. EVs isolated from parasite-infected host cells or plasma contain both host and parasite molecules, disseminate the pathogens, and regulate the host immune systems (140). EVs participate in the pathogenesis of various diseases, including osteoarthritis, atherosclerosis, thromboembolism, chronic renal disease, pulmonary hypertension, periodontitis, gastric

ulcers and bacterial infections (141). Tumor-derived EVs deliver causative molecules such as microRNAs or oncogenes to recipient cells, resulting in the change of biological functions, gene expression pattern, and overt tumorigenic conversion in recipient cells (142). The levels of EVs are increased in atherosclerotic lesions and they may lead to the progress of atherosclerosis (143). The level of plasma EVs is higher in patients with cardiovascular diseases compared with healthy individuals. Therefore the circulating EVs could be used as a prognostic marker for diagnosis of cardiovascular (144). EVs cause extracellular matrix degradation and inflammation by carrying catabolic proteases, antigens and miRNA, thus contributing to the pathophysiology of osteoarthritis and rheumatoid arthritis (145). Given the fact that EVs mediate disease pathogenesis, the potential use of EVs as diagnostic and therapeutic biomarkers has been explored in various diseases. Developmental endothelial locus-1 protein in circulating EVs is a promising biomarker, which could be potentially used to identify early-stage breast cancer (146). Specific miRNA in salivary exosomes has the potential to be utilized as a biomarker for diagnosis of salivary gland diseases such as Sjögren's syndome (102).

1.5 Dietary exosomes or exosome-like nanoparticles (ELNs)

Dietary exosomes or ELNs have been demonstrated to participate in interspecies communication (34). For example, grape-derived ELNs induced $Lgr5^{hi}$ intestinal stem cells and by doing so protected mice from DSS-induced colitis (30). Grapefruit-derived ELNs attenuated DSS-induced colitis by inhibiting the production of IL-1β and Tumor necrosis factor-α (TNF-α), suggesting their immune modulatory role in colitis (147). In addition, grapefruit-derived ELNs have been utilized as a nano-vector to deliver

therapeutic compounds to tumor sites (148). Compared with synthetic nanoparticles, grapefruit-derived ELNs do not cause cytotoxic reactions (149).

Besides grape-derived and grapefruit-derived ELNs, milk exosomes gain extensive interest. The exosome surface markers such as MHC, CD63, CD81 and CD86 are detectable in exosomes isolated from human breast milk, and these exosomes inhibit the release of interleukin-2 (IL-2) and interferon- γ (IFN-γ), suggesting the immune regulatory functions of these exosomes (103). Immune-related miRNAs are found in breast milk exosomes and can be transferred to infants and thereby affect the development of infant immune system (150). Bovine milk exosomes (BMEs) are taken up by human and rate intestinal cells through endocytosis and glycoproteins on the surfaces of exosome play important role in uptake of BMEs (151). The bioactivity of BMEs has been demonstrated *in vivo* and in *vitro*. BMEs deliver miRNAs into macrophages and therefore alter gene expression of inflammatory cytokine such as IL-1, IL-6 and IL-10, and increase phagocytosis (152). Depletion of BMEs in the mouse diet not only alters the gene expression pattern and the profiles of amino acid in skeletal muscle of mice, but also alters their microbial communities in gut, suggesting that BMEs facilitate the interspecies communication among cow milk, host, and microbiomes (153,154). Compared with the distribution of intact BMEs, the distribution of specific miRNA cargoes from BMEs has a distinct pattern in mice (155). Intact BMEs are mainly accumulated in liver, spleen and brain, while their fluorescence-labeled microRNA cargos distribute in intestinal mucosa, heart, liver, spleen and brain (155). BMEs regulate lung inflammatory responses through promoting M1 macrophage polarization and increasing the levels of IL-6 and TNF-α (156). BMEs were used for the delivery of

chemotherapeutic/chemopreventive agents in mice and did not cause adverse immune and inflammatory response (157). Therefore, BMEs have high potential to serve as a drug delivery platform.

1.6 Effects of dietary molecules on activation of the NLRP3 inflammasome

1.6.1 Saturated fatty acids

Many foods are abundant in saturated fatty acids (SFAs), including fast foods, meats and dairy products (158). Dietary SFAs trigger NLRP3 inflammasome activation in adipocytes and macrophages via several possible mechanisms, including JNK signaling pathways, toll-like receptor 2/4 (TLR 2/4) (159-162), or an AMPK-autophagy-ROS signaling pathway (163). It was also suggested that SFAs-induced NLRP3 inflammasome activation involves in Na, K-ATPase disruption and K+ efflux (164). SFAs such as free fatty acids (FFAs) activate the NLRP3 inflammasome and result in IL-1β secretion, which inhibits insulin signaling through serine-threonine kinase phosphorylation and thereby leads to insulin resistance (163). A recent study shows that the reduction of inter-endothelial tight junction proteins ZO1/2 and increased permeability of endothelial monolayers were associated with FFAs-induced NLRP3 inflammasome activation because these impairments were reversed by NLRP3 inflammasome-related inhibitors such as HMGB1 inhibitor and lysosomal cathepsin B inhibitor (165). Palmitate (PA), a long-chain SFA, activates NLRP3 inflammasome and thus promotes inflammatory responses in cardiac fibroblasts (55). A new mechanism of SFA-induced NLRP3 inflammasome activation was proposed recently. SFAs became crystallized inside of macrophages, subsequently caused lysosomal dysfunction, and therefore led to activation of the NLRP3 inflammasome (54).

1.6.2 Unsaturated fatty acids

Unlike SFAs, unsaturated fatty acids (UFAs) inhibit NLRP3 inflammasome activation in macrophages or monocytes (55). UFAs improve insulin resistance by reducing NLRP3 inflammasome-dependent IL-1β secretion in high fat diet (HFD)-induced obese mice (166). Fish oil enriches in omega-3 polyunsaturated fatty acids (PUFAs), which are beneficial for human health. Interestingly, omega-3 PUFAs also inhibit activation of the NLRP3 inflammasome and reduce transient expression of the *Il1b* gene in peripheral blood mononuclear cells (167-169). In the traumatic brain injury model, omega-3 PUFAs prevented mitochondrial localization of the sensor NLRP3 and by doing so inhibited NLRP3 inflammasome activation and reduced IL-1β secretion (170). Omega-3 PUFAs downregulated expression of inflammatory genes including *Nlrp3* and *Il1b* genes in adipocytes and monocytes/macrophages stimulated with LPS plus INF-γ (171). IL-1β secretion and caspase-1 autocleavage were reduced in macrophages and hepatocytes from dietary PUFAs-fed mice and it seems that dietary PUFAs attenuated NLRP3 inflammasome activation through increasing autophagy pathway (172). 17-oxo-DHA, a derivative of omega-3 fatty acids Docosahexaenoic acid (DHA), inhibited NLRP3 inflammasome activation through suppressing mROS production and ERK signaling in macrophages (173).

1.6.3 Other dietary components

Components extracted from various foods have anti-inflammatory properties. Some of them inhibit activation of the NLRP3 inflammasome, though the underlying mechanism is poorly understood. Epigallocatechin-3-gallate (EGCG) is a polyphenolic component extracted from green tea. It has been shown that EGCG inhibited expression

of *Nlrp3* gene in mice with lupus nephritis (174). Another polyphenolic compound from turmeric called curcumin inhibited caspase-1 autocleavage and IL-1β secretion upon NLRP3 inflammasome activation, possibly by suppressing K+ efflux (175). Purple sweet potato color (PSPC) is a flavonoid extracted from purple sweet potato. It inhibited NF-kB nuclear translocation and NLRP3 inflammasome activation, and therefore protected high-fat diet mice against hepatic inflammation (176). Fruits enriched in procyanidins, such as grapes, have been reported to have inhibitory effects on inflammatory response and inflammatory diseases (177,178). Later, Yang group revealed that procyanidin blocked the transcriptional activity of activator protein-1 pathway, which led to the inhibition of ROS production, and subsequently suppressed NLRP3 inflammasome activation in endothelial cells (57). Mangiferin isolated from mangos inhibited activity of the transcription factor NF-kB, which regulates transcription of many inflammatory genes including cytokines and chemokines (179). Magniferin also inhibited NLRP3 inflammasome activity. As a result, Mangiferin alleviated severity of some diseases including mastitis, traumatic brain injury and liver injury (180-183). Resveratrol is enriched in fruits such as grapes, raspberries, blueberries and mulberries (184). Resveratrol treatment helped to maintain mitochondrial integrity, reduce the mROS production and therefore attenuate activation of the NLRP3 inflammasome (185,186). Sulforaphane, a compound enriched in broccoli, inhibited NLRP3 inflammasome activation and ameliorated Ischemia/reperfusion injury and acute gout in animal models (187,188). It was demonstrated that Taiwanese green propolis suppressed NLRP3 inflammasome activation and its bioactive compound called propolin G likely mediated this inhibitory effect (189). In summary, these dietary

components represent a novel and alternative strategy to inhibit activation of the NLRP3 inflammasome and thus curb or treat chronic low-grade inflammation in a variety of diseases.

1.7 Medical functions of mushrooms

1.7.1 Anti-inflammatory property

Mushrooms have been reported to have anti-inflammatory functions. A mushroom called Huaier (*Trametes robiniophila* Murr.) inhibited NLRP3 inflammasome activation via the autophagy-lysosome degradation pathway, and therefore attenuated symptoms of DSS-induced colitis (190). Glucans extracted from Indian oyster (*Pleurotus pulmonarius*) also suppressed inflammation in DDS-induced colitis, suggesting its potential role in the treatment of inflammatory bowel disease (191). Ergosterol peroxide (a bioactive compound found in hen of the woods (*Grifola frondosa*)), cauliflower mushroom (*Sparassis crispa*) and lingzhi mushroom (*Ganoderma lucidum*), suppressed cytokine production by inhibiting mitogen-activated protein kinases (MAPKs) signaling pathways or NF-κB signaling in macrophages (192-194). Oyster mushroom (*Pleurotus ostreatus*) also showed anti-inflammatory function because it suppressed TNF-α and IL 6 secretion both *in vitro* and *in vivo* (195). Addition of portobello (*Agaricus bisporus*) and shiitake mushrooms (*Lentinula edodes*) in the diet protected $Ldlr^{-/-}$ mice from high-fat diet-induced atherosclerosis (50).

