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Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders

Tao Gong,* Xiaqiong Wang,* Yanqing Yang,* Yiqing Yan,* Chenggong Yu,† Rongbin Zhou,*,‡,§,¶ and Wei Jiang*,§

Plant-derived dietary lectins have been reported to be involved in the pathogenesis of several inflammatory diseases, including inflammatory bowel disease, diabetes, rheumatoid arthritis, and celiac disease, but little is known about the molecular mechanisms underlying lectin-induced inflammation. In this study, we showed that plant lectins can induce caspase-1 activation and IL-1β secretion via the NLRP3 inflammasome. Lectins were internalized and subsequently escaped from the lysosome and then translocated to the endoplasmic reticulum. Endoplasmic reticulum–loaded plant lectins then triggered Ca^{2+} release and mitochondrial damage, and inhibition of Ca^{2+} release and mitochondrial reactive oxygen species by chemical inhibitors significantly suppressed NLRP3 inflammasome activation. In vivo, plant lectin–induced inflammation and tissue damage also depended on the NLRP3 inflammasome. Our findings indicate that plant lectins can act as an exogenous "danger signal" that can activate the NLRP3 inflammasome and suggest that dietary lectins might promote inflammatory diseases via the NLRP3 inflammasome. ***The Journal of Immunology*****, 2017, 198: 000–000.**

Lectins are a heterogeneous group of proteins that were first discovered in plants and subsequently in other species, from microorganisms to humans. They share an important biological propriety and specifically bind to carbohydrates in a reversible way. This ability makes them act as a participator of a wide range of biological events that are involved in protein–carbohydrate recognition, such as cell development, cell recognition, tumor metastasis, host defense, and inflammation (1). Most research efforts on lectins have been focused on plant lectins. Plant lectins are distributed in various kinds of foods, such as grains, legumes, fruits, vegetables, and nuts (2). For some plants, they make up as much as 10% of the total nitrogen in mature seed extracts. Most importantly, plant lectins are more resistant to heat denaturation and digestion than the animal proteins (3). Dietary lectins can increase intestinal permeability and allow for the increased translocation of both dietary and microbial Ags to the periphery. Peripheral circulatory lectins not only provoke IgG and IgM Ab production, but they also bind to cell surface glycoproteins, such as epidermal growth factor receptor and insulin receptor, and disrupt their functions (4–6). Therefore, lectins may contribute to the pathogenesis of food intolerance, food allergy, and other inflammatory diseases, such as inflammatory bowel disease, insulin-dependent diabetes, rheumatoid arthritis, and IgA nephropathy (6–9). Specifically, lectins from plants and microorganisms have been considered as modulators of leukocyte function (10–13). Although the inflammatory effects of lectins are emerging, the mechanisms are still poorly understood.

The innate immune system initiates inflammation and protects us through pattern recognition receptors. Several members of pattern recognition receptors act as sensor molecules, together with ASC and procaspase-1, to form cytosolic protein complexes named the "inflammasomes." The inflammasomes are assembled in response to microbial infection or "danger signals." In contrast to NLRC4, AIM2, NLRP1, and Pyrin inflammasomes, which are triggered by a limited number of pathogen-associated molecular patterns (PAMPs), the NLRP3 inflammasome not only can be activated by several PAMPs, including viral RNA, bacterial surface protein, and microbial toxins, but also by the widest array of danger-associated molecular patterns (DAMPs), such as extracellular ATP, monosodium urate (MSU), silica, aluminum adjuvant, hyaluronan, and amyloid-β (14, 15). Owing to the diverse nature of PAMPs and DAMPs that activate the NLRP3 inflammasome, how the NLRP3 inflammasome is activated is not clearly understood. The NLRP3 inflammasome is responsible for the maturation and release of proinflammatory cytokines, such as IL-1β and IL-18, and has been proposed to be involved in the pathogenesis of various inflammatory diseases, including gout, diabetes, Alzheimer's disease, atherosclerosis, and arthritis (14, 16). However,

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Abbreviations used in this article: ALT, alanine aminotransferase; Alum, aluminum hydroxide; 2-APB, 2-aminoethoxydiphenyl borate; BiP, IgH-binding protein; BMDM, bone marrow–derived macrophage; DAMP, danger-associated molecular pattern; ER, endoplasmic reticulum; FAK, focal adhesion kinase; LCA, *Lens culinaris* agglutinin; MBL, mannose-binding lectin; MnTBAP, manganese (III) tetrakis (4-benzoic acid) porphyrin chloride; MSU, monosodium urate; PAMP, pathogen-associated molecular pattern; poly(dA:dT), poly(deoxyadenylic-thymidylic) acid; ROS, reactive oxygen species; SBA, soybean agglutinin; siRNA, small interfering RNA; WGA, wheat germ agglutinin.

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whether the NLRP3 inflammasome contributes to diet-induced inflammatory diseases is still unclear.

In this study we found that plant lectins activated the NLRP3 inflammasome through the endoplasmic reticulum (ER) stress–mitochondira axis. Furthermore, plant lectins induced NLRP3 inflammasome–dependent proinflammatory cytokine release and tissue damage in vivo, suggesting that the NLRP3 inflammasome may contribute to diet-induced inflammatory diseases.

Materials and Methods

Mice

Nlrp3$^{-/-}$, *Asc*$^{-/-}$, *Caspase-1*$^{-/-}$, *Ipaf*$^{-/-}$, *Nod2*$^{-/-}$, and *Caspase-2*$^{-/-}$ mice have been described (17–20). *Nlrp6*$^{-/-}$ and *Nod1*$^{-/-}$ mice were provided by Millennium Pharmaceuticals. *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice were crossed to generate *Nod1/2*$^{-/-}$ double-knockout mice. *Itgal*$^{-/-}$ mice were from The Jackson Laboratory. All mice were in a C57BL/6 background. The age- and sex-matched wild-type mice were used as controls. All animal procedures were approved by the Ethics Committee of the University of Science and Technology of China.

Reagents

Lectin from *Canavalia ensiformis* (Con A), *Lens culinaris* (*L. culinaris* agglutinin [LCA]), *Triticum vulgaris* (wheat germ agglutinin [WGA]), *Glycine max* (soybean agglutinin [SBA]), *Arachis hypogaea* (peanut agglutinin), *Ulex europaeus* (*U. europaeus* agglutinin), *Artocarpus integrifolia* (jacalin), nigericin, MSU, aluminum hydroxide (Alum), ATP, FITC-labeled Con A, FITC-labeled WGA, bafilomycin A1, brefeldin A, 2-aminoethoxydiphenyl borate (2-APB), manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), and PMA were from Sigma-Aldrich. PF-431396 and CA-074-Me were from Selleck Chemicals. Ultrapure LPS, poly(deoxyadenylic-thymidylic) acid [poly(dA:dT)], MitoTracker Red, LysoTracker Red, and MitoSOX Red were from InvivoGen. Ab to IgH-binding protein (BiP) were from Proteintech. Ab to mouse IL-1β was from R&D Systems. Abs to mouse caspase-1 (p20) was from Adipogen International. Anti-calreticulin was from Abcam. Ab to human IL-1β was from Sangon Biotech. Anti-human caspase-1 was from Cell Signaling Technology.

Cell preparation and stimulation

Human THP-1 cells were obtained from American Type Culture Collection. THP-1 cells were grown in RPMI 1640 medium plus 10% (v/v) FBS and 50 μM 2-ME. THP-1 cells were treated for 3 h with 100 nM PMA and then incubated overnight to differentiate into macrophages. Bone marrow–derived macrophages (BMDMs) were derived from tibia and femoral bone marrow cells as described and were cultured in DMEM supplemented with 10% FBS, in the presence of L929 cell culture supernatants (21).

