JLB

Article

Syk is involved in NLRP3 inflammasome-mediated caspase-1 activation through adaptor ASC phosphorylation and enhanced oligomerization

Ying-Cing Lin, Duen-Yi Huang,*,† Jang-Shiun Wang,*,† Yi-Ling Lin,‡ Shie-Liang Hsieh,§,¶ Kuo-Chin Huang,‖,1,2 and Wan-Wan Lin*,†,1,2*

*Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan; †Graduate Institute of Medical Sciences and ¶Institute for Cancer Biology and Drug Discovery, Taipei Medical University, Taipei, Taiwan; ‡Institute of Biomedical Sciences and §Genomics Research Center, Academia Sinica, Taipei, Taiwan; and ‖Department of Family Medicine, National Taiwan University Hospital, Taipei, Taiwan

RECEIVED AUGUST 4, 2014; REVISED NOVEMBER 3, 2014; ACCEPTED NOVEMBER 30, 2014. DOI: 10.1189/jlb.3HI0814-371RR

ABSTRACT

NLRP3 is the most crucial member of the NLR family, as it detects the existence of pathogen invasion and self-derived molecules associated with cellular damage. Several studies have reported that excessive NLRP3 inflammasome-mediated caspase-1 activation is a key factor in the development of diseases. Recent studies have reported that Syk is involved in pathogen-induced NLRP3 inflammasome activation; however, the detailed mechanism linking Syk to NLRP3 inflammasome remains unclear. In this study, we showed that Syk mediates NLRP3 stimuli-induced processing of procaspase-1 and the consequent activation of caspase-1. Moreover, the kinase activity of Syk is required to potentiate caspase-1 activation in a reconstituted NLRP3 inflammasome system in HEK293T cells. The adaptor protein ASC bridges NLRP3 with the effector protein caspase-1. Herein, we find that Syk can associate directly with ASC and NLRP3 by its kinase domain but interact indirectly with procaspase-1. Syk can phosphorylate ASC at Y146 and Y187 residues, and the phosphorylation of both residues is critical to enhance ASC oligomerization and the recruitment of procaspase-1. Together, our results reveal a new molecular pathway through which Syk promotes NLRP3 inflammasome formation, resulting from the phosphorylation of ASC. Thus, the control of Syk activity might be effective to modulate NLRP3 inflammasome activation and treat NLRP3-related immune diseases. ***J. Leukoc. Biol.*** **97: 825–835; 2015.**

Abbreviations: $^{-/-}$ = deficient, Alum = aluminum adjuvant, ASC = apoptosis-associated speck-like protein, BMDC = bone marrow-derived dendritic cell, BMDM = bone marrow-derived macrophage, CARD = caspase recruitment domain, CLR = C-type lectin receptor, FLICA = fluorescent-labeled inhibitor of caspase assay, h = human, HEK = human embryonic kidney, IKK = IκB kinase, KD = kinase deficiency, MSU = monosodium urate, NLR = nucleotide-binding

(*continued on next page*)

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

The innate immune response triggered by PRRs is the first line of a host's defense against microbial pathogens. NLRs are a pivotal family of PRRs that can detect intracellular pathogens and self-damage molecules and coordinate with TLRs to regulate immune functions [1]. Among the 22 hNLRs, NLRP3 is the most crucial receptor in mediating various pathogenic infections and it has been studied more frequently than other NLR family members [2]. Previous research has shown that numerous chemically and structurally diverse stimuli from microbes and danger signals are NLRP3 ligands. Sources of these stimuli include bacterial pore-forming toxins (e.g., nigericin), silica, ATP, MSU crystals, Alum, and fatty acids [3–7]. These ligands trigger NLRP3 to recruit the pivotal adapter protein ASC for the formation of inflammasome and the activation of caspase-1 [1]. ASC associates with procaspase-1 via CARD interaction and interacts with NLRP3 via pyrin domain interaction [8].