1.7.2 Anti-cancer property

The anti-cancer property of mushrooms has been widely studied. A protein FVE isolated from Enoki mushroom (*Flammulina velutipes*) and grifolin isolated from Sheep Polypore mushroom (*Albatrellus confluens*) and white button mushroom *(Agaricus*

bisporus) inhibited tumor growth, indicating that these mushrooms contain anti-tumor agents (51,196,197). In most cases, mushrooms induce apoptosis of cancer cells. For example, Chaga mushroom (*Inonotus obliquus*), lection isolated from *Kurokawa* mushroom (*Boletopsis leucomelas*), and grifolin isolated from Sheep Polypore mushroom (*Albatrellus confluens*) are all capable of inducing apoptosis of cancer cells (197-199). Some studies have demonstrated that some of the mushrooms or their extracts, such as willow bracket (*phellinus igniarius*) and polysaccharide lentinan from shiitake mushroom (*Lentinus edodes*) caused cell cycle arrest at G_0/G_1 phase and prevented tumor cell proliferation (200,201). White button mushroom (*Agaricus bisporus*) inhibited the proliferation of both prostate cancer cells and breast cancer cells (51,202). Song gen mushroom (*Phellinus linteus*) caused either cell-cycle arrest or apoptosis in lung cancer cells depending the dosage used, suggesting it has an anti-cancer effect (203). Ergosterol peroxide isolated from Chaga mushroom (*Inonotus obliquus*) inhibited proliferation of colon cancer cells *in vitro* and tumor growth in mice (204).

1.7.3 Other properties

Besides anti-inflammatory and anti-cancer properties, mushrooms also show other beneficial effects such as enhancement of immunity, antioxidant function, and anti-microbial function (204-206). Mushrooms enhance immunity through stimulating natural killer cell or increasing IFN γ and TNFα secretion (207). Consumption of dried shiitake mushroom (*Lentinus edodes*) enhanced immunity in humans by promoting proliferation of gamma delta T (γδ-T) cells and natural killer T (NK-T) cells and increasing secretory immunoglobulin A in saliva (208). Lectin isolated from mushroom *Fomitella fraxinea* has an immunomodulatory function. It increased major

histocompatibility complex (MHC)-restricted antigen presentation and protected chicken from coccidiosis (52,209). Oyster mushroom (*Pleurotus ostreatus*) extracts protected rats from CCl_4-induced liver injury by decreasing the levels of liver-related enzymes, including glutamic oxaloacetic transaminase, alkaline phosphatase and glutamic pyruvate transaminase, and improved antioxidant activity of liver by increasing antioxidant activity-related enzymes, such as catalase, glutathione peroxidase and superoxide dismutase (210). Extracts (using ethanol) from crab-of-the-woods mushroom (*Laetiporus sulphureus*), extracts (using water, methanol, acetone, and ethyl acetate) from Enoki mushroom (*Flammulina velutipes*), white and brown shimeji variants (*Hypsizygus tesselatus*) inhibited the growth of bacteria. These findings suggest that these mushrooms contain antimicrobial agents, which could be potentially used for the treatment of infection (46,211). Extracts (using water) from Almond mushroom (*Agaricus blazei* Murill) protected mice against *streptococcus pneumoniae* infection (53).

1.8 Conclusion

The NLRP3 inflammasome is a multiprotein complex that consists of the sensor NLRP3, the adaptor ASC, and the effector caspase-1. Both endogenous DAMPs or exogenous PAMPs can trigger NLRP3 inflammasome activation, thereby leading to caspase-1 autocleavage and subsequent IL-1β secretion. Several models have been proposed to explain how NLRP3 inflammasome activation is triggered. Intracellular K+ efflux, ROS production, lysosomal destabilization and rupture were indicated as the upstream events of NLRP3 inflammasome activation. Dysregulated activation of the NLRP3 inflammasome is involved in a variety of chronic diseases, such as type 2 diabetes,

atherosclerosis and liver disease. EVs are phospholipid bilayer-enclosed tiny particles released from eukaryotic and prokaryotic cells. They play an important role in intercellular communication and even interspecies communication by delivering and releasing cargos such as microRNAs in the target cells. Exosomes, a subtype of EVs, are nanoparticles that contain lipids, RNAs, and proteins. Dietary exosomes or exosome-like nanoparticles (ELNs) are taken up by consumers, distributed in different tissues, and have bioactive functions in animal models. Mushrooms have showed a range of medical benefits to human health and therefore their bioactive components represent promising candidates for the treatment of diseases. Therefore, in our studies, we aimed to evaluate the effects of ELNs extracted from mushrooms on NLRP3 inflammasome activity.

CHAPTER 2. EXPERIMENTS AND RESULTS

2.1 Introduction

The NLRP3 inflammasome is a multiprotein complex composed of NLRP3, ASC and caspase-1 (10,11). A variety of exogenous PAMPs or endogenous DAMPs stimulate the NLRP3 inflammasome, leading to caspase-1 autocleavage and IL-1β and IL18 secretion (65). Activation of the NLRP3 inflammasome requires two signals: the first signal stimulates expression of the *Nlrp3* and *Il1b* genes; the second signal causes the assembly of inflammasome complex, which activates the autocleavage of caspase-1 to produce caspase-1 p10 and p20, and then cleave pro-IL-1β and pro-IL18 to generate active IL-1β and IL 18 (65). IL-1β is a major cause of acute and chronic inflammation and thus involved in the pathological processes of various inflammatory diseases (212). Therefore, activation of the NLRP3 inflammasome contributes to the pathogenesis of these diseases. Emerging evidence demonstrates that inhibition of the NLRP3 inflammasome suppresses the secretion of IL-1β, and therefore improves the conditions of chronic inflammatory diseases, including diabetics, Alzheimer's disease and gout (213-215). In short, the NLRP3 inflammasome is suggested to be a promising therapeutic target for treatment of NLRP3 inflammasome-related diseases.

Exosomes are biolayer membrane-enclosed nanoparticles released from cells (20). They participate in intercellular communication and even interspecies communication by delivering and releasing their cargos of RNAs, proteins and lipids to target cells, thereby regulating the bioactivity of target cells, including gene expression and functions (106). Dietary exosomes or exosome-like nanoparticles (ELNs) have been obtained from diet sources. A variety of dietary ELNs have been identified from edible plants such as ginger,

carrots, grapefruit and grapes (34). In particular, dietary ELNs have therapeutic potential for the treatment of diseases (30). Mushrooms have been utilized as medicines in certain countries because of their anti-inflammatory, anti-cancer, antioxidant and anti-microbial properties (42-47). We are highly interested in mushrooms due to their medical properties. To the best of our knowledge, mushroom-derived ELNs have never been studied and the effects of mushroom-derived ELNs on NLRP3 inflammasome activation has never been evaluated.

In this study, shitake mushroom-derived ELNs (S-ELNs) were isolated and characterized for the first time. We discovered the inhibitory effects of S-ELNs on NLRP3 inflammasome activation in macrophages. Furthermore, we identified that lipids in S-ELNs as active biomolecules that suppressed NLRP3 inflammasome activation. Collectively, S-ELNs represent a promising agent that blocks NLRP3 inflammasome activation.

2.2 Central Hypothesis, Purpose, and Specific Aims

A. Purpose of the Study

The purpose of the study is to investigate the effects of S-ELNs on the NLRP3 inflammasome activation in bone marrow-derived macrophages (BMDM) and identify active biomolecules in S-ELNs with anti-inflammasome functions.

B. Central Hypothesis

S-ELNs inhibit NLRP3 inflammasome activation in macrophages.

C. Specific Aims

Specific Aim 1: Evaluate the effects of S-ELNs on NLRP3 inflammasome activation.

Specific Aim 2: Identify which category of cargos (RNAs, proteins and lipids) is the active

biomolecules that inhibit the NLRP3 inflammasome in macrophages.

2.3 Materials and Methods

2.3.1 Preparation of S-ELNs

Mushrooms were washed with water and gently dried using tissues. After washing, mushrooms were weighted and cut into small pieces. The mushroom pieces were put in cold phosphate buffered saline (PBS) and homogenized in a kitchen blender for 15 seconds. The juice was collected and centrifuged at 500g, 10 minutes 4 ℃ and then at 2000g, 20 minutes, 4 ℃, respectively. The juice was then collected and ultracentrifuged at 10,000g, 30 minutes, 4 ℃ and at 100,000g, 2 h, 4 ℃, respectively. The pelleted ELNs were resuspended in 2 ml PBS and passed through a 0.45 μm filter and then 0.2 μm filter. The concentration of ELNs was measured using the Nanosight NS300 machine (Malvern Panalytical).

2.3.2 RNA depletion of S-ELNs

0.2 μl RNase (10 ng/ml) was added to 200 μl S-ELNs ($3.55*10^{12}$ /ml). The solution underwent bath sonication for 90 minutes at room temperature using a bath sonication machine (Branson CPX5800H). After sonication, the solution was incubated at 37 ℃ water bath for another 1 h.

2.3.3 Liposome preparation

Lipids in S-ELNs were extracted based on the Folch method (30). 1000 μl S-ELNs ($1.12*10^{12}$ /ml) were added to 3800 μl mixture of CHCl3 (1267 μl) : MeOH (2533 μl) at a 1:2 ratio (v/v) and vortex. An aliquot (1267 μl) of $CHCl_3$ was added to the mixture. The mixture was vortexed and stirred vigorously for 1 h. After stirring, 1267 μl molecular water was added to the mixture. The mixture was vortexed and stirred vigorously for another 1

h. The mixture was centrifuged at 2,000 g for 10 minutes at room temperature, and then organic phase containing lipids was collected. The organic phase was dried under nitrogen gas (0.2-0.5 psi) at 60 ℃. Dried lipids were suspended in 200 µl molecular water, and subsequently sonicated for 5 minutes. 200 µl buffer (40 mM HEPES, 308 mM NaCL, Ph 7.4) was added and sonicated for another 5 minutes to obtain liposomes. The liposomes were used immediately or stored at – 80 ℃.

2.3.4 Scanning electron microscopy (SEM)

S-ELNs were purified using sucrose gradient methods. 1000 µl crude S-ELNs were added on top of sucrose gradient with density of 2M, 1.8M, 1.6M, 1.4M, 1.2M, 1M, 0.8M, 0.6M, 0.4M, 0.2M and then were ultracentrifuged at 100,000g, 12 h, 4 ℃. The layer contains S-ELNs was collected and fixed with glutaraldehyde (final concentration 2.5%, EMS#16320) for 30 minutes at room temperature. Subsequently, S-ENLs were fixed with tannic acid (final concentration 1%, EMS#21700) for 30 minutes at room temperature. After fixation, S-ELNs were loaded on the Nucleopore track-etched membrane (100 nm pore size, Whatman), dried overnight, and sputter-coated using a Desk Vsputter (Denton Vacuum InC). S-ELNs were visualized using scanning electron microscope (Hitachi S4700 FE).