For induction of inflammasome activation, 6×10^5 macrophages were plated overnight in 12-well plates and then the cells were primed for 3 h (using Opti-MEM supplemented with 1% FBS and 100 ng/ml ultrapure LPS). After that, the cells were stimulated with various lectins, including MSU (300 mg/ml) and Alum (300 mg/ml) for 6 h, or stimulated with ATP (1 mM) and nigericin (5 μM) for 45 min. For poly(dA:dT) transfection, poly (dA:dT) (1 μg/ml) was transfected by using Lipofectamine 2000 per the manufacturer's protocol (Invitrogen). Precipitated culture supernatants and cell extracts were analyzed by Western blotting.

Small interfering RNA–mediated gene silencing in THP-1 cells

PMA-differentiated THP-1 cells were plated in 12-well plates (at a density of 6×10^5 cells per well) and then were transfected with 50 nM negative control small interfering RNA (siRNA) or 25 nM of two different ITGAL (encodes LFA-1α) siRNAs by using Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions (Invitrogen). The siRNA sequence are as follows: 5′-CGCCAATGTGACCTGTAACAA-3′ and 5′-CCCATCAATGTTTCCCTGAAT-3′. All siRNA was chemically synthesized by GenePharma.

ELISA

Mouse IL-1β, IL-6 (R&D Systems), and IL-18 (eBioscience) in supernatants of cell culture or peritoneal lavage fluid were quantified by ELISA according to the manufacturers' guidelines.

Confocal microscopy

THP-1 cells or BMDMs plated on coverslips (12-well plates, 2×10^5) overnight were used for stimulation or staining with MitoTracker Red

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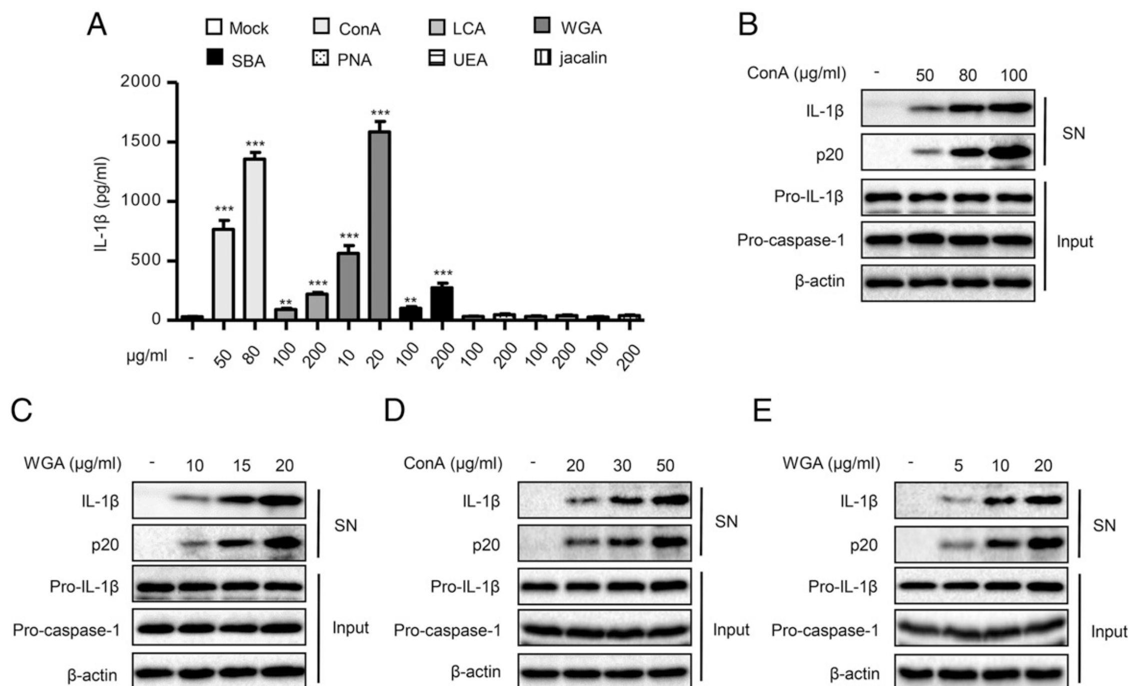

FIGURE 1. Plant lectins induce caspase-1 activation and IL-1β secretion. (**A**) ELISA of IL-1β in culture supernatants from LPS-primed BMDMs stimulated with different kinds of plant lectins for 6 h. (**B** and **C**) Immunoblot analysis of cleaved IL-1β and caspase-1 (p20) in culture supernatants (SN) and inactive precursor molecule (pro–IL-1β, pro–caspase-1) in cell lysates (Input) of LPS-primed BMDMs stimulated with different doses of Con A (B) or WGA (C) for 6 h. (**D** and **E**) Immunoblot analysis of cleaved IL-1β and caspase-1 (p20) in culture supernatants (SN) and inactive precursor molecule (pro–IL-1β, pro–caspase-1) in cell lysates (Input) of PMA-differentiated THP-1 cells stimulated with different doses of Con A (D) or WGA (E) for 6 h. Data are shown as mean ± SEM values and represent three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

(50 nM), LysoTracker Red (50 nM), or MitoSOX Red (1 μM) for 30 min. After being washed twice, the cells were fixed for 20 min with 4% paraformaldehyde and then were rinsed in PBST. For Ab staining, the cells were permeabilized with 0.1% Triton X-100, blocked with 10% goat serum in PBST, and then incubated overnight at 4˚C with anti-calreticulin in 10% goat serum in PBST. After being washed with PBST, cells were incubated for 1 h with Alexa Fluor 546–conjugated secondary Ab (Invitrogen) in 10% goat serum in PBST and then were washed three times with PBST. Nuclei were stained with DAPI (Invitrogen).

Real-time PCR

BMDM extraction was performed using TRIzol reagent (Invitrogen), and cDNA was synthesized with the PrimeScript RT reagent kit (Takara Bio). Quantitative real-time PCR was performed by AB StepOne real-time PCR system (Applied Biosystems) and using SYBR Premix Ex TaqTM II (Takara Bio). The primer sequences are as follows: BiP, 5′-TCATCGGACG-CACTTGGAA-3′ (sense) and 5′-CAACCACCTTGAATGGCAAGA-3′ (antisense); Gapdh, 5′-GGTGAAGGTCGGTGTGAACG-3′ (sense) and 5′-CTCGCTCCTGGAAGATGGTG-3′ (antisense). The expression levels of BiP were normalized to the housekeeping gene Gapdh (ΔCt). The results were calculated by the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry

LPS-primed THP-1 cells or BMDMs were stimulated with FITC-labeled Con A or FITC-labeled WGA for 1 h. After being washed twice, the cells were digested and resuspended in cold PBS solution containing 1% FBS. Live cells were gated and the mean fluorescence intensity of FITC was analyzed using FACSVerse flow cytometry (BD Immunocytometry Systems). Data were analyzed using FlowJo software (Tree Star).

Con A–induced acute hepatitis

Con A was dissolved in PBS and i.v. injected into wild-type and NLRP3-deficient mice via the tail vein. For survival experiments, mice were monitored for 72 h after Con A (25 mg/kg) injection. For serum alanine aminotransferase (ALT) assay and H&E staining analysis, Con A (15 mg/kg) was injected. Serum was collected at 8 h to measure the levels of ALT with the enzymatic assay kit. Liver specimens were collected at 24 h and fixed with 4% paraformaldehyde for 1 d and then embedded in paraffin. Paraffin-embedded liver sections were cut into 4-μm slices, and all slices were deparaffinized prior to staining with H&E.

WGA-induced cytokine release in vivo

Wild-type and NLRP3-deficient mice were injected i.p. with WGA (10 mg/kg, dissolved in PBS). Six hours later, mice were sacrificed by exposure to CO_2 and then peritoneal cavities were washed with 1 ml of cold PBS and the concentrations of IL-1β, IL-18, or IL-6 were determined in the supernatant of peritoneal lavage fluid by ELISA or Western blotting.

Statistical analyses

The data are expressed as the mean ± SEM. Statistical significance analyses were performed with the t test for two groups or two-way ANOVA (GraphPad Prism software) for multiple groups. A p value <0.05 was considered significant.