One of functions of the NLRP3 inflammasome is to promote the maturation of IL-1β, which is essential for mounting a protective host response against invasive bacteria, fungi, and viruses in immune system [1, 9–11]. Two intracellular steps are generally required for IL-1β maturation. The first step (Step 1) is the induction of pro-IL-1β and NLRP3 expression through NF-κB-dependent gene transcription, and the second step (Step 2) is the activation of the NLRP3 inflammasome, leading to the proteolytic processing of procaspase-1 [12–14]. Under stimulation with NLRP3 ligands, ASC can oligomerize and form a large macromolecular ASC oligomer that constitutes the large multiprotein assemblies, called NLRP3 inflammasome [15–17]. The formation of NLRP3 inflammasome triggers caspase-1 activation, resulting in the cleavage of the proenzyme into active p20 and

1. These authors contributed equally to this work.

2. Correspondence: W.-W.L.: Dept. of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan. E-mail: wwllaura1119@ntu.edu.tw; K.-C.H.: Dept. of Family Medicine, National Taiwan University Hospital, Taipei, Taiwan. E-mail: bretthuang@ntu.edu.tw

p10 subunits that subsequently mediate the cleavage of pro-IL-1β and secretion of mature IL-1β [8, 18, 19].

Syk is a nonreceptor protein tyrosine kinase that is expressed in numerous cell types, particularly in immune cells, such as monocytes and macrophages [20]. The most frequently identified cellular function of Syk is its role in activated immunoreceptors that recruit adaptor proteins containing ITAMs [21, 22]. Moreover, Syk has also been observed in the signaling of specific innate immune receptors, such as TLRs, CLRs, and NLRs [23–27]. Upon activation, Syk transmits multiple crucial signals and regulates inflammatory cytokine secretion, cell migration, and pathogen clearance. Recent studies indicate that Syk might control NLR inflammasome activation [28]. Genetic deletion or pharmacological inhibition of Syk in BMDCs has been shown to abrogate selectively NLRP3 inflammasome activation caused by *Candida albicans* but not NLRC4 inflammasome activation caused by *Salmonella typhimurium*. $Syk^{-/-}$ in BMDCs does not affect pro-IL-1β production and NLRP3 inflammasome activation by nigericin or ATP as well [29]. However, Syk activation by MSU and hemozoin crystals can activate NLRP3 inflammasome and promote IL-1β secretion in macrophages and dendritic cells [6, 16, 30]. Controversially, He et al. [31] demonstrated that Syk is involved in gene induction of IL-1β but not NLRP3 inflammasome activation in BMDMs. Specifically, they found that pro-IL-1β and pro-IL-18 expression caused by LPS stimulation is regulated differentially by Syk.

As described above, several reports have suggested the involvement of Syk in NLRP3 inflammasome activation under various stimulus conditions; however, the detailed analyses of the effect and the mechanism of how Syk regulates pro-IL-1β induction (the Step 1 signaling) and NLRP3/ASC/caspase-1 inflammasome formation (the Step 2 signaling) are missing. We also wonder whether Syk is involved in the signaling of common NLRP3 stimuli besides autoimmune antigens and pathogens, such as MSU and *C. albicans*. To this end, we selected primary mouse macrophages as a cell model to address the roles of Syk in NLRP3-mediated caspase-1 activation. Herein, we used genetic and pharmacological approaches to investigate further the roles of Syk in regulating NLRP3 inflammasome activation. Our data in this study demonstrate that Syk can positively regulate NLRP3 inflammasome formation and caspase-1 activity. We show that Syk can be activated by 4 NLRP3 ligands and recruited to the NLRP3 and ASC. Meanwhile, activated Syk phosphorylates hASC at Tyr146 and Tyr187 and promotes ASC oligomerization and caspase-1 activation. Whereas Syk positively regulates the inflammasome formation (Step 2 signaling), our data indicate that Syk negatively regulates LPS-elicited pro-IL-1β and NLRP3 gene up-regulation (Step 1 signaling). Taken together, we demonstrated that Syk plays a critical role in NLRP3 inflammasome activation, indicating that inhibition of Syk could be a candidate target for treating diseases caused by the excessive activation of caspase-1.