2.3.5 Agarose gel, SDS-PAGE gel and Thin-layer Chromatography (TLC) analysis

Total RNAs were extracted from S-ELNs using commercial miRNeasy mini kit (Qiagen). 700 µl QIAzol lysis reagent was added to S-ELN pellets. The RNAs were eluted in 30 µl RNase-free molecular biology grade water (Corning). 400 ng RNAs were loaded into 2% agarose gels to separate. Proteins extracted from S-ELNs were separated using SDS-PAGE gels (Novex) and gels were stained using Coomassie Brilliant Blue. Lipids

were extracted from S-ELNs using the Folch method mentioned above. The lipids were separated on the thin-layer chromatography (TLC) plate (Millipore Sigma) using a solvent system (chloroform : methanol : acetic acid at a ratio of 190:9:1, v/v/v) and visualized using $CuSO_4$ phosphric acid solution.

2.3.6 Macrophage cell culture and ELNs treatment

Mouse bone marrow-derived macrophages (BMDMs) were cultured using the bone marrow cells from the femurs and tibias of C57BL/6 mice. The bone marrow cells stored at -80 ℃ were thawed and cultured in growth media (61% RPMI 1640 media (Corning), 1% sodium pyruvate (Corning), 1% L-glutamine (Corning), 1% penicillin/streptomycin (Corning), 1% HEPES (Corning), 25% L929 media and 10% fetal bovine serum (FBS, Atlanta Biologicals)). Cells were incubated at 37 ℃ in 5% CO_2 for 6-8 days until a uniform monolayer of macrophages was established. Macrophages were split and seeded into a 12-well plate with growth media overnight. The culture media were changed to plating media (76% RPMI 1640 media (Corning), 1% sodium pyruvate (Corning), 1% L-glutamine (Corning), 1% penicillin/streptomycin (Corning), 1% HEPES (Corning), 10 % L929 media and 10% precleared-fetal bovine serum (FBS, Atlanta Biologicals)). 4 h later, S-ELNs were added to cells. After 16 h S-ELN incubation, Lipopolysaccharide (LPS (10 ng/ml), InvivoGen, tlrl-peklps) was added to cells and incubated for 3 h. Free fatty acid sodium palmitate (FFA) (Sigma) was added to cells and incubated for another 12 h. The culture medium was collected and centrifuged at 300 g, 5 minutes, 4℃ to remove cells and big cell debris. The precleared culture medium was used to measure IL-1β release using ELISA kit (eBioscience). The cell lysates were collected using Sodium Dodecyl Sulfate (SDS) lysis buffer and subject to western blot to measure capase-1 autocleavage. Macrophages

were also stimulated using Alum (Thermo Scientific), nigericin (Enzo Life Sci) or ATP (Sigma) to activate the NLRP3 inflammasome.

2.3.7 ELISA and western blot

The level of IL-1β in the culture medium was measured using ELISA kit (eBioscience). Proteins in cell lysates were separated using 4-12% SDS-PAGE gels (Novex) and transferred into polyvinylidene difluoride (PVDF) membrane (0.45 μm, GE Healthcare). Primary antibodies against tubulin, caspase-1 p10, pro-IL-1β, ASC, NLRP3 and Nek7 were used to incubate with membrane overnight. The secondary antibodies including anti-rabbit-HRP, anti-mouse-HRP or anti-goat-HRP (Cell Signaling) were used to detect the levels of proteins. Tubulin antibody was purchased from Santa Cruz Biotechnology. Primary antibodies against NLRP3, ASC, caspase-1 p10 were purchased from Adipogen. Primary antibody against Nek7 was purchased from Abcam. Anti-pro-IL-1β antibody was purchased from R&D systems.

2.3.8 Uptake studies of S-ELNs

RNAs in S-ELNs were labeled using ExoGlow^{TM}-RNA EV labeling kit (SBI System Biosciences). 8.3 μl S-ELNs ($1.83 * 10^{12}$ /ml) were added to incubation buffer to make a 50 μl solution. 3 μl ExoGlow RNA probe was added to the mixture and then the mixture was incubated at 37 ℃ 1 h in dark. The mixture was added into 35 ml PBS and ultracentrifuged at 100,000g, 2 h to remove free probes. The pellet of RNA-labeled S-ELNs was suspended in 2 ml PBS. Lipids in S-ELNs were labeled using DiI dye (Thermo Fisher Scientific) as per manufacturer's protocol. After labeling, the mixture was added into 35 ml PBS and ultracentrifuged at 100,000g, 2 h to remove free DiI dye. The pellet of lipid-labeled S-ELNs was suspended in 2 ml PBS.

Glass coverslips were pre-plated in a 24-well plate, and then macrophages were seeded and cultured using growth media overnight. 4 h before S-ELNs were added to cells, the culture medium was changed to plating medium. RNA-labeled or lipid-labeled S-ELNs were added to macrophages. After 16 h incubation, macrophages were washed with PBS for 3 times and fixed with 4% paraformaldehyde (Sigma) on ice for 20 minutes. After 20 minutes, macrophages were washed with PBS for 3 times. Nucleus was subsequently stained using DAPI (Fisher Scientific). The uptake of fluorescence-labeled S-ELNs by macrophages was evaluated using a confocal fluorescence microscope (Nikon A1R).

2.3.9 ASC speck detection

Glass coverslips were pre-plated in a 24-well plate, and then macrophages were seeded and cultured using growth media overnight. The culture medium was changed to plating media. 4 h later, S-ELNs were added to cells. After 16 h incubation, macrophages were incubated with LPS (10 ng/ml) for 3 h, followed by incubation of caspase-1 inhibitor VX765 (10 μM) for 0.5 h. Finally macrophages were stimulated with FFA for 12 h. After 12 h incubation, macrophages were washed with PBS for 3 times and fixed with 4% paraformaldehyde (Sigma) on ice for 20 minutes. After 20 minutes, macrophages were washed with PBS for 3 times. Rabbit anti-ASC antibody (Adipogen) was used to incubate with macrophages overnight at 4 ℃. Secondary antibody anti-rabbit Alexa Fluor 594 (Invitrogen) was used. Nucleus were stained using DAPI (Fisher Scientific). Immunofluorescence was detected using a confocal microscope (Nikon A1R).

2.3.10 RNA extraction and real time quantitative polymerase chain reaction (qPCR)

Macrophages were treated with S-ELNs for 16 h, incubated with LPS for 3 h, and then activated with FFA for 12 h or ATP for 0.5 h. mRNAs were extracted from

macrophages using RNA bee (Tel-Test) and then the concentration of mRNA was measured. 1 μg mRNAs were used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed to assess expression of the genes including *Nlrp3*, *Caspase1*, *Pycard* and *Il1b* using a real time qPCR machine (Bio-Rad). All genes were normalized to a hypoxanthine guanine phosphoribosyl transferase (*Hprt)* gene. Primers of these genes were designed in advance and ordered from Integrated DNA Technologies.

2.3.11 Statistical analysis

Comparisons of treatment group with control group were analyzed using two-tailed t-test. P values were calculated: * $P < 0.05$, ** $P < 0.01$. Both * and ** were consided as significant.

2.4 Results

2.4.1 S-ELNs strongly inhibited caspase-1 autocleavage and IL-1β secretion upon NLRP3 inflammasome activation

Activation of the NLRP3 inflammasome leads to autocleavage of caspase-1 to produce the enzymatic active caspase-11 p10 and p20, which process pro-IL1β and pro-IL18 to produce active IL-1β and IL18. Very often, caspase-1 autocleavage and IL-1β secretion are used as indicators of NLRP3 inflammasome activation (65). To screen the effects of different mushroom-derived ELNs on activation of the NLRP3 inflammasome, six mushrooms were chosen to extract ELNs: White beach mushroom (*White buna shimeji*), Brown beach mushroom (*Brown buna shimeji*), White common mushroom (*White agaricus bisporus*), Brown common mushroom (*Brown agaricus bisporus*), King mushroom (*Pleurotus* eryngii) and Shiitake mushroom (*Lentinus edodes*). Macrophages

were treated with mushroom-derived ELNs for 16 h. Mushroom-derived ELNs-primed macrophages were treated with LPS for 3 h and then were activated with FFA for 12 h. Caspase-1 autocleavage and IL-1β secretion were measured using western blot and ELISA, respectively. Among six mushroom-derived ELNs we tested, shiitake-derived ELNs (S-ELNs) strongly inhibited both caspase-1 autocleavage and IL-1β secretion in a dose-dependent manner (Figure 2F). In contrast with S-ELNs, white beach-derived ELNs and brown beach-derived ELNs had mild effects on caspase-1 autocleavage and IL-1β secretion (Figure 2A, 2B). Although king-derived ELNs mildly inhibited IL-1β secretion at high dose, it increased IL-1β at low dose and had no effects on caspase-1 autocleavage (Figure 2C). White common-derived ELNs and brown common-derived ELNs suppressed IL-1β secretion, but they slightly increased caspase-1 production (Figure 2D, 2E). These results suggested that ELNs from shiitake mushroom were the only mushroom-derived ELNs that strongly inhibited caspase-1 autocleavage and IL-1β secretion upon NLRP3 inflammasome activation. Therefore, S-ELNs were chosen for further studies.

2.4.2 Characterization of S-ELNs

The size and morphology of S-ELNs were first characterized using Nanosight NS300 instrument and scanning electron microscope (SEM). Nanosight NS300 measurement showed that the size of S-ELNs was distributed from 80 nm to 300 nm in diameter and most of them were accumulated at 115 nm in diameter (Figure 3A). Consistently, we further confirmed that S-ELNs had different sizes under SEM (Figure 3B). S-ELNs were intact and round under SEM (Figure 3B), suggesting S-ELNs had similar shape and size as those of mammalian exosomes (35).

The cargos of S-ELNs were characterized by running RNA gel, protein gel, and TLC plate. Based on RNA electrophoresis result, the size of RNA in S-ELNs is less than 50 nucleotides (nt) (Figure 3C). After adding RNase to RNA samples, the band was not detected (Figure 3C), indicating that the nucleic acids we extracted were RNAs. The proteins of S-ELNs were separated using SDS-PAGE gels. The results showed that three main bands were visualized after Coomassie Brilliant Blue staining. Their sizes were around 50 KDa, 30 KDa and 13 KDa, respectively (Figure 3D). The TLC plate results showed that lipids in S-ELNs were separated into three bands, and the majority of lipids in S-ELNs was accumulated on the top of the TLC plate (Figure 3E). These results demonstrated that the ELNs we isolated from shiitake mushroom were nanoparticles that contain cargos including RNAs, proteins, and lipids.