Results

Plant lectins induce caspase-1 activation and IL-1β secretion in macrophages

To investigate whether lectins could induce IL-1β maturation, LPS-primed BMDMs were stimulated with various kinds of plant lectins, including Con A, LCA, WGA, SBA, peanut agglutinin, *Ulex europaeus* agglutinin, and jacalin. Secretion of IL-1β was indeed detected after treatment with Con A, SBA, WGA, and LCA (Fig. 1A), which belong to mannose or *N*-acetylglucosamine–binding lectins. Because IL-1β secretion is controlled by caspase-1 activity, we then tested whether Con A or WGA could activate caspase-1. Treatment of BMDMs with Con A or WGA induced caspase-1 activation and IL-1β maturation in a dose-dependent manner (Fig. 1B, 1C). These results were also confirmed in human THP-1 cells (Fig. 1D, 1E). Thus, our results indicate that

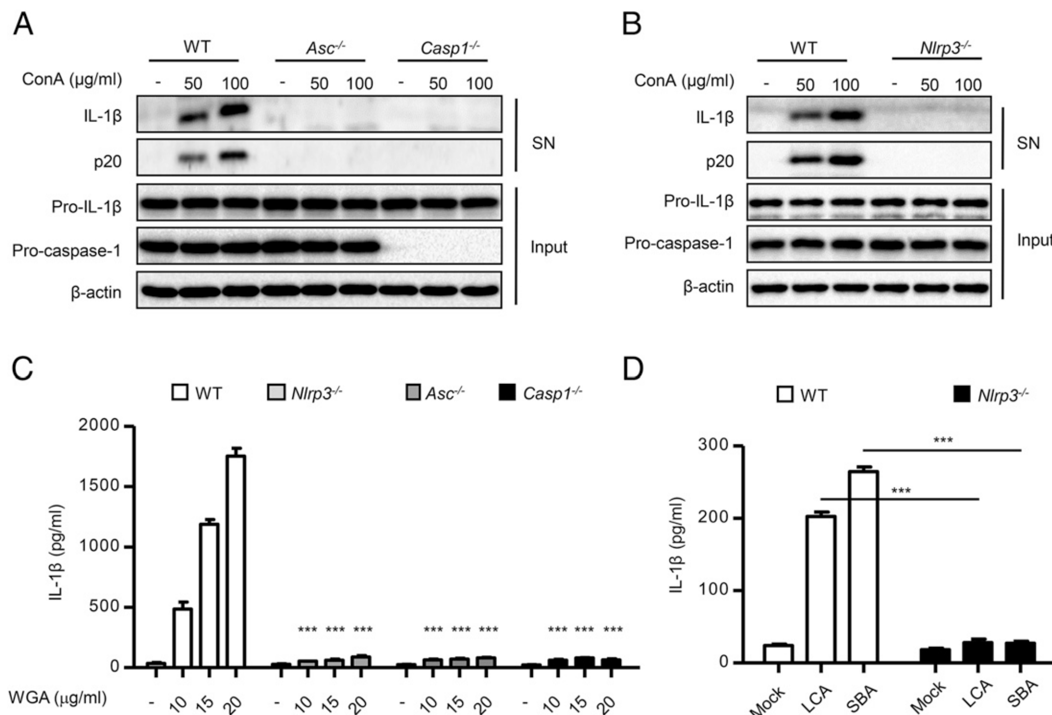

FIGURE 2. The NLRP3 inflammasome is required for plant lectin–induced IL-1β maturation. (**A**) Immunoblot analysis of cleaved IL-1β and caspase-1 (p20) in culture supernatants (SN) and inactive precursor molecule (pro–IL-1β, pro–caspase-1) in cell lysates (Input) of LPS-primed wild-type (WT), *Asc*$^{-/-}$, or *Caspase-1*$^{-/-}$ (*Casp1*$^{-/-}$) BMDMs stimulated with different doses of Con A for 6 h. (**B**) Immunoblot analysis of cleaved IL-1β and caspase-1 (p20) in culture supernatants (SN) and inactive precursor molecule (pro–IL-1β, pro–caspase-1) in cell lysates (Input) of LPS-primed WT or *Nlrp3*$^{-/-}$ BMDMs stimulated with different doses of Con A for 6 h. (**C**) ELISA of IL-1β in culture supernatants from LPS-primed WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, or *Casp1*$^{-/-}$ BMDMs stimulated with different doses of WGA for 6 h. (**D**) ELISA of IL-1β in culture supernatants from LPS-primed WT and *Nlrp3*$^{-/-}$ BMDMs stimulated with different lectins (200 μg/ml) for 6 h. Data are shown as mean ± SEM values and represent three independent experiments. ***$p < 0.001$.

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mannose or *N*-acetylglucosamine–binding plant lectins, especially Con A or WGA, can induce caspase-1 activation and IL-1β secretion in mouse and human macrophages.

Plant lectins activate the NLRP3 inflammasome

Next we explored the pathway responsible for caspase-1 activation and IL-1β maturation. BMDMs from $Asc^{-/-}$ or $caspase\text{-}1^{-/-}$ mice failed to release IL-1β after stimulation with Con A (Fig. 2A), suggesting that lectin-induced IL-1β production depends on inflammasomes. Next we sought to determine which inflammasome was involved in the lectin-induced IL-1β release. The NLRP3 inflammasome is well conserved in mice and humans and can be activated by various DAMPs. Con A could induce caspase-1 activation and IL-1β in wild-type BMDMs, but could not in NLRP3-deficient cells (Fig. 2B). Similar to Con A, WGA-induced IL-1β release was totally dependent on NLRP3, ASC, and caspase-1 (Fig. 2C). LCA- or SBA-induced IL-1β release was also dependent on the NLRP3 inflammasome (Fig. 2D). Other Nod-like receptors were not involved in IL-1β maturation induced by Con A or WGA (Supplemental Fig. 1). Taken together, these results indicate that plant lectins activate the NLRP3 inflammasome, which then promotes caspase-1 activation and subsequent maturation of IL-1β.

Plant lectin Con A–induced NLRP3 inflammasome activation in human macrophages depends on LFA-1

We next investigated the mechanism involved in lectin-induced NLRP3 inflammasome activation. Lectins bind to numerous carbohydrate-containing receptors enriched on the cell surface, which may trigger cell signal transduction and physiological responses. For example, FimH, a bacteria lectin, can be recognized by gp2, which is specifically expressed on the apical plasma membrane of M cells among enterocytes and initiates mucosal immune response (22). The cell surface receptors for Con A and WGA still remain elusive. Integrin $\alpha_L\beta_2$ (also called LFA-1) is a leukocyte surface glycoprotein composed of α and β subunits. A previous study has shown that LFA-1 binds to mammalian lectin Galectin-8 (23), and we therefore investigated whether LFA-1–mediated plant lectins induced NLRP3 inflammasome activation. Con A colocalized with LFA-1α in plasma membrane when THP-1 cells were incubated with FITC–Con A (Fig. 3A). Inhibition of LFA-1α expression by small interfering RNA (siRNA) significantly suppressed the binding of Con A with THP-1 cells and the subsequent inflammasome activation (Fig. 3B–D). In contrast, inhibition of LFA-1α expression had no effect on the binding of WGA with THP-1 cells or WGA-induced inflammasome activation (Fig. 3B–