(*continued from previous page*)

oligomerization domain-like receptor, NLRP3 = nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3, pro-IL-1β = precursor form of IL-1β, PRR = pattern recognition receptor, RIPA = radioimmunoprecipitation assay, Syk = spleen tyrosine kinase, SykI = Syk inhibitor I, TAK1 = TGF-β -activated kinase 1, TRAF6 = TNFR-associated factor 6, WT = wild-type, z-VAD-FMK = Z-Val-Ala-Asp-fluoromethylketone

MATERIALS AND METHODS

Mice

WT (C57BL/6) and $Syk^{+/-}$ mice (backcrossed to C57BL/6 mice for at least 6 generations) [32] were bred under pathogen-free conditions at the Animal Center of National Taiwan University College of Medicine. The animal experiments were conducted in accordance with institute regulations after receiving approval from the Ethics Committee of the National Taiwan University College of Medicine (No. 20110047).

Cell culture

We cultured primary macrophages from $Syk^{+/+}$ or $Syk^{-/-}$ fetal liver cells in accordance with our previous study [25], and the HEK293T cells were cultured in DMEM. All pure media were supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reagents and antibodies

We purchased nigericin (Cat.: tlrl-nig) and MSU (tlrl-msu) from InvivoGen (San Diego, CA, USA); Imject Alum from Thermo Scientific (Rockford, IL, USA); SykI, BAY61-3606, and z-VAD-FMK from Calbiochem (Darmstadt, Germany); R406 from Selleck Chemicals (Houston, TX, USA); and LPS, ATP, protease inhibitor cocktails, anti-Flag M2 agarose beads, anti-Flag M2 mAb, active GST-rhSyk protein, as well as various other chemicals from Sigma-Aldrich (St. Louis, MO, USA). We also purchased specific antibodies for phosphorylated Syk (Y525/526), Myc-tag, and human caspase-1 p20 from Cell Signaling Technology (Danvers, MA, USA); antibodies directed against mouse caspase-1 p10 (M-20), Syk (N-19), and HRP-coupled second antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies directed against Myc-tag (Cat.: GTX115046) from GeneTex International (Hsinchu City, Taiwan); antibody directed against β-actin from Millipore (Billerica, MA, USA); antibody directed against mouse IL-1β and a mouse IL-1β ELISA kit (Cat.: DY401) from R&D Systems (Minneapolis, MN, USA); and antibodies directed against NLRP3 and ASC, as well as a mouse caspase-1 ELISA kit (Cat.: AG-45B-0002-KI01) from Adipogen International (San Diego, CA, USA). In addition, we bought recombinant caspase-1 p10/p20 complex (ALX-201-056-U100; Enzo Life Science, Farmingdale, NY, USA); rhGST-ASC, rhGST-caspase-1, and rhGST-NLRP3 (Abnova, Taipei City, Taiwan); and DMEM, Opti-MEM, trypsin-EDTA, and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The protein concentration was determined by use of a Bio-Rad protein assay (Hercules, CA, USA). The ECL reagent was purchased from Millipore and PerkinElmer (Waltham, MA, USA). Finally, we also obtained TriPure Isolation Reagent, FastStart SYBR Green Master, and Genopure Plasmid Maxi Kit (Roche Diagnostics, Indianapolis, IN, USA) and γ-^{32}P-ATP (PerkinElmer).

ELISA assays of IL-1β and caspase-1 p10

Following incubation with the ligands, the concentrations of mouse IL-1β and mouse caspase-1 in the culture medium were determined by use of ELISA (in accordance with the manufacturer's instruction).

Immunoblotting analysis

Following reagent stimulation, the media were collected to detect the secretion of cytokine. The cells were rinsed twice under ice-cold PBS and then harvested and lysed directly with 1× sample loading buffer or RIPA lysis buffer [25]. The samples were heated at 95°C for 5 min, and equal amounts of whole-cell lysates were electrophoresed on 8–12% SDS-PAGE and then transferred to Immunobilon-P (Millipore). The specific protein bands were detected by use of the ECL detection reagent.

Immunoprecipitation

To analyze the protein complex association, cells were treated and extracted in 500 μl RIPA lysis buffer. The following method of immunocomplex was described previously [25].

FLICA

A FLICA provided the whole-cell caspase activity detection. All experimental procedures were conducted in accordance with the manufacturer's instructions. In brief, WT and Syk$^{-/-}$ macrophages were primed with LPS for 6 h and then were stimulated with the NLRP3 ligands. After stimulation, caspase-1-specific FLICA probe (Immunochemistry, Bloomington, MN, USA) was added and incubated for 2 h to induce probe/caspase-1 complex formation. The samples were fixed, and the green fluorescence was detected by use of a fluorescence microscope with a bandpass filter (excitation 490 nm, emission >520 nm).