2.4.3 S-ELNs were taken up by macrophages

To assess whether S-ELNs were taken up by macrophages, we treated macrophages with either RNA-labeled S-ELNs or lipid-labeled S-ELNs at different doses. The results showed that the fluorescence of both RNA-labeled S-ELNs and lipid-labeled S-ELNs were detected in macrophages and fluorescence intensity was increased with dose of S-ELNs (Figure 4A, 4B), suggesting that S-ELNs, including the cargos inside of S-ELNs, were taken up by macrophages and the uptake was increased in a dose-dependent manner.

2.4.4 S-ELNs suppressed the NLRP3 inflammasome activated by a range of stimuli

Besides FFA, the NLRP3 inflammasome can be activated by other stimuli, including alum, nigericin and ATP (70,216,217). We further tested whether S-ELNs had effects on the NLRP3 inflammasome activated by alum, nigericin or ATP. Macrophages were treated with S-ELNs for 16 h. S-ELNs-primed macrophages were treated with LPS

for 3 h and then were activated with nigericin or ATP for 30 minutes, or alum for 5 h. Caspase-1 autocleavage and IL-1β secretion were measured using western blot and ELISA, respectively. Results showed that S-ELNs significantly suppressed caspase-1 autocleavage and IL-1β secretion when the NLRP3 inflammasome is activated by alum, nigericin and ATP (Figure 5A, 5B, 5C). Taken together, S-ELNs had broadly inhibitory effects on the NLRP3 inflammasome activated by different stimuli.

2.4.5 S-ELNs blocked ASC speck formation

ASC subunit is a key component involved in the assembly of NLRP3 inflammasome and ASC contains CARD domain which is responsible for recruiting caspase-1 (61). After stimulation, ASC is recruited by NLRP3 and subsequently form specks in the perinuclear area of the cell. Therefore, ASC specks could be used as an indicator of inflammasome assembly (62,63). To determine the effects of S-ELNs on inflammasome assembly, we assessed ASC speck formation with or without S-ELNs treatment in macrophages. ASC specks were formed when NLRP3 inflammasome was activated by FFA (Figure 6A, 6B). However, the number of ASC specks decreased significantly when macrophages were pre-incubated with S-ELNs (Figure 6A, 6B). This result indicated that S-ELN inhibited assembly of the NLRP3 inflammasome.

2.4.6 S-ELNs decreased both protein and mRNA levels of the *Il1b* gene and decreased transcription levels of some inflammasome subunits

Activation of the NLRP3 inflammasome requires two signals: the first signal stimulates transcription of the *Nlrp3* and *I1b* genes; the second signal activates the assembly of NLRP3 inflammasome subunits including NLRP3, ASC and caspase-1, leading to the caspase-1 autocleavage and generation of mature IL-1β (61). Here, we

assessed the effects of S-ELNs on both protein and mRNA levels of the *Il1b*, *Nlrp3*, *Pycard* (*Asc*), and *caspase-1* genes. After 16 h incubation with S-ELNs at different concentrations, macrophages were treated with LPS plus FFA to activate the NLRP3 inflammasome. We first measured the protein levels of NLRP3 inflammasome subunits using western blot. As shown in Figure 7A, S-ELN treatment significantly suppressed the protein levels of pro-IL1β, but had marginal impact on protein levels of inflammasome subunits NLRP3, caspase-1, ASC or inflammasome mediator NIMA-related kinases 7 (Nek7) (218). We further tested whether S-ELNs have any inhibitory effect on mRNA levels of the *Nlrp3*, *Pycard*, *caspase1*, *and Il1b* genes after NLRP3 inflammasome was activated using LPS plus FFA. The qPCR results showed that S-ELNs suppressed transcription of the *Il1b*, *caspase1* and *Nlrp3* genes (Figure 7B, 7D, and 7E). S-ELNs treatment slightly decreased expression of the *Pycard* gene, but the effects were not statistically significant (Figure 7C). When the NLRP3 inflammasome was activated using LPS plus ATP, S-ELNs dramatically inhibited the protein level of pro-IL1β, but had no impact on protein levels of inflammasome subunits or mediator (Figure 8A). At the transcription level, S-ELNs strongly inhibited transcription of the *Il1b*, *Pycard* and *caspase1* genes (Figure 8C, 8D, 8E), but did not affect expression of the *Nlrp3* gene (Figure 8B). Collectively, these results indicated that S-ELNs decreased both protein and mRNA levels of the *Il1b* gene and decreased transcription levels of some inflammasome subunits.

2.4.7 Lipids in S-ELNs were identified as active agents that inhibit activation of the NLRP3 inflammasome

To identify which category of biomolecules in S-ELNs has biological activity on inhibiting activation of the NLRP3 inflammasome, S-ELNs were subjected to different

treatment. S-ELNs were heated at 95 °C for 10 min to denature proteins. RNAs in S-ELNs were depleted by bath sonication plus RNase treatment. Bath sonication disrupts membrane of ELNs and subsequently RNase can reach RNAs in the vesicles to catalyze the degradation of RNAs (156,219,220). To study the functional significance of lipids in S-ELNs, the total lipids of S-ELNs were extracted, dried, and assembled into liposomes. Macrophages were treated with protein-denatured S-ELNs, RNA-depleted S-ELNs and liposomes prepared from S-ELN lipids for 16 h, respectively. Afterwards, LPS plus FFA were used to activate the NLRP3 inflammasome in macrophages. Protein-denatured S-ELNs still had inhibitory effects on caspase-1 autocleavage and IL-1β secretion (Figure 9A), indicating proteins in S-ELNs are not the active cargos that inhibit activation of the NLRP3 inflammasome. The inhibition of caspase-1 autocleavage and IL-1β secretion was not dramatically affected after removal of most RNAs in S-ELNs (Figure 9B), which suggested that RNAs in S-ELNs are not necessary for the inhibition of NLRP3 inflammasome activation. The inhibitory effect of S-ELN derived liposomes was as strong as that of intact S-ELNs on caspase-1 autocleavage and IL-1β secretion (Figure 10). These results suggested that the active biomolecules in S-ELNs with anti-inflammasome functions were lipids rather than proteins and RNAs.

2.5 Discussion

In this study, we isolated and characterized ELNs from shiitake mushroom. We showed that S-ELNs are nanoparticles that contain cargos including RNAs, proteins, and lipids for the first time. Thus, S-ELNs had similar size and content as the mammalian exosomes (35). S-ELNs were taken up by macrophages in a dose-dependent manner. Importantly, we demonstrated the inhibitory effects of S-ELNs on caspase-1 autocleavage

and IL-1β secretion when NLRP3 inflammasome was activated by FFA. S-ELNs also inhibited the NLRP3 inflammasome activated by a range of stimuli, including alum, nigericin and ATP. S-ELNs blocked assembly of the NLRP3 inflammasome. In addition, S-ELNs inhibited the protein and mRNA levels of the *Il1b* gene. Furthermore, we identified lipids in S-ELNs as the activate cargos that inhibit caspase-1 autocleavage and IL-1β secretion. Collectively, our results showed that S-ELNs strongly inhibited the NLRP3 inflammasome activation.

Mushrooms have been demonstrated to have immune-modulatory properties to go against various diseases including diabetes, cancer, chronic hepatitis and arteriosclerosis (221). The effects of mushrooms or their extracts on the NLRP3 inflammasome activation have also been studied. Some mushrooms, such as almond mushroom (*Agaricus blazei* Murill), Turkey tail (*Trametes Versicolor*) and lentinan from shiitake mushroom (*Lentinus edodes*), have been reported to activate the NLRP3 inflammasome, and therefore lead to inflammatory cell death (222-224). However, some of other mushrooms like golden oyster mushroom (*Pleurotus citrinopileatus*) inhibited NLRP3 inflammasome activation (225). Thus, we decided to investigate the role of mushroom-derived ELNs in the NLRP3 inflammasome activation. We evaluated the effects of six mushroom-derived ELNs on the NLRP3 inflammasome activation. Interestingly, different kinds of mushroom-derived ELNs have different effects on the NLRP3 inflammasome activation. Among the mushroom-derived ELNs we tested, S-ELNs were the only one that strongly inhibited caspase-1 autocleavage and IL-1β secretion (Figure 2F). Unlike S-ELNs, White beach-derived ELNs, brown beach-derived ELNs and king-derived ELNs did not show significant effects on both caspase-1 autocleavage and IL-1β secretion (Figure 2A, 2B, 2C). We

reasoned that these three mushroom-derived ELNs might lack some specific biomolecules that only present in S-ELNs. Although both white common-derived ELNs and brown common-derived ELNs significantly inhibited IL-1β secretion, they had no effects on caspase-1 autocleavage (Figure 2D, 2E).

S-ELNs have similar shape and size (Figure 3A, 3B) as those of other dietary ELNs (34). RNAs, proteins, and lipids in S-ELNs were extracted and characterized in detail (Figure 3C, 3D, 3E). Thus, we considered S-ELNs as exosome-like nanoparticles (ELNs) that contain RNAs, protein, and lipids. Furthermore, we confirmed that macrophages were able to take up RNA-labeled and lipid-labeled S-ELNs (Figure 4A, 4B). However, the mechanism of this uptake is unknown. It has been suggested that exosomes are taken up by cells through endocytosis and micropinocytosis (226). In the future study, we will explore the mechanism underlying how macrophages take up S-ELNs.

The NLRP3 inflammasome is activated by a broad range of stimuli including FFAs, alum, nigericin and ATP (70,216,217). Here, we demonstrated the inhibitory effects of S-ELNs on the NLRP3 inflammasome activated by FFA, alum, nigericin and ATP (Figure 2F, 5A, 5B, 5C), indicating S-ELNs have a broad inhibitory effect on the NLRP3 inflammasome activation. Importantly, S-ELNs seem to have multiple targets that converge on the NLRP3 inflammasome and thus synergistically or additively suppress NLRP3 inflammasome activity. First, S-ELNs inhibited assembly of the inflammasome (Figure 6A, 6B), Second, S-ELNs inhibited both protein and mRNA levels of the *Il1b* gene when macrophages were activated by LPS plus FFA or LPS plus ATP (Figure 7A, 7E, 8A, 8E), suggesting the potential role of S-ELNs in attenuating NF-κB pathway. Finally, S-ELNs inhibited expression of some inflammasome subunits (Figure 7B, 7D, 8C, 8D).