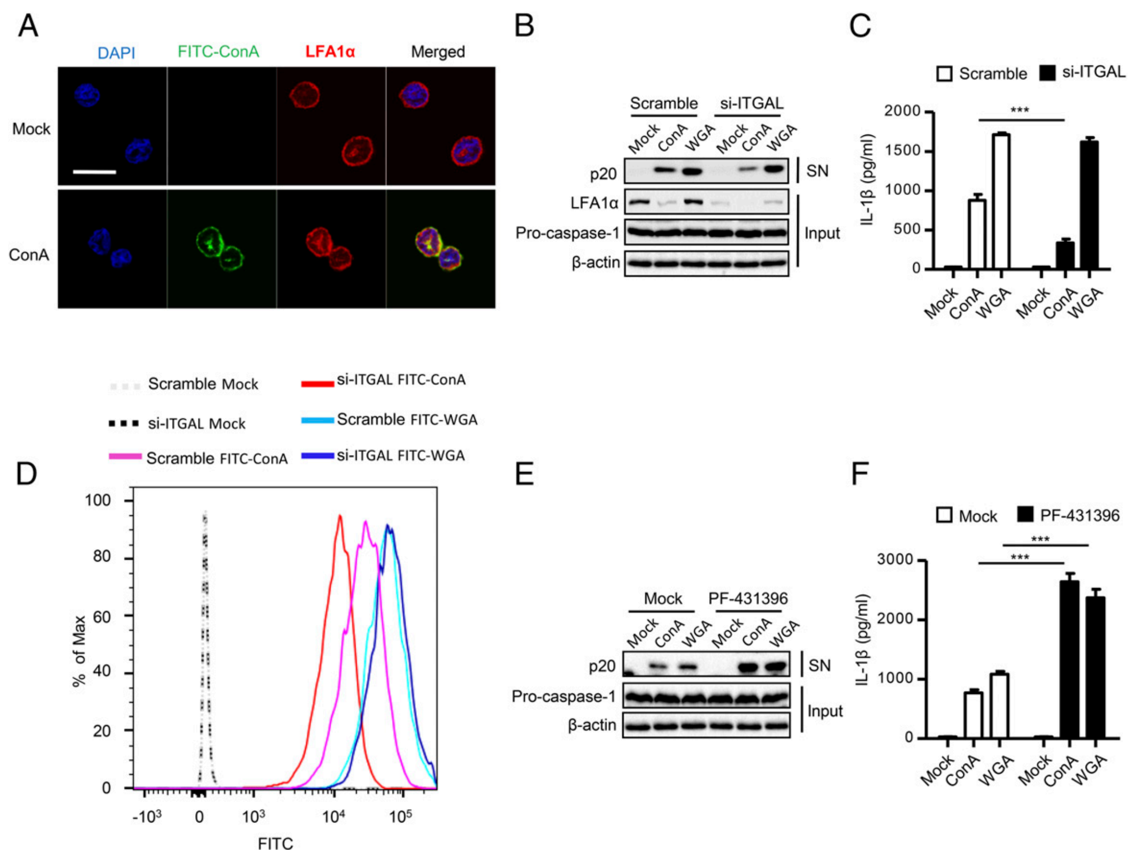

FIGURE 3. Cell surface receptor is required for NLRP3 inflammasome activation by lectins. (**A**) Confocal microscopy analysis of PMA-differentiated THP-1 cells stimulated with FITC-labeled Con A (10 μg/ml) for 1 h and then stained with anti–LFA-1α Ab. Scale bar, 20 μm. (**B** and **C**) PMA-differentiated THP-1 cells were transfected with control siRNA or ITGAL (encodes LFA-1α)-specific siRNA for 48 h. siRNA-transfected cells were primed with LPS and then stimulated with Con A (30 μg/ml) or WGA (10 μg/ml) for 6 h. (B) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (pro–caspase-1) in cell lysates (Input). (C) ELISA of IL-1β in culture supernatants. (**D**) PMA-differentiated THP-1 cells were transfected with control siRNA or ITGAL-specific siRNA for 48 h. FACS analysis is shown of siRNA-transfected cells primed with LPS and then stimulated with FITC-labeled Con A (10 μg/ml) or FITC-labeled WGA (5 μg/ml) for 1 h. (**E** and **F**) PMA-differentiated THP-1 cells were pretreated for 0.5 h with FAK inhibitor (PF-431396, 10 μM) and then stimulated with Con A (30 μg/ml) or WGA (10 μg/ml) for 6 h. (E) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (pro–caspase-1) in cell lysates (Input). (F) ELISA of IL-1β in culture supernatants. Data are shown as mean ± SEM values and represent three independent experiments. $^{***}p < 0.001$.

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D). Additionally, we found that Con A, but not WGA, treatment resulted in the degradation of LFA-1α (Fig. 3B), suggesting that Con A binding might promote the internalization and degradation of LFA-1. However, Con A– or WGA-induced NLRP3 inflammasome activation or the binding of Con A or WGA to BMDMs was normal in *Itgal*$^{-/-}$ BMDMs, indicating that the mouse LFA-1 receptor is not involved in Con A–induced NLRP3 inflammasome activation (Supplemental Fig. 2A, 2B). Ligand binding of the integrin receptor promotes clustering of the integrin and subsequent recruitment of focal adhesion kinase (FAK) (24). We asked whether FAK was involved in lectin-induced inflammasome activation. Indeed, we found that PF-431396, an inhibitor of FAK, could not inhibit Con A, WGA, or other inflammasome agonist-induced caspase-1 activation or IL-1β production in both human THP-1 cells or mouse BMDMs (Fig. 3E, 3F, Supplemental Fig. 2C–E), suggesting that the downstream signaling of LFA-1 was not required for lectin-induced NLRP3 inflammasome activation. Taken together, these results suggest that plant lectin–induced NLRP3 activation depends on different integrin receptors, but not its downstream signaling.

Lysosomal degradation negatively regulates plant lectin–induced NLRP3 inflammaosme activation

Because the downstream signaling of LFA-1 is not required for lectin-induced inflammasome activation, we then investigated how cytosolic lectins induce NLRP3 inflammasome activation. Confocal analysis indicated that intracellular FITC–Con A not only aggregated in the perinuclear space, but also diffusely distributed in cytoplasm (Fig. 4A). Indeed, internalized integrins and ligands can traffic to the late endosomes and lysosomes for degradation or can detach with ligands and then return to the plasma membrane (25). Consistent with this, staining for lysosomes with LysoTracker Red revealed aggregated Con A colocalized with lysosomes (Fig. 4B). Several crystal-induced lysosomal damage and rupture can activate the NLRP3 inflammasome, which can be blocked by inhibition of phagosomal acidification (26). To test whether lysosome-localized Con A contributes to inflammasome activation, we used bafilomycin A1 to inhibit the vacuolar H^+ ATPase, which is required for the acidification. Pretreatment with bafilomycin A1 before lectin stimulation did not inhibit, but enhanced, caspase-1 activation and IL-1β maturation (Fig. 4C, 4D). Additionally, inhibition of cathepsin B had no effect on lectin-induced NLRP3 inflammasome activation (Supplemental Fig. 3A, 3B). These results indicate that lysosome-located lectins are not responsible for NLRP3 inflammasome activation and that lysosomal degradation negatively regulates lectin-induced NLRP3 inflammaosme activation.

Involvement of ER stress and mitochondrial damage in plant lectin–induced NLRP3 inflammasome activation

We next examined whether diffusely distributed lectins activated the NLRP3 inflammasome. First, we found that the diffuse FITC–