MTT assay and crystal violet method

Primary macrophages were plated in 24-well plates and incubated at 37°C with the NLRP3 ligands. After incubating the MTT (5 μg/ml) for 1 h at 37°C, the supernatants were aspirated, and the formazan granules generated by the live cells were dissolved in DMSO. The OD values at 550 and 630 nm were measured by use of a microplate reader. The net absorbance (OD_{550}–OD_{630}) indicated the enzymatic activity of the mitochondria and cell viability. In certain experiments, the crystal violet method was used to assess the stimulated cells and measure the cellular proteins in accordance with our previous research [33].

RT and real-time quantitative PCR

To measure specific gene expressions, the primer sequences for IL-1β, NLRP3, and β-actin were synthesized (**Table 1**). Following LPS treatment, the cells were homogenized with 300 μl TriPure Isolation Reagents (Roche Diagnostics), and 2 μg total RNA was reverse transcribed with a RT-PCR kit (Promega, Heidelberg, Germany), in accordance with the manufacturer's instructions. The FastStart SYBR Green Master was used to perform the real-time PCRs in 96-well plates, and an ABI Prism 7900 was used to determine the PCR products.

Plasmids and site-directed mutagenesis

The WT, deleted domains, and KD of the Syk plasmids and Myc-tagged NLRP3 were generated in our laboratory [25]. The mutant Myc-tagged hASC plasmids were generated by mutating Tyr146, Tyr187, and Tyr146/Tyr187 to phenylalanine (Y146F, Y187F, and Y146/187F). The point mutation was achieved by use of the QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). All plasmids were confirmed by DNA sequencing.

In vitro Syk-mediated phosphorylation

To identify the phosphorylation site(s) on the ASC, the WT and mutant ASCs were transiently transfected to the HEK293T cells, followed by immunoprecipitation in accordance with previous research [24]. The immunocomplex was washed 3 times in cold kinase lysis buffer containing 400 mM NaCl and then washed once in 1× kinase reaction buffer. The beads were combined with active GST-hSyk (100 ng/sample) and then incubated at 30°C for 30 min in 40 μl kinase reaction buffer (supplemented with 10 μCi γ-^{32}P-ATP). In certain experiments, 200 ng rhGST-ASC, rhGST-caspase-1, and rhGST-NLRP3 were incubated with 100 ng active GST-hSyk at 30°C for 30 min in the kinase reaction buffer. Reaction products were detected by use of 12% SDS-PAGE, followed by autoradiography to determine the phosphorylation status. The autophosphorylation was applied as an index for Syk activation.

Reconstituted caspase-1 activation in a HEK293T cell system

The HEK293T cells were transiently transfected with 10 ng Flag-tagged human procaspase-1, 100 ng Myc-tagged hASC, 100 ng Flag-tagged hNLRP3, and 150 ng Flag-tagged mouse pro-IL-1β plasmids. Lipofectamine 2000 and Opti-MEM were used to transfect the plasmids. After 6 h, the cells were transferred to complete DMEM for 24 h. Subsequently, the supernatants were collected to detect the mouse IL-1β by use of the ELISA method.

In vitro caspase-1 activity assay

The activity of caspase-1 in the lysates and supernatants were measured by use of the fluorometric substrate acetylated Tyr-Val-Ala-Asp-amino-4-trifluoromethyl-coumarin in accordance with previous research [34]. The fluorescence intensities of treated samples were compared with those of the untreated control samples to determine the fold increase in caspase-1 activity.

ASC oligomerization cross-linking assay

The treated primary macrophages and transfected HEK293T cells were used to perform the ASC oligomerization experiment in accordance with previous research [35]. The cells were resuspended briefly in ASC lysis buffer A, and the lysates were sheared by passing them through a 27-G needle, 10 times. The cell lysates were centrifuged at 340 g for 8 min to remove the intact cells and nuclei. The supernatants were added with 1 vol CHAPS buffer, and the pellets containing the ASC were collected by centrifugation at 2650 g for 8 min. Subsequently, the crude pellets were resuspended in CHAPS buffer containing chemical cross-linked reagent (4 mM disuccinimidyl suberate; Cayman Chemical, Ann Arbor, MI, USA), incubated at room temperature for 30 min.