Lentinan extracted from shiitake mushroom has been shown to inhibit AIM2 inflammasome activation, but not NLRP3 inflammasome (227). In the future study, we will evaluate the effects of S-ELNs on activation of other inflammasomes. Together, these results provide a basis to further study the mechanisms underlying how S-ELNs inhibit NLRP3 inflammasome activation.

Exosomes and dietary ELNs participate in the intercellular communication or even interspecies communication by delivering their cargoes to target cells (34). Evidence has demonstrated that the RNA cargos in exosomes or ELNs have biological activity (154). We found that the inhibitory effect of lipids from S-ELNs was as strong as that of intact S-ELNs (Figure 10). Further identification of specific lipid components in S-ELNs that inhibit NLRP3 inflammasome activation is needed. In addition, further study is warranted to demonstrate the inhibitory effects of lipids in S-ELNs on NLRP3 inflammasome activation *in vivo*.

CHAPTER 3. DISCUSSION, LIMITATION, AND FUTURE STUDIES

3.1 Discussion

The NLRP3 inflammasome is a multiprotein complex containing NLRP3, ASC and capase-1 (65). It is activated by a variety of exogenous PAMPs and endogenous DAMPs and therefore plays an important role in immune system (65). However, dysregulated or excessive activation of the NLRP3 inflammasome contributes to the pathogenesis of many diseases including Alzheimer's disease, type 2 diabetes, and gout (213-215). Accumulating evidence has demonstrated that inhibition of the NLRP3 inflammasome has high therapeutic potential for treatment of NLRP3 inflammasome-related diseases (213,214). Exosomes are bilayer membrane-enclosed nanoparticles containing RNAs, proteins and lipids (23). They play an important role in intercellular communication (34,106). Besides endogenous exosomes, exosomes or exosome-like nanoparticles (ELNs) obtained from dietary sources have been identified and characterized (34,228), and their therapeutic potential for treatment of diseases has also been studied (30). A few studies have suggested that RNAs in bovine milk exosomes affect the biological activities of target cells (220,229), but the functional significance of cargos from edible plant-derived ELNs in consumers are still controversial. In addition, only a few edible plant-derived ELNs have been studied and many more dietary ELNs await further investigation.

3.1.1 Inhibition of the NLRP3 inflammasome

Inflammation protects body against exogenous pathogens and this function is beneficial to human health (230). However, aberrant inflammation contributes to the pathogenesis of diseases (230). Dysregulated or excessive activation of the NLRP3 inflammasome leads to continuous release of IL-1β, which is associated with a variety of

chronic inflammatory diseases, including diabetes and atherosclerosis (11,231). IL-1β inhibitors have been utilized in treating these diseases in clinical trials (232), but these inhibitors showed modest effects possibly because they only inhibit one downstream event of the NLRP3 inflammasome (61). Some molecules have been discovered to inhibit NLRP3 inflammasome activation *in vivo* or *in vitro*. For example, the neurotransmitter dopamine inhibited NLRP3 inflammasome activation via dopamine D1 receptor in macrophages. This study provides a promising approach for preventing neuroinflammation (233). Similarly, nitric oxide has been demonstrated to inhibit NLRP3 inflammasome activation through stabilizing mitochondria (234,235). A small-molecule chemical called MCC950 inhibited NLRP3 inflammation activation and protected mice from autoimmune diseases and cognitive decline (18,236). BAY11-7082 protected mice from spinal cord injury-induced lung injury by inhibiting NLRP3 inflammasome activation (237). Other pharmaceutical NLRP3 inhibitors including IFM-514, IFM-632 and CRID3 have also been investigated for their efficacy in the retinal pigment epithelium cells, thereby providing promising agents for treatment of atrophic age-related macular degeneration (238). Aspirin has been widely used in clinical treatment due to its anti-inflammatory effect. It has been demonstrated to have inhibitory effects on activation of the NLRP3 inflammasome via suppression of ROS (239).

Besides endogenous molecules and pharmacological molecules, some natural molecules from herbs or foods have also been reported to have inhibitory effects on NLRP3 inflammasome activation. Kaempferol found in herbs has been identified as a potential inhibitor that suppresses NLRP3 inflammasome activation via ubiquitin-autolysosome pathway (240). As a result, kaempferol treatment reduced neuroinflammation and

protected mice against neurodegeneration in Parkinson's disease (240). Another food-borne molecule naringenin also inhibited NLRP3 inflammasome activation and therefore protected against LPS-induced dopamine neurotoxicity in Parkinson's disease (241). *Laurus nobilis* leaf extract suppressed NLRP3 inflammasome activation in macrophages possibly through 1,8-cineole, a major component of this leaf extract (242). When L. nobilis leaf extract were administered to mice, they reduced expression of proinflammatory cytokine genes in an acute lung injury murine model (242). Despite these interesting findings, the efficacy and safety of these molecules for long-term usage in chronic diseases such as diabetes have not been evaluated. Therefore, there is a need to expand the pool of inflammasome inhibitors. Mushrooms have potential in inhibition of NLRP3 inflammasome activation (225), but more direct evidence is needed to support such functions. Our results demonstrate for the first time that S-ELNs strongly inhibit NLRP3 inflammasome activation in macrophages (Figure 2F). Thus, S-ELNs represent a new promising agent that blocks NLRP3 inflammasome activation.

3.1.2 Therapeutic potential of dietary exosome-like nanoparticles (ELNs)

Dietary ELNs have been reported as promising therapeutic approaches for treatment of diseases. Grape-derived ELNs were taken up by intestinal macrophages and intestinal stem cells, indicating a possible role of dietary ELNs in mediating interspecies communication (34). Grape-derived ELNs promoted the growth of Lgr5+ intestinal stem cells, therefore protecting mice against DSS-induced colitis (30). Ginger-derived ELNs also show anti-inflammatory property, because ginger-derived ELNs ameliorated disease symptoms in DDS-induced colitis mouse models (243). In addition, ginger-derived ELNs mediated nuclear factor erythroid 2-related factor (Nrf2) activation in liver cells, and

therefore protected mice against alcohol-induced liver injury (244). Unlike grape-derived ELNs, ginger-derived ELNs were taken up by intestinal epithelial cells (243). Bovine milk exosomes (BMEs) regulated gene expression and the levels of amino acids in skeletal muscles in mice (227). In addition, BMEs affected the cognitive performance and gut microbiome in mice (38,245). BMEs enhanced mucin production by increasing goblet cell activity (246). In addition, BMEs promoted protein synthesis by inducing expression of GRP94, a intraluminal endoplasmic reticulum chaperones involved in protein folding (246). As a result, BME treatment protected mice against necrotizing enterocolitis (NEC)-induced intestinal injury (246). Recently, a study from our laboratory suggested that ginger-derived ELNs had strong inhibitory effect on NLRP3 inflammasome activation (247). Together, dietary ELNs could regulate gene expression and activate functions of certain molecules at the molecular level (219,246) and promote cell proliferation at the cellular level (30,246). More importantly, dietary ELNs could protect mice against DSS-induced colitis, alcohol-induced liver injury and NEC-induced intestinal injury at the pathophysiological level (30,244,246).

3.1.3 Biological activities of cargos in dietary ELNs

ELNs participate in the intercellular or interspecies communications by carrying RNAs, proteins and lipids (34,106). RNA cargos in BMEs regulated gene expression in target cells (220,229). The glycoproteins on the surface of BMEs play an important role in the uptake process in human and rat intestinal cells (151). MicroRNAs in BMEs are mainly distributed in intestinal mucosa, spleen, liver, heart and brain (155). This distribution pattern is distinctive from intact BMEs (155). In our study, we confirmed S-ELNs as exosome-like nanoparticles (ELNs) containing RNAs, proteins and lipids (Figure 3A-E).

we also discovered that S-ELNs, including the cargos inside of S-ELNs, were taken up by macrophages in a dose-dependent manner (Figure 4A, 4B). However, whether glycoproteins of S-ELNs play a critical role in the uptake process has not been studied. MicroRNAs in ginger-derived ELNs regulated the functions and motility of gut microbiota in the mice (248), while lipids in grape-derived ELNs promoted proliferation of intestinal stem cells and liposome-like nanoparticles assembled from lipids of grape-derived ELNs play a crucial role in targeting intestinal stem cells (30). In addition, lipids from grapefruit-derived ELNs have been utilized as a vector to deliver therapeutic agents for cancer treatment, because these lipids do not cause cytotoxicity and inflammatory response (149). Recently, lipids in ginger-derived ELNs have been demonstrated as active biomolecules that inhibit NLRP3 inflammasome activation (247). Similarly, lipids in S-ELNs have also been identified as bioactive agents that inhibit NLRP3 inflammasome activation in macrophages (Figure 10A).

3.2 Limitation and future studies

Here, we list four limitations in the current study and how we plan to address them in the future studies. Firstly, although we demonstrated the inhibitory effects of S-ELNs on NLRP3 inflammasome activated by a range of stimuli in macrophages, the mechanism underlying how S-ELNs inhibit NLRP3 inflammasome activation has not been studied. We mentioned three upstream events involved in NLRP3 inflammasome activation in the chapter 1: intracellular K+ efflux, reactive oxygen species (ROS) production, and lysosomal destabilization and rupture. We will assess intracellular KCl, the production of ROS and lysosomal dysfunction to demonstrate how S-ELNs inhibited NLRP3 inflammasome activation at the cellular level. Secondly, we identified lipids as the active

agents that inhibit NLRP3 inflammasome activation. S-ELNs contain many different kinds of lipids (Figure 3E). The specific lipid that is responsible for anti-inflammasome functions has not been identified. To address this question, we plan to perform lipidomics analysis of lipids in S-ELNs. Among six mushroom-derived ELNs we tested, five mushroom-derived ELNs have no effects on caspase-1 autocleavage or IL-1β secretion (Figure 2A, 2B, 2C, 2D, 2E). We plant to choose one of these five inactive mushroom-derived ELNs as a negative control. The lipidomes of active S-ELNs and inactive mushroom-derived ELNs will be compared. The lipids specifically enriched in S-ELNs could be the potential candidates, which will be tested individually for their effects on NLRP3 inflammasome activation. Thirdly, we found that macrophages take up S-ELNs. Whether S-ELNs can be taken up by intestinal cells and how S-ELNs are distributed in mice have not been determined. Uptake, delivery and distribution of S-ELNs will be investigated in the future. Finally, we confirmed the inhibitory effects of S-ELNs on NLRP3 inflammasome activation *in vitro*, but the effects of S-ELNs on NLRP3 inflammasome activation *in vivo* have not been evaluated. We will determine whether oral administration or intravenous injection of S-ELNs could protect mice from NLRP3 inflammasome-related diseases. If S-ELNs reduce inflammation and NLRP3 inflammasome activity in mice, we will further use *Nlrp3*$^{-/-}$ mice to confirm that anti-inflammatory functions of S-ELNs are mediated through the NLRP3 inflammasome.