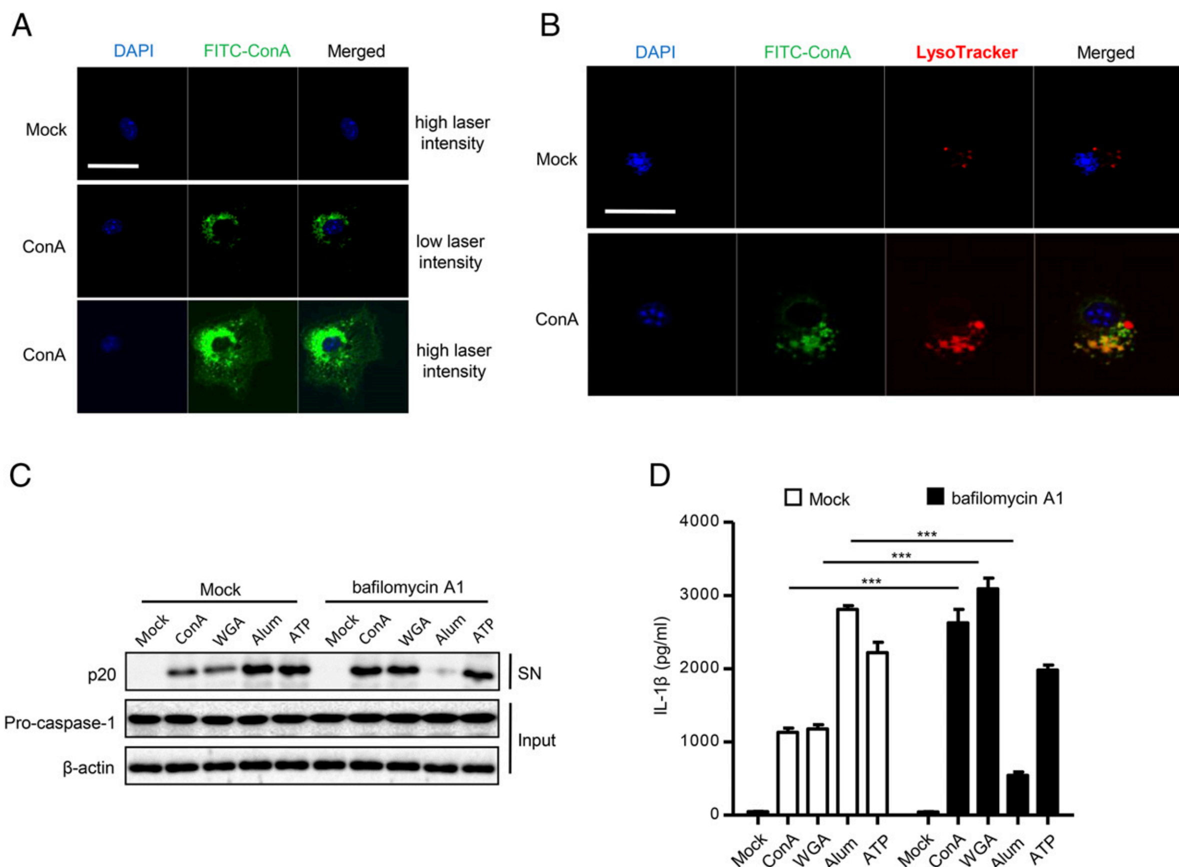

FIGURE 4. Lysosomal degradation negatively regulates plant lectin–induced NLRP3 inflammaosme activation. (**A**) Confocal microscopy analysis of LPS-primed BMDMs stimulated with FITC-labeled Con A (30 μg/ml) for 1 h. Scale bar, 20 μm. (**B**) Confocal microscopy analysis of LPS-primed BMDMs stimulated with FITC-labeled Con A (30 μg/ml) for 1 h, followed by staining with LysoTracker Red (50 nM). Scale bar, 20 μm. (**C** and **D**) LPS-primed BMDMs were pretreated for 0.5 h with lysosome inhibitor (bafilomycin A1, 200 nM) and then stimulated with Con A (80 μg/ml), WGA (20 μg/ml), or Alum (300 μg/ml) for 6 h or ATP (1 mM) for 45 min. (C) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (pro–caspase-1) in cell lysates (Input). (D) ELISA of IL-1β in culture supernatants. Data are shown as mean ± SEM values and represent three independent experiments. $***p < 0.001$.

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Con A was colocalized with calreticulin, a marker for ER (Fig. 5A). Inhibition of lysosomal acidification by bafilomycin A1 resulted in fewer Con A aggregates and more Con A colocalization with ER (Fig. 5B). Because bafilomycin A1 enhanced caspase-1 activation and IL-1β maturation (Fig. 4C, 4D), we speculated that inhibition of lysosomal acidification led to the escape of lectins from lysosomes and then activated the NLRP3 inflammasome via an ER-dependent manner.

The accumulation of misfolded proteins in the ER lumen lead to ER stress, which then promotes mitochondrial damage, mitochondrial reactive oxygen species (ROS) production, and NLRP3 inflammasome activation (27, 28). To test whether ER-located lectins induced ER stress and inflammasome activation, we measured the expression of BiP, a monitor of ER stress. The expression of BiP was increased after Con A or WGA stimulation in BMDMs (Fig. 5C, Supplemental Fig. 3C), suggesting that lectins