Statistical evaluation and image quantification

The values were expressed as the mean ± SEM of at least 3 independent experiments that were performed in duplicate. A Student's *t*-test was used to determine whether the differences were statistically significant ($P < 0.05$). Western blotting images were quantified with ImageJ software.

RESULTS

Syk activity is required for NLRP3-mediated caspase-1 activation

To understand whether Syk plays a crucial role in regulating NLRP3 inflammasome activation, we first treated macrophages with several NLRP3 stimuli and found that Syk is phosphorylated and activated by ATP, nigericin, MSU, and Alum adjuvant (**Fig. 1A**). Next, we tested the effects of specific SykI kinase on NLRP3 inflammasome activation. As the NF-κB activation pathway is a critical step to induce pro-IL-1β protein expression and for mature IL-1β production, we conducted experiments in LPS-primed macrophages. After LPS priming, we found that SykI and BAY61-3606 caused a reduction in the secretion of IL-1β induced by ATP (Fig. 1B, left), nigericin (Fig. 1B, right), and Alum and MSU (Fig. 1B, middle). Note that this reduction was not resulting from the reduced protein expressions of pro-IL-1β

TABLE 1. List of primer sequences used for real-time RT-PCR analysis

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
IL-1β	GCTTCAGGCAGGCAGTATCAC	CGACAGCACGAGGCTTTTT
NLRP3	AGAGAATGAGGTCCTCTTTACCATGT	AGCCCCGTGCACACAATC
β-Actin	CGGGGACCTGACTGACTACC	AGGAAGGCTGGAAGAGTGC