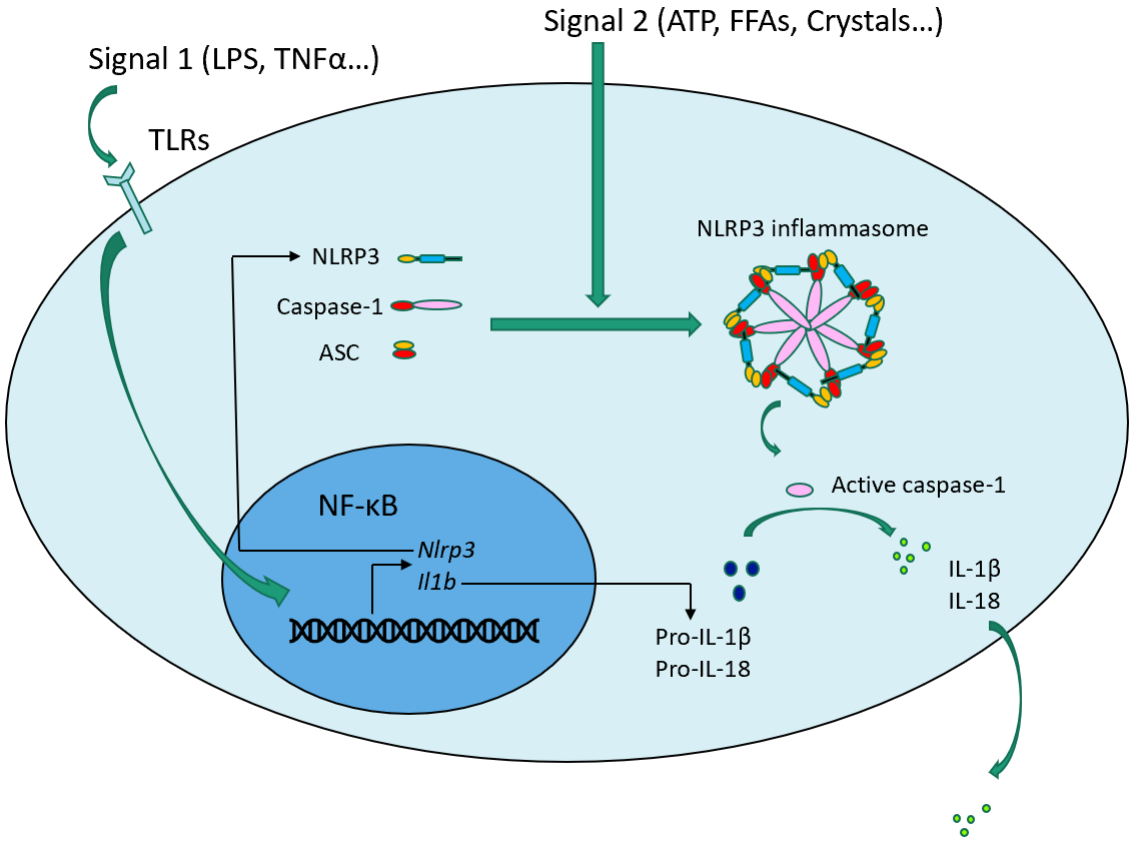

Figure 1. Activation of the NLRP3 inflammasome. Activation of the NLRP3 inflammasome requires two signals. Signal 1, such as LPS and TNFα, induces expression of the *Nlrp3* and *Il1b* genes through toll-like receptor (TLR)/nuclear factor (NF)-κB pathway; Signal 2, such as ATP, FFAs and crystals, triggers assembly of the NLRP3 inflammasome. Activation of the NLRP3 inflammasome causes autocleavage of caspase-1 to produce the active caspase-1, which cleaves pro-IL-1β and pro-IL18 into active IL-1β and IL-18. The mature cytokines IL-1β and IL-18 are released outside of cells.

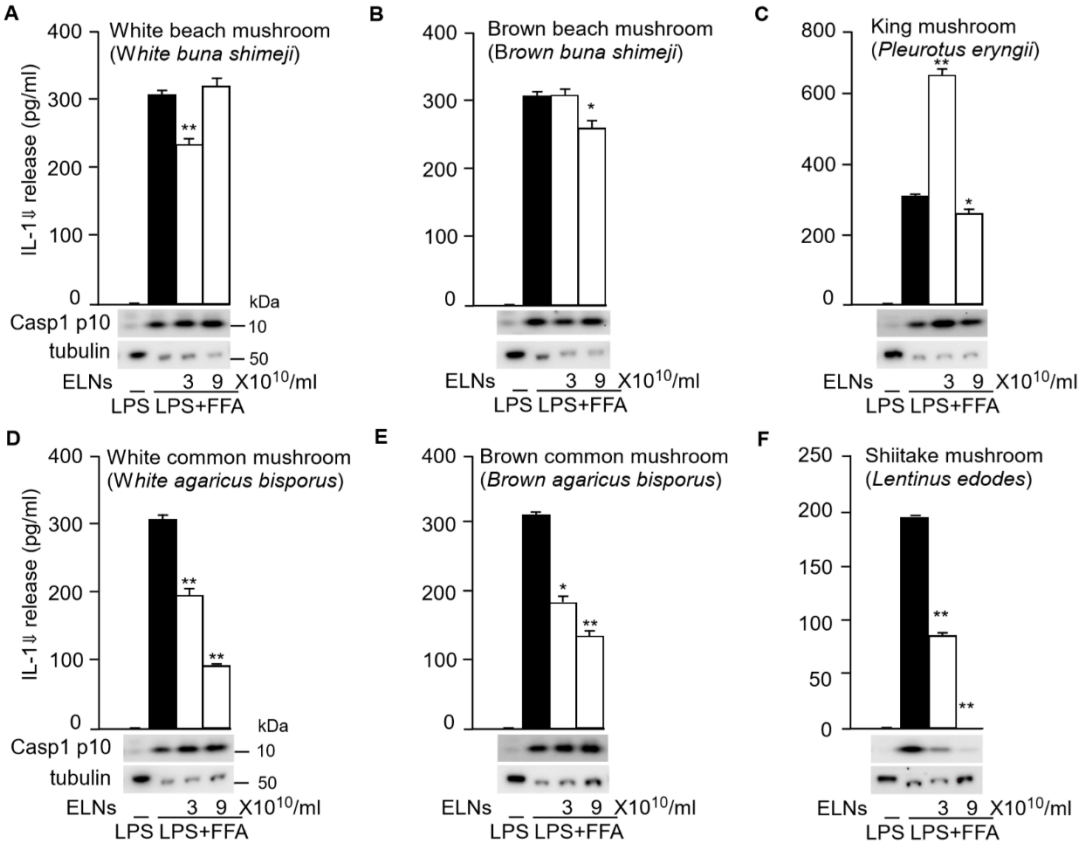

Figure 2. S-ELNs strongly inhibited caspase-1 autocleavage and IL-1β secretion upon NLRP3 inflammasome activation. A-F. Macrophages were treated with mushroom-derived ELNs for 16 h and then treated with LPS for 3 h, and finally activated with FFA for 12 h. Caspase-1 autocleavage and IL-1β were measured using western blot and ELISA, respectively. Tubulin is used as a control to show equivalent loading. P values were calculated: * $P < 0.05$; ** $P < 0.01$. Treatment groups (mushroom-derived ELNs+LPS+FFA, white bars) were compared with the control group (LPS+FFA, black bar). A. White beach mushroom (*White buna shimeji*). B. Brown beach mushroom (*Brown buna shimeji*). C. King mushroom (*Pleurotus* eryngii). D. White common mushroom (*White agaricus bisporus*). E. Brown common mushroom (*Brown agaricus bisporus*). F. Shiitake mushroom (*Lentinus edodes*).

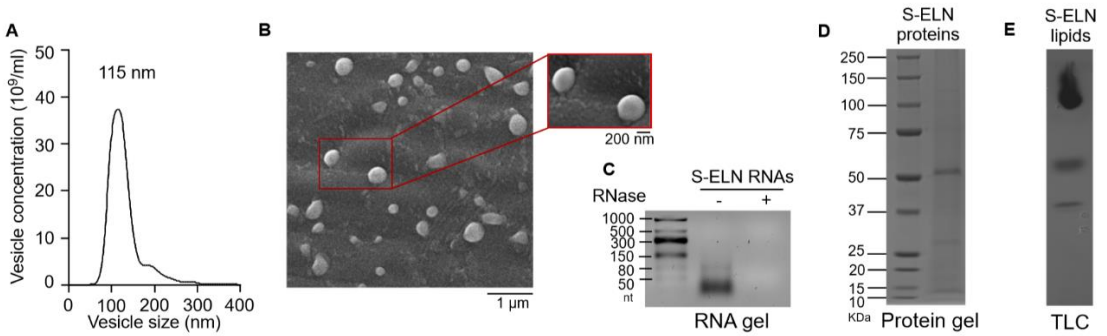

Figure 3. Characterization of S-ELNs

A. Size distribution and vesicle concentration of S-ELNs. B. scanning electron microscope images of S-ELNs. C. RNAs of S-ELNs with or without RNase treatment were separated in the 2% agarose gel. D. Proteins extracted from S-ELNs were separated on the SDS-PAGE gel and stained using Coomassie Brilliant Blue. E. lipids extracted from S-ELNs were separated on the TLC plate.