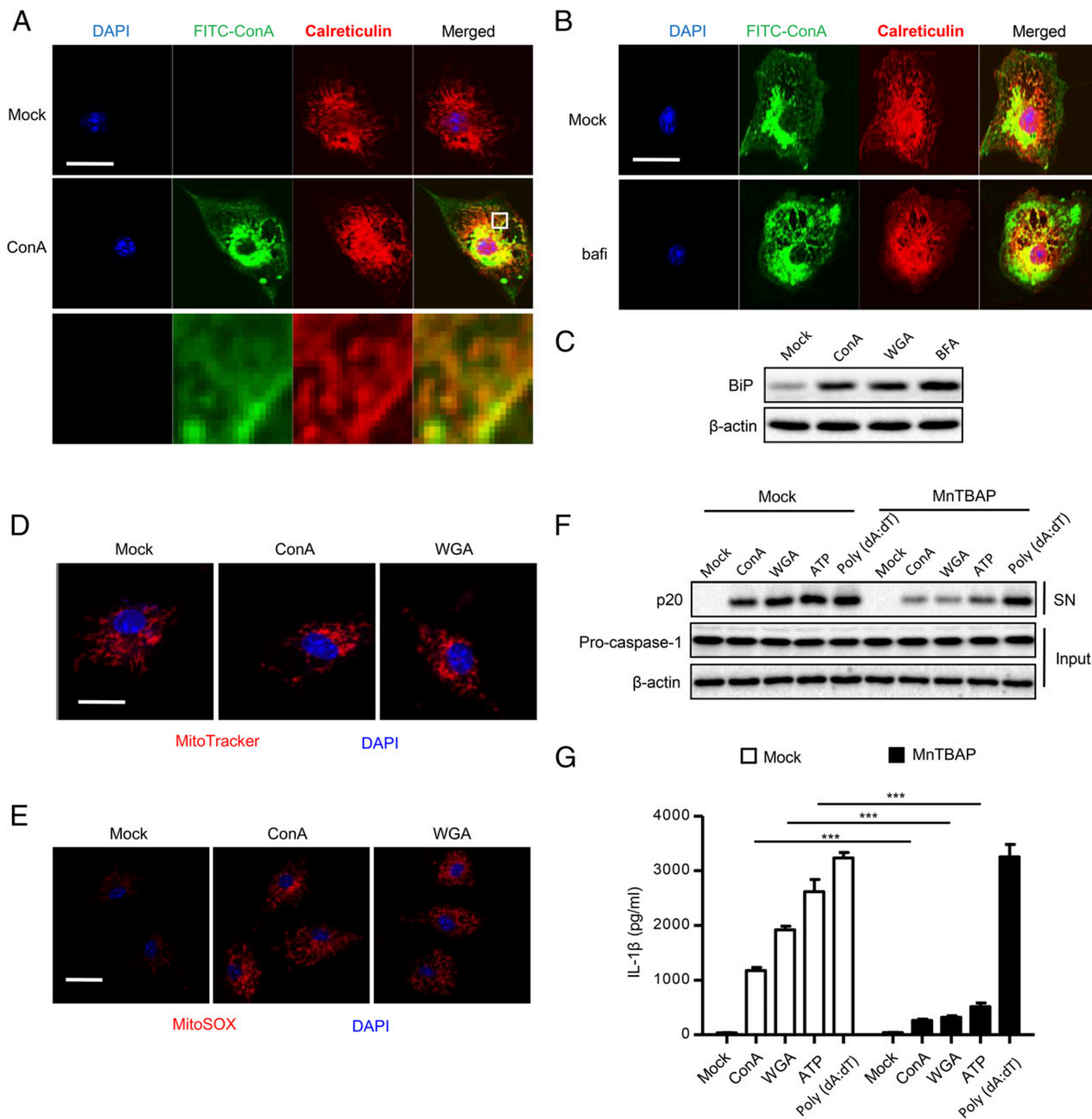

FIGURE 5. ER-loaded plant lectins induce ER stress and mitochondrial damage to activate the NLRP3 inflammasome. (**A**) Confocal microscopy analysis of LPS-primed BMDMs stimulated with FITC-labeled Con A (30 μg/ml) for 1 h and then stained with anti-calreticulin Ab. Scale bar, 20 μm. (**B**) Confocal microscopy of LPS-primed BMDMs given no pretreatment (Mock) or pretreated for 0.5 h with lysosome inhibitor (bafilomycin A1, 200 nM), followed by treatment and staining as in (A). Scale bar, 20 μm. (**C**) Immunoblot analysis of BiP in lysates of BMDMs stimulated with Con A (80 μg/ml), WGA (20 μg/ml), or brefeldin A (BFA; 2 μg/ml) for 4 h. (**D**) Confocal microscopy of LPS-primed BMDMs left untreated or treated for 2 h with Con A (30 μg/ml) or WGA (10 μg/ml) and then stained with mitochondrial dye MitoTracker Red (50 nM) and with the DNA-binding dye DAPI (blue). Scale bar, 20 μm. (**E**) Confocal microscopy of LPS-primed BMDMs left untreated or treated for 4 h with Con A (30 μg/ml) or WGA (10 μg/ml) and then stained with MitoSOX Red (1 μM) and with the DNA-binding dye DAPI (blue). Scale bar, 20 μm. (**F** and **G**) LPS-primed BMDMs were pretreated for 0.5 h with ROS inhibitor (MnTBAP, 150 μM) and then stimulated with Con A (80 μg/ml), WGA (20 μg/ml), or poly(dA:dT) (1 μg/ml) for 6 h or ATP (1 mM) for 45 min. (F) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (procaspase-1) in cell lysates (Input). (G) ELISA of IL-1β in culture supernatants. Data are shown as mean ± SEM values and represent three independent experiments. $***p < 0.001$.

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induced ER stress in macrophages. Furthermore, treatment with Con A or WGA promoted mitochondrial fission and aggregate formation in the perinuclear space of BMDMs (Fig. 5D). Mitochondrial damage was accompanied by mitochondrial ROS production (Fig. 5E). Inhibition of ROS produced by damaged mitochondria with chemical inhibitor diminished lectin-induced caspase-1 activation and IL-1β maturation (Fig. 5F, 5G), suggesting that mitochondrial damage and ROS production are involved in lectin-induced inflammaosme activation. These results suggest that ER-located lectins induce ER stress, which then promotes mitochondrial damage and NLRP3 inflammasome activation.

Inhibition of Ca^{2+} release by chemical inhibitor suppresses lectin-induced mitochondrial damage and inflammasome activation

We further investigated how ER stress promoted mitochondrial damage and inflammasome activation. Published reports have suggested that ER stress may promote mitochondrial damage through different signal pathways. One study suggested ER stress induces activation of caspase-2 and the proapoptotic factor Bid, and then triggers mitochondrial dysfunction and NLRP3 inflammasome activation (28). An alternative model is that Ca^{2+} release from the ER mediates mitochondrial damage and amplifies NLRP3 inflammasome activation (29). To assess the role of caspase-2 in lectin-induced NLRP3 inflammasome activation, we treated BMDMs from caspase-2–deficient mice with Con A or WGA and found that IL-1β release was similar in wild-type and caspase-2–deficient BMDMs (Supplemental Fig. 4A), suggesting that caspase-2 is not involved in lectin-induced inflammasome activation. We next investigated whether Ca^{2+} release from the ER was responsible for the damage of mitochondria and subsequent inflammasome activation. We found that inhibition of Ca^{2+} release from the ER by 2-APB, the IP3 receptor antagonist, prevented mitochondrial damage and ROS production (Fig. 6A, Supplemental Fig. 4B). Moreover, 2-APB blocked lectin-induced caspase-1 processing and IL-1β release in a dose-dependent manner (Fig. 6B–E). Additionally, similar to ER stress-induced NLRP3 inflammasome activation, lectin-induced NLRP3 inflammasome activation could be suppressed by extracellular potassium (Supplemental Fig. 4C). Taken together, our results suggest that Ca^{2+} signaling is involved in lectin-induced mitochondrial damage and inflammasome activation.

Plant lectin-induced NLRP3 inflammasome activation promotes inflammation in vivo

Abundant evidence indicates that plant lectins cause or contribute to the current epidemic of chronic inflammatory illness and autoimmune disease (30, 31). We then asked whether the NLRP3 inflammasome was involved in lectin-induced inflammation in vivo. Con A–induced acute liver injury is a well-known mouse model of hepatitis. In this model, Th cells as well as macrophages are the major effector cells (32). To test whether the NLRP3 inflammasome contributed to this pathological process, we challenged wild-type and NLRP3-deficient mice with Con A. Survival analysis suggested that NLRP3-deficient mice exhibited delayed

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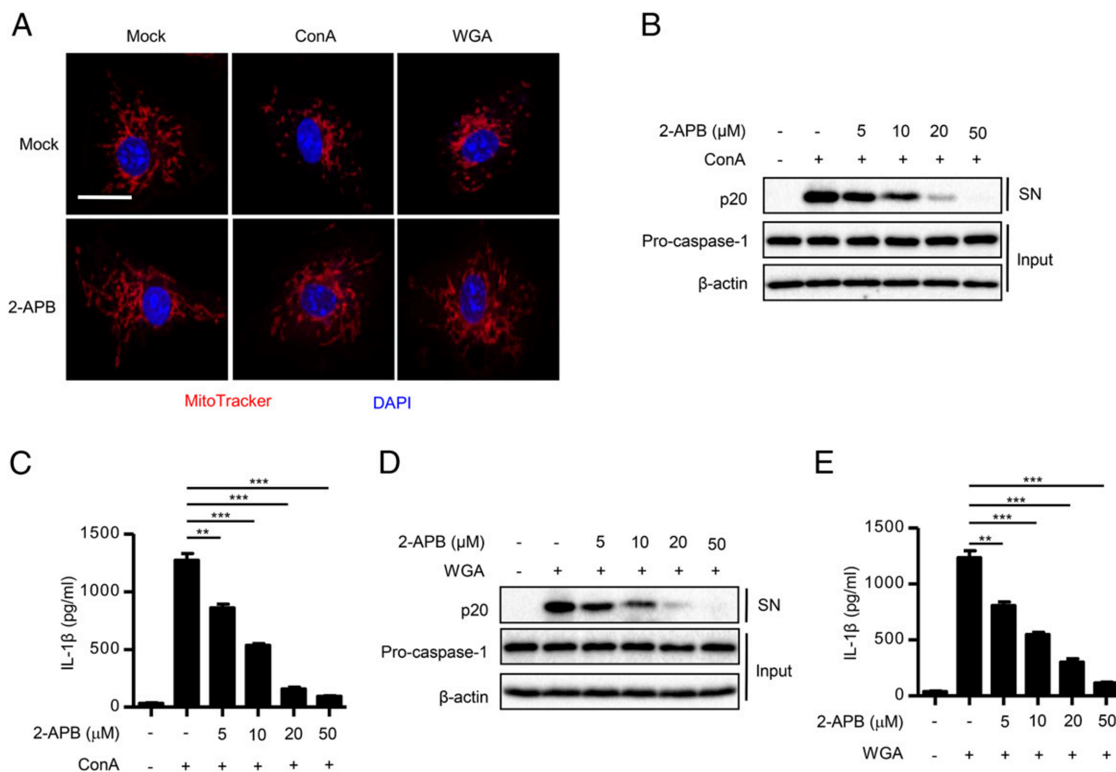

FIGURE 6. ER-loaded plant lectins induce ER stress and mitochondrial damage to activate the NLRP3 inflammasome through Ca^{2+} signaling. (**A**) Confocal microscopy of LPS-primed BMDMs given no pretreatment (Mock) or pretreated for 0.5 h with IP3R inhibitor (2-APB, 50 μM), followed by treatment with Con A (30 μg/ml) for 2 h and then staining with the mitochondrial dye MitoTracker Red (50 nM) and with the DNA-binding dye DAPI (blue). Scale bar, 20 μm. (**B** and **C**) LPS-primed BMDMs were pretreated for 0.5 h with various doses of 2-APB and then stimulated with Con A (80 μg/ml) for 6 h. (B) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (pro–caspase-1) in cell lysates (Input). (C) ELISA of IL-1β in culture supernatants. (**D** and **E**) LPS-primed BMDMs were pretreated for 0.5 h with various doses of IP3R inhibitor (2-APB) and then stimulated with WGA (20 μg/ml) for 6 h. (D) Immunoblot analysis of cleaved caspase-1 (p20) in culture supernatants (SN) and inactive precursor of caspase-1 (pro–caspase-1) in cell lysates (Input). (E) ELISA of IL-1β in culture supernatants. Data are shown as mean ± SEM values and represent three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

fatal injury (Fig. 7A). NLRP3-deficient mice also had diminished serum ALT and reduced hepatic injury after Con A injection (Fig. 7B, 7C). Consistent with this, the IL-1β levels in serum or liver tissue were also compromised (Fig. 7D, 7E). These results indicate that Con A–induced NLRP3 inflammasome activation contributes to Con A–induced acute liver injury.