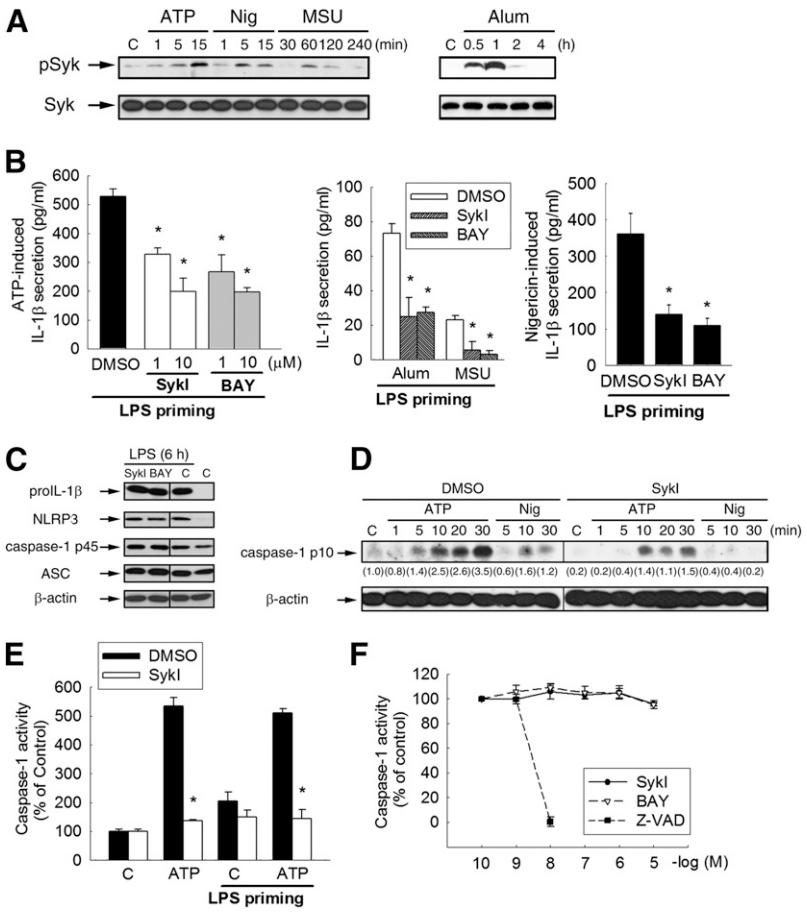

Figure 1. NLRP3 stimuli induce Syk activation, and Syk inhibitors suppress NLRP3 inflammasome activation. (A) Macrophages were treated with ATP (5 mM), nigericin (Nig; 10 μM), MSU (100 μg/ml), or Alum (150 μg/ml) for the indicated time. Total cell lysates were collected and then analyzed by immunoblotting with the phosphorylated (p)Syk (Y525/526) antibody. C, Control. (B) Macrophages were primed with LPS (1 μg/ml) for 6 h and followed by treatment with SykI (1 or 10 μM) or BAY61-3606 (BAY; 1 or 10 μM) for another 1 h. After treatment with inhibitors, macrophages were stimulated with ATP (left; 5 mM), Alum (middle; 150 μg/ml), MSU (middle; 100 μg/ml), and nigericin (right; 10 μM), as indicated, for 20 min, 9 h, 9 h, and 20 min, respectively. The media were collected to determine the concentrations of secreted IL-1β. (C) Macrophages were stimulated with vehicle (C) or LPS (1 μg/ml) for 6 h and then were treated with SykI for another 1 h; the whole-cell lysates were collected and were analyzed by immunoblotting. (D) Macrophages were pretreated with DMSO or SykI (10 μM) for 1 h and then were stimulated with ATP (5 mM) or nigericin (10 μM) for indicated times. The whole-cell lysates were analyzed for cleaved caspase-1 p10 and β-actin by immunoblotting. The numbers in parentheses are the quantification values for p10/β-actin, which was expressed as fold of the DMSO control group. Data are means from 3 independent experiments. (E) After LPS (1 μg/ml) priming for 6 h, macrophages were treated with ATP (5 mM) for 20 min, and the whole-cell lysates were collected for in vitro caspase-1 activity assay. Data were normalized to protein concentration and then were expressed as percentages relative to the control (non-ATP-treated) sample. (F) Ten units of the caspase-1 p10/p20 recombinant protein complex were preincubated with vehicle (DMSO) or indicated concentrations of SykI, BAY61-3606, or z-VAD-FMK (Z-VAD) for 1 h at 4°C. Then, samples were performed in an in vitro caspase-1 activity assay. The fluorescence intensity of the DMSO group was set as 100% to express the relative values. (B and E) Data are means ± SEM from 3 independent experiments. *$P < 0.05$ when comparing DMSO with SykI.

(NLRP3 inflammasome-related components), including NLRP3, ASC, and procaspase-1, under SykI treatment (Fig. 1C). Furthermore, SykI markedly decreased the ATP- and nigericin-induced caspase-1 activation, as evidenced by either accessing p10 formation with immunoblotting (Fig. 1D) or determining caspase-1 enzymatic activity in whole-cell lysates (Fig. 1E). With the consideration that the inhibitory effects of SykI result from cytotoxicity, we measured the cell viability by MTT and lactate dehydrogenase assays. As a result, both SykI at 10 μM did not have cytotoxicity in LPS-primed cells under 20 min (ATP or nigericin) or 9 h treatment (Alum or MSU; data not shown).

To determine whether Syk inhibitors exert a direct effect on caspase-1 activation, we incubated recombinant caspase-1 p10/p20 (active form) with indicated inhibitors for 1 h, followed by performing the in vitro caspase-1 activity assay. Data shown in Fig. 1F indicated that the pan-caspase inhibitor z-VAD-FMK can abolish the caspase-1 enzyme activity at 10 nM, whereas SykI and BAY61-3606 exerted no effect on the activity of the purified recombinant enzyme. These results suggest that Syk is a positive regulator of NLRP3 inflammasome activation.

In addition to pharmacological inhibitors, furthermore, we used a genetic knockout approach to strengthen Syk, crucial in NLRP3-mediated caspase-1 activation. Syk$^{-/-}$ macrophages exhibited lower expression of caspase-1 (p10) after ATP treatment (**Fig. 2A**, upper, and Fig. 2B) and Alum (Fig. 2B). Likewise nigericin-induced p10 expression was reduced in Syk$^{-/-}$ macrophages (Supplemental Fig. 1, lower). As reported, LPS priming can enhance NLRP3 inflammasome assembly and activation through mechanisms of NF-κB-mediated NLRP3 induction [36]. However, previously, we have shown the negative role of Syk in TLR4-mediated signaling of MyD88/TRAF6/TAK/IKK [25]. To understand whether Syk-mediated NLRP3 inflammasome activation might be affected by the Step 1 signaling pathway under LPS priming, we also compared the caspase-1 p10 expression in LPS-primed WT and Syk$^{-/-}$ macrophages. As a result, we found that in LPS priming condition, the Alum (Fig. 2A, lower)- and nigericin (Supplemental Fig. 1, upper)-induced p10 expression was also reduced in Syk$^{-/-}$ macrophages. Based on these findings, we suggest that Syk is a direct regulator of NLRP3 inflammasome.