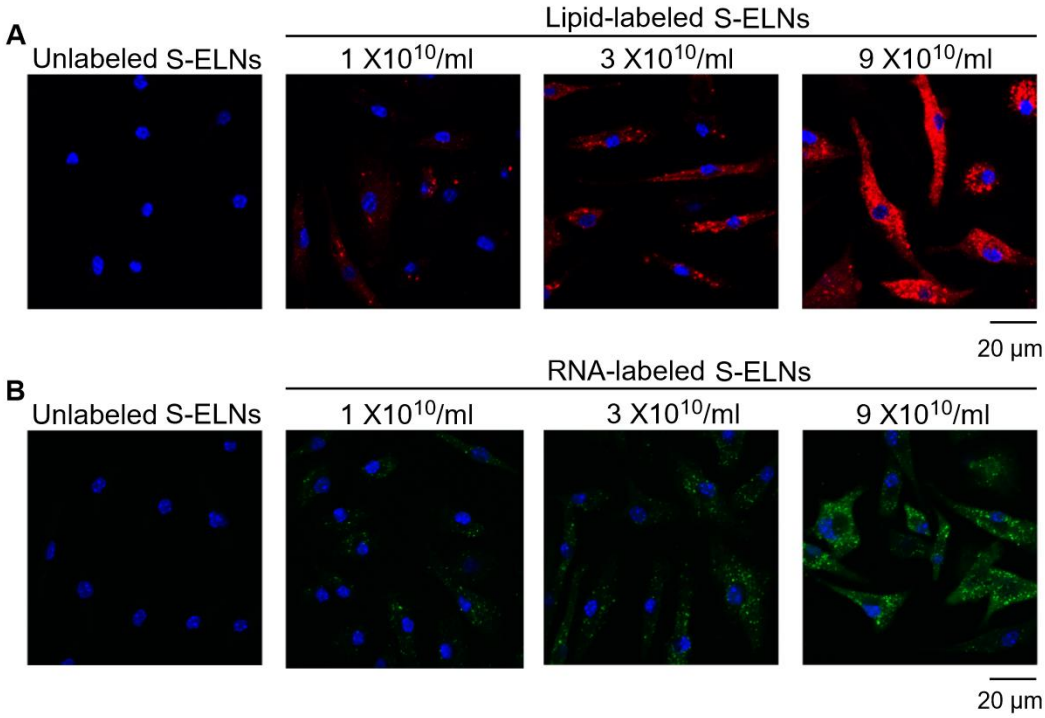

Figure 4. S-ELNs were taken up by macrophages

A.B. Macrophages were incubated with RNA-labeled or lipid-labeled S-ELNs at different doses for 16 h. The fluorescence was detected by confocal microscopy. A. Lipid-labeled S-ELNs were taken up by macrophages. B. RNA-labeled S-ELNs were taken up by macrophages.

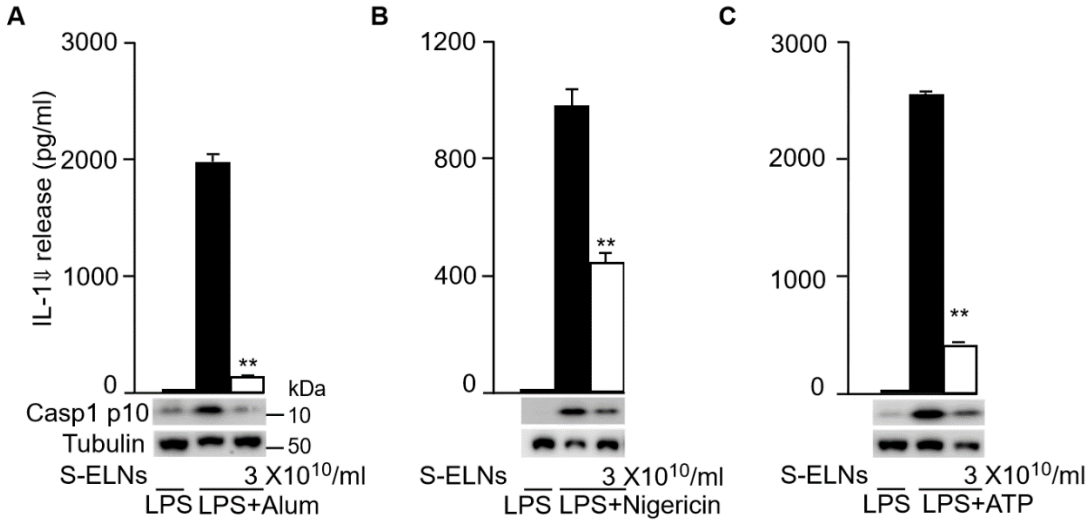

Figure 5. S-ELNs suppressed NLRP3 inflammasome activated by a range of stimuli

A. S-ELNs suppressed alum-induced NLRP3 inflammasome activation. S-ELNs-primed BMDMs were treated with LPS for 3 h, and then activated with Alum for 5h. B. S-ELNs suppressed nigericin-induced NLRP3 inflammasome activation. S-ELNs-primed BMDMs were treated with LPS for 3 h, and then activated with nigericin for 30 minutes. C. S-ELNs suppressed ATP-induced NLRP3 inflammasome activation. S-ELNs-primed BMDMs were treated with LPS for 3 h, and then activated with ATP for 30 minutes. Tubulin is used as a control to show equivalent loading. * $P < 0.05$; ** $P < 0.01$. Treatment group (S-ELNs+LPS+stimuli, white bar) was compared with the control group (LPS+stimuli, black bar).

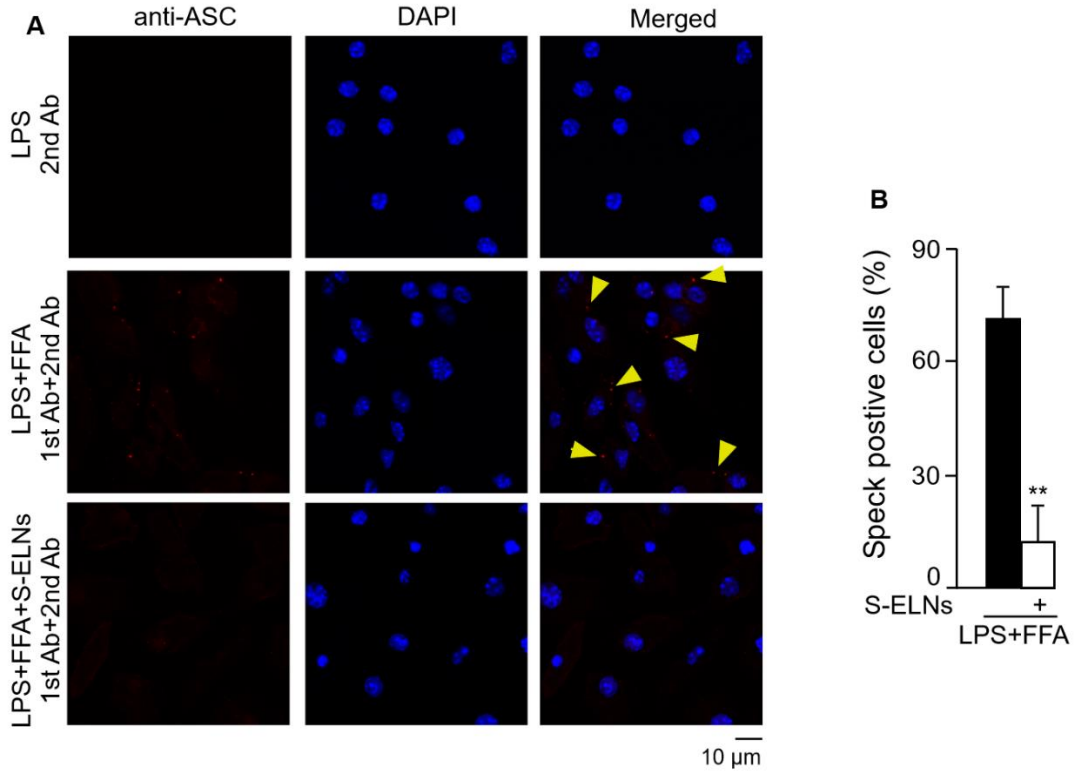

Figure 6. S-ELNs blocked ASC speck formation

A. Immunofluorescence of ASC speck formation in macrophages stimulated with LPS+FFAs with or without S-ELNs incubation. Caspase-1 inhibitor VX765 (10 μM) were added 30 min before FFAs was incubated with cells. Yellow arrows indicate ASC specks. Anti-ASC rabbit antibody was used as primary antibody (1st Ab) and Alexa Fluor 594 anti-rabbit antibody was used as secondary antibody (2nd Ab). B. Percentage of ASC speck positive cells. * $P < 0.05$; ** $P < 0.01$. Treatment group (LPS+FFA+S-ELNs, white bar) was compared with control group (LPS+FFA, black bar).

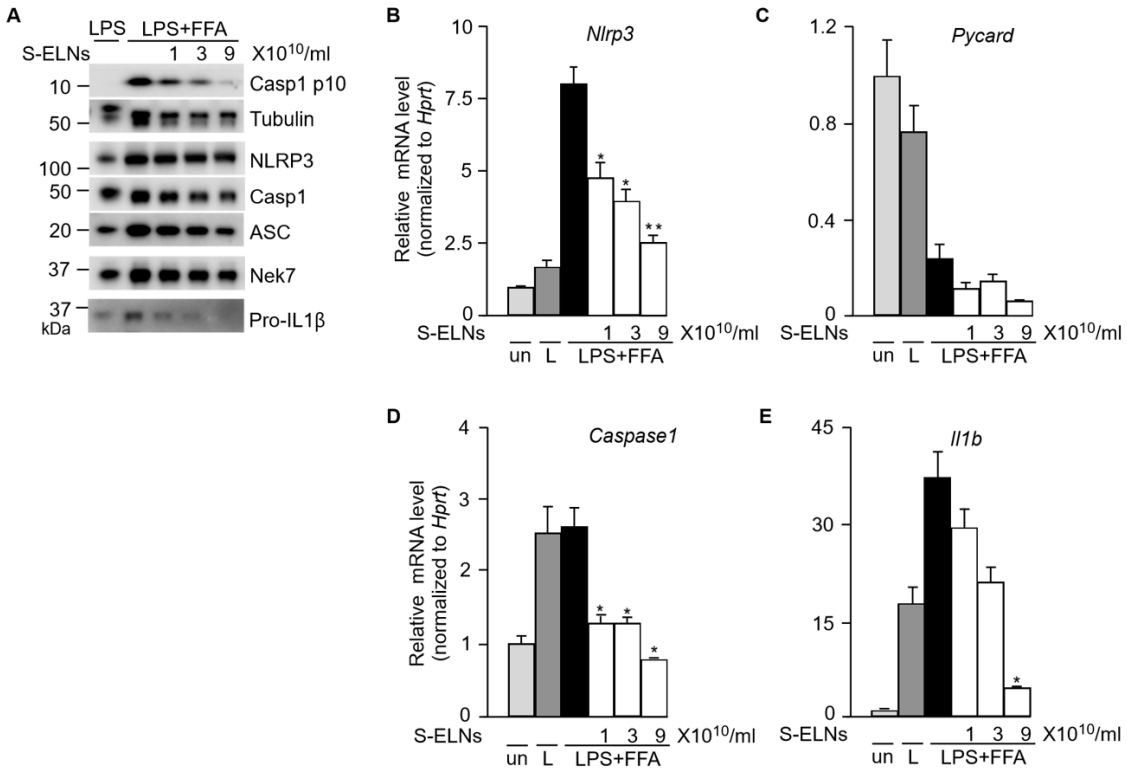

Figure 7. S-ELNs suppressed pro-IL-1β protein level and expression of the *Il1b, Nlrp3, and caspase1* genes when inflammasome is activated using LPS+FFA.