Among the food lectins, WGA is the most common one, which is found in wheat germ (up to 0.5 g/kg) (33). Wheat is the staple food for >35% of the global population (34). Published studies have suggested that WGA increases intestinal permeability (35). In the serum of healthy individuals, Abs to WGA have been found (36). Significantly higher Ab levels to WGA were detected in patients with celiac disease, and WGA may be involved in the pathogenesis of this disease (37). When wild-type and NLRP3-deficient mice were i.p. injected with WGA, the production of IL-1β and IL-18 in the peritoneal lavage fluid was impaired in NLRP3-deficient mice (Fig. 8A–C). However, the production of IL-6, which is released independent of the inflammasome, was similar in wild-type and NLRP3-deficient mice (Fig. 8D). Taken together, these results indicate that plant lectins can induce NLRP3 inflammasome activation and promote inflammation in vivo.

Discussion

Food intolerances or allergies lead to chronic digestive and autoimmune diseases. One of the key causes contributing to food-induced illness may be plant lectins that are present in all foods, such as grain lectins, legume lectins, and dairy lectins. In this study, we found that plant lectins acted as an exogenous "danger signal" and activated the NLRP3 inflammasome. Thus, our results suggest that the NLRP3 inflammasome might play an important role in diet-induced inflammatory disorders.

Lectins have been reported to bind to numerous carbohydrate-containing receptors enriched on the cell surface. We identified human LFA-1, a glycoprotein that belongs to integrin families, as the receptor for Con A. LFA-1 was responsible for the binding and internalization of Con A in human macrophages. Knockdown of LFA-1α significantly suppressed Con A–induced inflammasome activation, whereas it had no effect on WGA-induced inflammasome activation in human macrophage. Additionally, we found that LFA-1 was not involved in Con A– or WGA-induced NLRP3 infalmmaosme activation in mouse BMDMs. One explanation for this is that human or murine LFA-1α has a different affinity for binding with Con A. Although human LFA-1α shares 72% of its amino acids with its murine counterpart, human ICAM-1 can bind to human, but not murine, LFA-1α, suggesting the existence of species differences between mice and humans for this protein (38, 39). Another explanation is that other integrins compensate the deficiency of LFA-1α in mouse macrophages. Indeed, Con A can bind to several other integrins, including LFA-1β, Mac-1α, and CD11c (40–42). The role of these intergrins or other Con A–binding proteins in plant lectin–induced NLRP3 inflammasome activation needs to be further investigated. We also found that some lectins, including jacalin, could not activate inflammasome in macrophages, and this might be due to the lack of receptors for these lectins in BMDMs. However, we cannot exclude the possibility that jacalin might activate inflammasome in intestine, because it can bind glycoproteins, such as mucins and IgA1, which are abundant in intestinal barrier (43, 44).

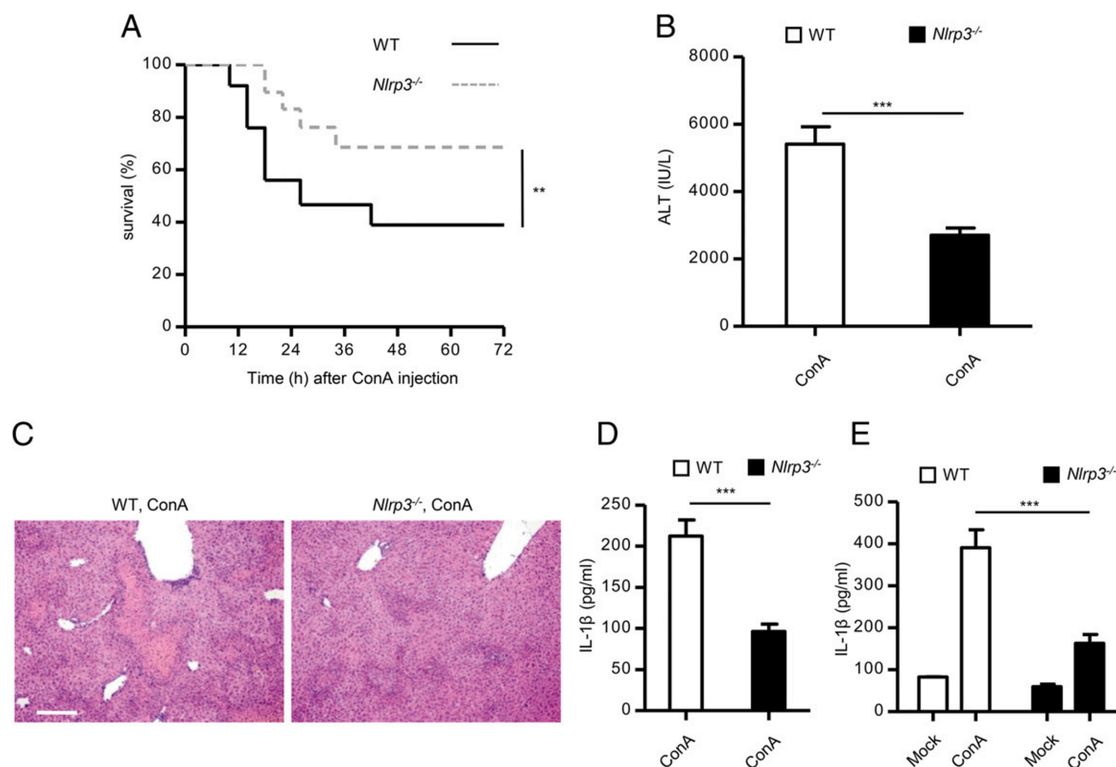

FIGURE 7. NLRP3 inflammasome activation contributes to Con A–induced acute liver injury. (**A**) Wild-type (WT) or *Nlrp3*$^{-/-}$ mice were injected with a lethal dose of Con A (25 mg/kg). Survival was monitored every 4 h. (**B**) Serum ALT levels of WT or *Nlrp3*$^{-/-}$ mice 8 h after Con A injection (15 mg/kg). (**C**) Liver H&E staining of WT or *Nlrp3*$^{-/-}$ mice 24 h after Con A injection (15 mg/kg). Scale bar, 200 μm; original magnification, ×100. (**D**) ELISA of IL-1β in the serum of WT or *Nlrp3*$^{-/-}$ mice 8 h after Con A injection (15 mg/kg). (**E**) WT or *Nlrp3*$^{-/-}$ mice were injected with Con A (15 mg/kg) for 8 h. Liver was isolated and cultured for 24 h, and supernatants were analyzed by ELISA for IL-1β. Data are shown as mean ± SEM of six to eight mice per group and represent three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