Furthermore, the data from the ELISA and in vitro enzymatic assay demonstrated that the NLRP3 stimuli-induced, mature caspase-1 secretion and caspase-1 activity were reduced significantly in the Syk$^{-/-}$ macrophages, with or without LPS priming (Fig. 2C and D). Likewise, the in vitro fluorescence-based FLICA caspase-1 activity assay also revealed lower caspase-1 activities upon 4 NLRP3 stimuli treatment in the Syk$^{-/-}$ macrophages (Fig. 2E). Collectively, these results indicate that Syk plays an important role for NLRP3 inflammasome activation and caspase-1 activity.

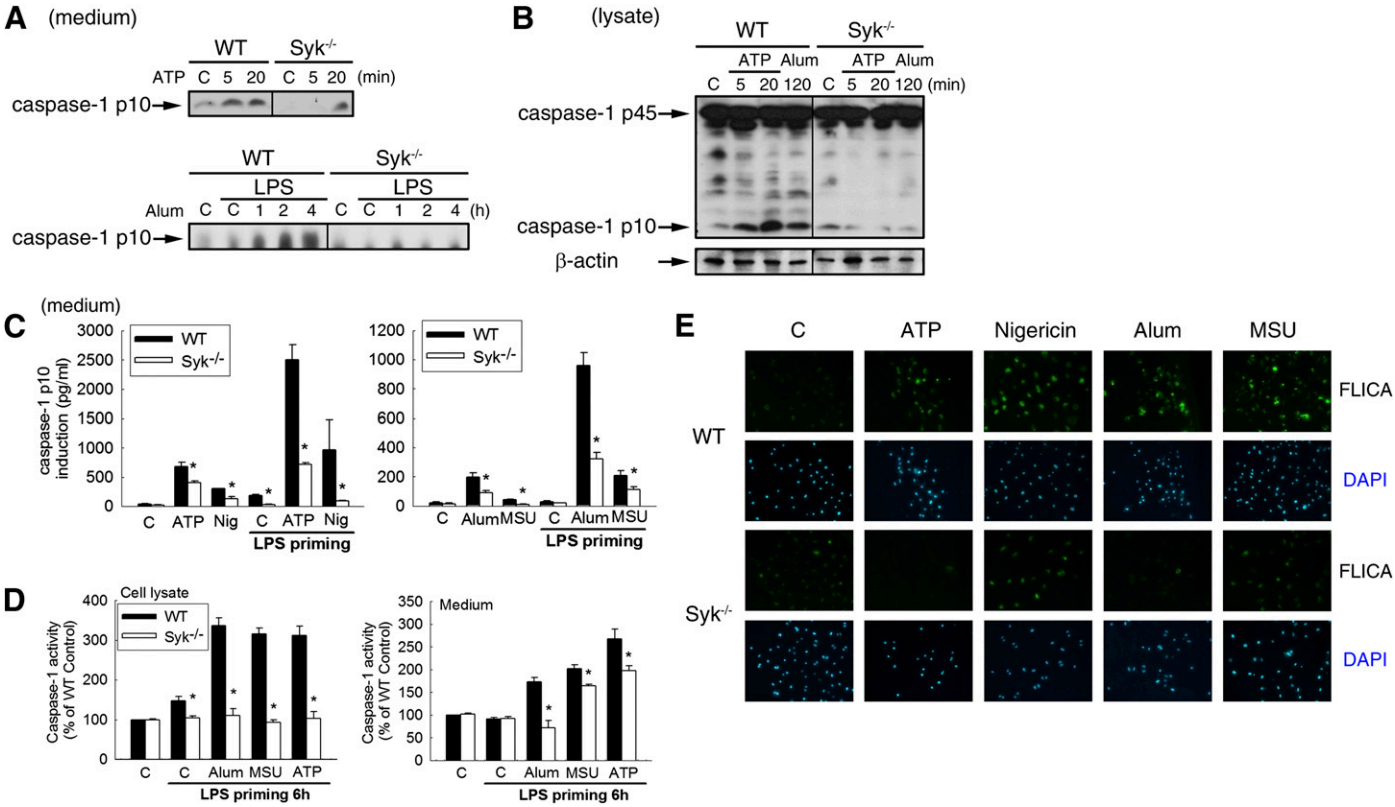

Figure 2. Syk mediates caspase-1 activation upon NLRP3 ligand stimulation. (A) Macrophages were stimulated with ATP (5 mM) for 5 or 20 min (upper). In some studies, macrophages were primed with LPS (1 μg/ml) for 6 h, followed by stimulation with Alum (150 μg/ml) for 1, 2, or 4 h (lower). After incubation, the media were collected for immunoblotting with caspase-1 p10 antibody. (B) Macrophages were stimulated with ATP or Alum for the indicated time, and then, whole-cell lysates were collected for immunoblotting with caspase-1 p10 antibody. (C and D) After treatment, with or without LPS, macrophages were treated with ATP (5 mM) or nigericin (10 μM) for 20 min; Alum (150 μg/ml) or MSU (100 μg/ml) for 9 h; and then media or whole-cell lysates, as indicated, were used to determine caspase-1 p10 by the ELISA method (C) or the in vitro caspase-1 activity assay (D). Data are means ± SEM from 3 independent experiments. $^*P < 0.05$ when comparing WT and Syk$^{-/-}$ macrophages. (E) After LPS priming for 6 h, WT and Syk$^{-/-}$ macrophages were treated with ATP or nigericin for 1 h or Alum or MSU for 4 h and then subjected to microscopic fluorescent staining with caspase-1 FLICA (green) and DAPI (blue).

Syk promotes NLRP3 stimuli-mediated ASC oligomerization and inflammasome formation