A-E. Macrophages were treated with S-ELNs for 16 h, and then treated with LPS for 3 h, and finally activated with FFAs for 12h. Cells were collected for analysis of protein and mRNA levels, respectively. A. S-ELN treatment significantly suppressed the protein level of pro-IL-1β. B. S-ELNs significantly suppressed transcription of the *Nlrp3* gene. C. S-ELNs slightly decreased expression of the *Pycard* gene. D. S-ELNs significantly suppressed transcription of the *caspase1* gene. E. S-ELNs significantly suppressed transcription of the *Il1b* gene. * $P < 0.05$; ** $P < 0.01$. Treatment groups (S-ELNs+LPS+FFA, white bars) were compared with the control group (LPS+FFA, black bar).

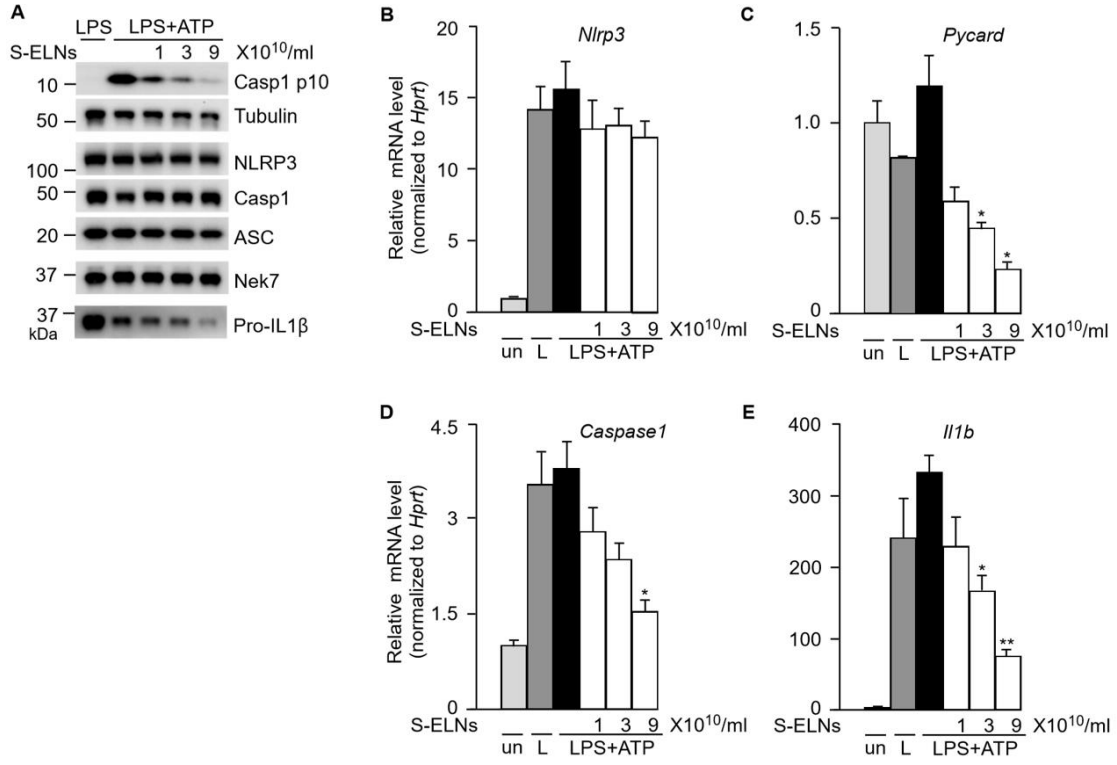

Figure 8. S-ELNs suppressed pro-IL-1β protein level and expression of the *Il1b, Pycard, and caspase1* genes when inflammasome is activated using LPS+ATP.

A-E. Macrophages were treated with S-ELNs for 16 h, and then treated with LPS for 3 h, and finally activated with ATP for 0.5 h. Cells were collected for analysis of protein and mRNA levels, respectively. A. S-ELNs dramatically inhibited the protein level of pro-IL-1 β. B. S-ELNs had no impact on expression of the *Nlrp3* gene. C. S-ELNs significantly inhibited transcription of the *Pycard* gene. D. S-ELNs significantly inhibited transcription of the *caspase1* gene. E. S-ELNs significantly inhibited transcription of the *Il1b* gene. * $P < 0.05$; ** $P < 0.01$. Treatment groups (S-ELNs+LPS+ATP, white bars) were compared with the control group (LPS+ATP, black bar).

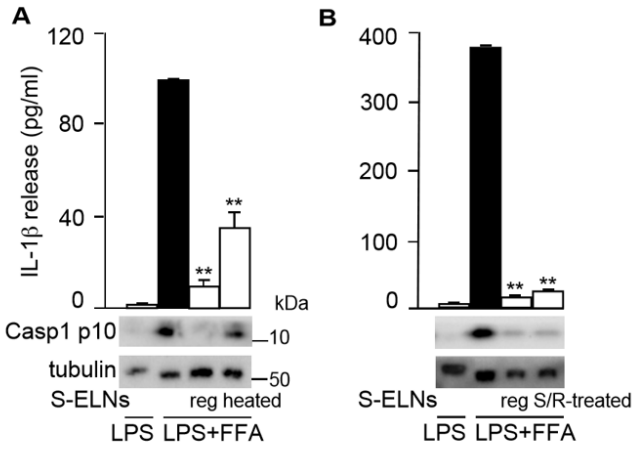

Figure 9. Protein and RNAs in S-ELNs were not the major bioactive agents that inhibit NLRP3 inflammasome activation. A.B. Macrophages were incubated with S-ELNs that were subjected to different treatments for 16 h, and then stimulated with LPS+FFA. A. Protein-denatured S-ELNs still had inhibitory effects on caspase-1 autocleavage and IL-1β secretion. Proteins in S-ELNs were denatured by heating at 95 °C for 10 min. Macrophages were treated with 6×10^{10}/ml of regular S-ELNs (reg) or protein-denatured S-ELNs (heated). B. Removal of the majority of RNAs did not affect the inhibitory effects of S-ELNs on caspase-1 autocleavage and IL-1β secretion. RNAs in S-ELNs were removed through bath sonication plus RNase (S/R) treatment. Macrophages were treated with 6×10^{10}/ml of regular S-ELNs (reg) or RNA-depleted S-ELNs (S/R-treated). Tubulin is used as a control to show equivalent loading. * $P < 0.05$; ** $P < 0.01$. Treatment groups (S-ELNs or protein-denatured S-ELNs or RNA-depleted S-ELNs +LPS+FFA, white bars) were compared with the control group (LPS+FFA, black bar).

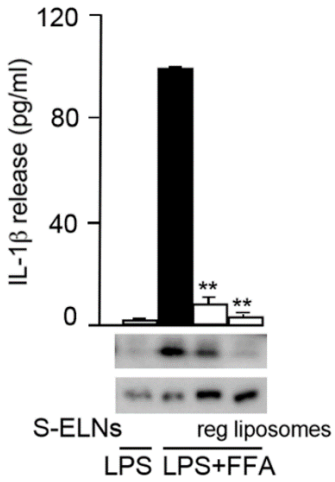

Figure 10. Lipids in S-ELNs were identified as bioactive agents that inhibit NLRP3 inflammasome activation. Lipids from S-ELNs strongly inhibited caspase-1 autocleavage and IL-1β secretion. Lipids were extracted from S-ELNs and then re-assembled into liposomes. Macrophages were treated with regular S-ELNs (reg) or liposomes. Tubulin is used as a control to show equivalent loading. * $P < 0.05$; ** $P < 0.01$. Treatment groups (S-ELNs or liposomes+LPS+FFA, white bars) were compared with the control group (LPS+FFA, black bar).

APPENDIX:

Abbreviations

ApoE—apolipoprotein E
ASC— Apoptotic speck protein containing a caspase recruitment domain
ATP—Adenosine triphosphate
BMDM-Bone marrow-derived macrophages
BMEs—Bovine milk exosomes
CARD—Caspase recruitment domain
DAMPs—Damage-associated molecular patterns
DAPI—4′,6-diamidino-2-phenylindole
DHA—Docosahexaenoic acid
DSS—Dextran sulfate sodium
EGCG—Epigallocatechin-3-gallate
ELNs—exosome-like nanoparticles
EVs—Extracellular vesicles
FBS—Fetal bovine serum
FFA—Free fatty acid sodium palmitate
FFAs—Free fatty acids
HEPES—4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD—High fat diet
Hprt—hypoxanthine guanine phosphoribosyl transferase
IFN γ— Interferon γ
IL-1β—Interleukin-1β
IL-2—Interleukin-2
IL-6—Interleukin-6
LPS—Lipopolysaccarides
LRRs—Leucine-rich repeats
MAPKs—mitogen-activated protein kinases
MHC—Major histocompatibility complex
NaCl—Sodium chloride
NEC—Necrotizing enterocolitis
NEK—NIMA-related kinases 7
NF-κB—Nuclear factor kappa B
NK-T cells—Natural killer T cells
NLRP3—NOD-like receptor family, pyrin domain containing 3
NLRs—NOD-like receptors
NOD/NACHT—Nucleotide-binding-and-oligomerization
Nrf2—nuclear factor erythroid 2-related factor
nt—Nucleotide
PAMPs—Pathogen-associated molecular patterns
PA—Palmitate
PBS—phosphate buffered saline
PI3K—phosphatidylinositol 3-kinase
Pro-IL-1β—Pro-interleukin-1β
PRRs— Pattern recognition receptors

PSPC—Purple sweet potato color
PUFAs—polyunsaturated fatty acids
PVDF—Polyvinylidene difluoride
PYD—pyrin domain
qPCR—Quantitative polymerase chain reaction
ROS—Reactive oxygen species
SDS-PAGE—sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS—Sodium dodecyl sulfate
S-ELNs—shiitake-derived exosome-like nanoparticles
SFAs—Saturated fatty acids
STAT3—Signal transducer and activator of transcription 3
TLC—thin-layer chromatography
TLR 2/4—toll-like receptor 2/4
TLRs—Toll-like receptors
TNFα—Tumor necrosis factor-α
UFAs—unsaturated fatty acids
γδ-T cells—gamma delta T cells

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