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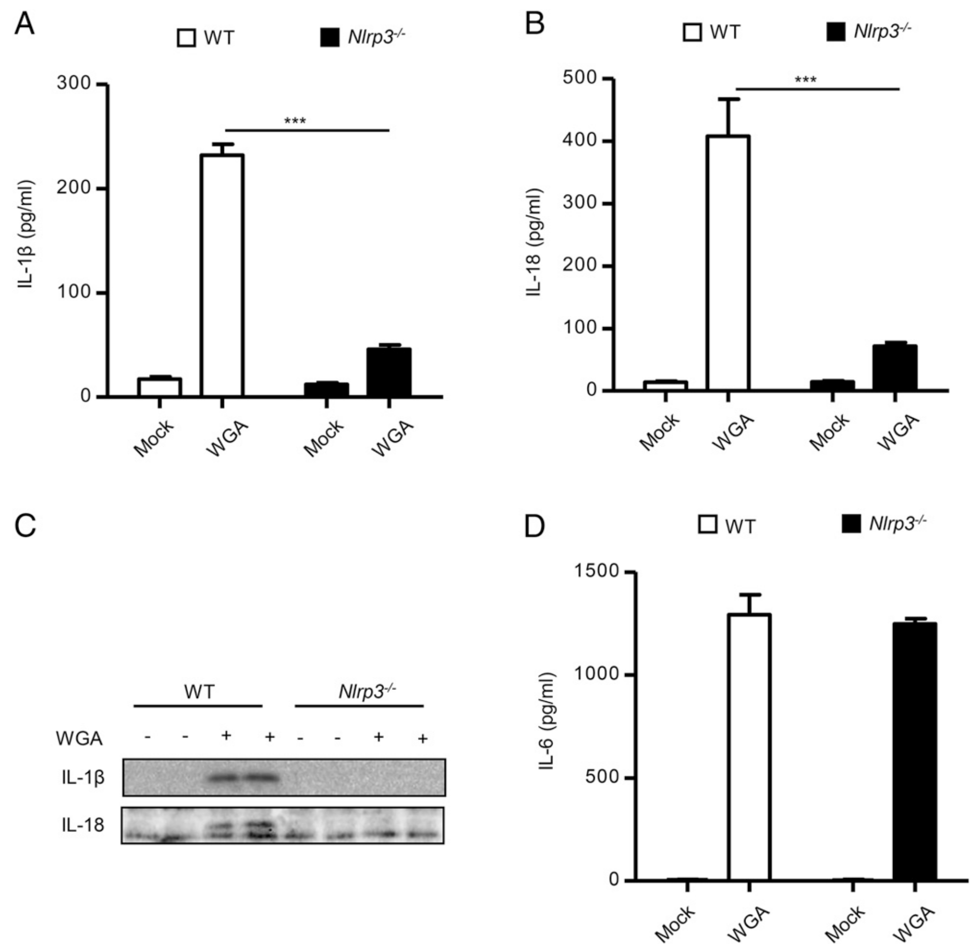

FIGURE 8. WGA induces NLRP3 inflammasome activation in vivo. (**A** and **B**) ELISA of IL-1β (A) and IL-18 (B) in the peritoneal cavity fluid of wild-type (WT) or *Nlrp3*$^{-/-}$ mice 6 h after i.p. injection with WGA (10 mg/kg). (**C**) Immunoblot analysis of cleaved IL-1β and IL-18 in the peritoneal cavity fluid of WT or *Nlrp3*$^{-/-}$ mice 6 h after i.p. injection with WGA (10 mg/kg). (**D**) ELISA of IL-6 in the peritoneal cavity fluid of WT or *Nlrp3*$^{-/-}$ mice 6 h after i.p. injection with WGA (10 mg/kg). Data are shown as mean ± SEM of six to eight mice per group and represent three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

Along with their receptors, internalized lectins traffic to the late endosomes and lysosomes for degradation. Our data indicated that internalized lectins colocalized with the lysosomes and ER. We speculated that lectins could escape from lysosomes and bind to ER. In line with this hypothesis, inhibition of lysosomal degradation led to more lectins colocalized with the ER. However, how lectins escape from lysosomes is still unknown. Because lectins are fairly resistant to proteolytic degradation (45), the accumulation of lectins in lysosomes might have resulted in the leakage of lectins from lysosomes. A cluster of guanylate-binding proteins (GTPases), which can induce the lysis of the pathogen-containing vacuole and release bacteria into the cytosol (46), may be involved in the escape of lectins. The exact mechanisms for the escape of lectins from lysosomes need to be further investigated.

Our results demonstrate that the downstream signaling of LFA-1 is not required for lecin-induced NLRP3 inflammaosme activation. Inhibition of FAK kinase could not suppress Con A–induced inflammasome activation. Additionally, we found that inhibition of FAK enhanced Con A–induced NLRP3 inflammasome activation. A possible reason for this is that FAK can control actin assembly via the Arp2/3 complex (47), and therefore inhibition of FAK would block the trafficking of the LFA-1/Con A complex to lysosomes and promote the escape of Con A from endosomes to activate the NLRP3 inflammasome.

Several reports have linked ER stress to NLRP3 inflammasome activation. Bronner et al. (28) reported that ER stress triggers mitochondrial dysfunction and inflammasome activation through an IRE1α–NLRP3–caspase-2–Bid signal pathway. However, they also found that some compounds induced ER stress and NLRP3 inflammasome activation independent this pathway. This suggests that other types of signaling may participate in ER stress-induced NLRP3 inflammasome activation. Murakami et al. (29) reported that Ca^{2+} released from the ER during ER stress promote mitochondrial damage and NLRP3 inflammasome activation. Multiple NLRP3 inflammasome activators induce Ca^{2+} mobilization, such as ATP, crystals, nigericin, and high levels of extracellular Ca^{2+}. Con A and WGA have also been reported to induce intracellular Ca^{2+} mobilization in different cell types (48, 49). Our results suggest that Ca^{2+} released from the ER is involved in the lectin-induced NLRP3 inflammasome activation, which is consistent with the latter model.

Lectins are widely distributed in almost all species, from microorganisms to humans. Our results showed that mannose or *N*-acetylglucosamine–binding plant lectins activated the NLRP3 inflammasome. Whether mannose or *N*-acetylglucosamine–binding lectins from other species, especially pathogens and humans, could activate the NLRP3 inflammasome needs to be further investigated. Indeed, the mannose-binding lectin (MBL), which is the most intensively studied human lectin, also can mediate ER stress and promote mitochondrial damage in renal tubular cells independent of complement activation (50). Clinical data indicate that MBL levels are higher in serum of patients suffering from autoimmune diseases, such as type 1 diabetes, rheumatic heart disease, and systemic lupus erythematosus (51–53). These results suggest that human MBL might have a proinflammatory role, but the role of the NLRP3 inflammasome needs to be further investigated.

Although we demonstrate that plant lectins can induce strong NLRP3 inflammasome activation in macrophages, the concentration needed for NLRP3 activation in vitro is high. Indeed, the

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physiological concentration of lectins should be much lower, because the intestinal barrier could prevent the entry of lectins to the body (54). However, in patients with inflammatory bowel disease, the concentrations of serum WGA Abs are much higher than in healthy controls (37), suggesting that more lectins can enter the body and trigger NLRP3-dependent inflammation when the intestinal barrier is disrupted. Additionally, the physiological concentration of lectins might trigger low-grade and persistent NLRP3-dependent inflammation, which then contributes to chronic inflammatory diseases.

Our results demonstrate that Con A–induced NLRP3 inflammasome activation contributes to Con A–induced hepatitis. We found that Con A treatment in *Nlrp3*$^{-/-}$ mice resulted in less severe liver injury. This result is not consistent with a previous report by Deutsch et al. (55) in which they showed that NLRP3 deficiency had no effects on Con A–induced liver injury. The differences might be caused by the different doses used in the experiments. Although NLRP3 is mainly expressed on monocytes, macrophages, and dendritic cells, and macrophages are critical for Con A–induced hepatitis (56, 57), we cannot exclude the possible contribution of NLRP3-dependent pyroptosis in hepatocytes to Con A–induced hepatitis, because constitutive NLRP3 activation in mutant NLRP3 knock-in mice can cause hepatocyte pyroptosis, which contributes to severe liver injury and inflammation (58).

Collectively, our results demonstrate that plant lectins can activate the NLRP3 inflammasome and promote secretion of proinflammatory cytokines, which may contribute to food-associated chronic inflammation and inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

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