Previous studies have reported that the oligomerization of ASC is a critical step for caspase-1 activation; therefore, we investigated whether the ASC oligomer formation is regulated by the activation of Syk. As a result, we showed that the ASC oligomerization induced by ATP and nigericin was decreased markedly in Syk$^{-/-}$ macrophages (**Fig. 3A**). Likewise, SykI and R406 mimic the effect of Syk$^{-/-}$ to diminish the ATP- and nigericin-induced ASC oligomerization (Fig. 3B). Collectively, the genetic and chemical approaches support the notion that Syk is required for the ASC oligomerization in macrophages.

Based on these observations that Syk regulates NLRP3 inflammasome activation, we next wanted to know whether Syk is recruited to the NLRP3 inflammasome complex following ligand stimulation. We performed immunoprecipitation experiments by use of Syk antibody and observed the association of Syk with ASC and NLRP3 after ATP stimulation for 20 min (**Fig. 4A**, middle). With the use of ASC antibody for immunoprecipitation, we also detected the association of ASC to Syk and NLRP3 following ATP treatment (Fig. 4A, right). In addition, we observed the association of Syk with procaspase-1 upon ATP or Alum treatment (Supplemental Fig. 2A). Subsequently, we used Syk$^{-/-}$ macrophages to confirm the function of Syk in NLRP3 inflammasome formation. The WT and Syk$^{-/-}$ macrophages were stimulated with ATP or Alum, and the immunoprecipitation was performed by incubation with a procaspase-1 antibody. As a result, we observed that the interaction between ASC and caspase-1 under ATP (Fig. 4B) or Alum (Supplemental Fig. 2B) treatment was reduced markedly in the Syk$^{-/-}$ macrophages. These results indicate that Syk is a new component of the NLRP3 inflammasome; moreover, Syk promotes the binding between ASC and caspase-1, which is a critical step to trigger caspase-1 activation.

To further confirm the association between Syk and NLRP3 inflammasome components in the NLRP3 ligand-stimulated macrophages, we used an overexpression system to determine the domains responsible for the protein–protein interactions between Syk and NLRP3 inflammasome. HEK293T cells were cotransfected with Myc-tagged NLRP3 or ASC, together with various Flag-tagged Syk-deletion mutants constructs then performed the immunoprecipitation against the Flag antibody. Our data indicated that the C-terminal kinase domain of Syk is

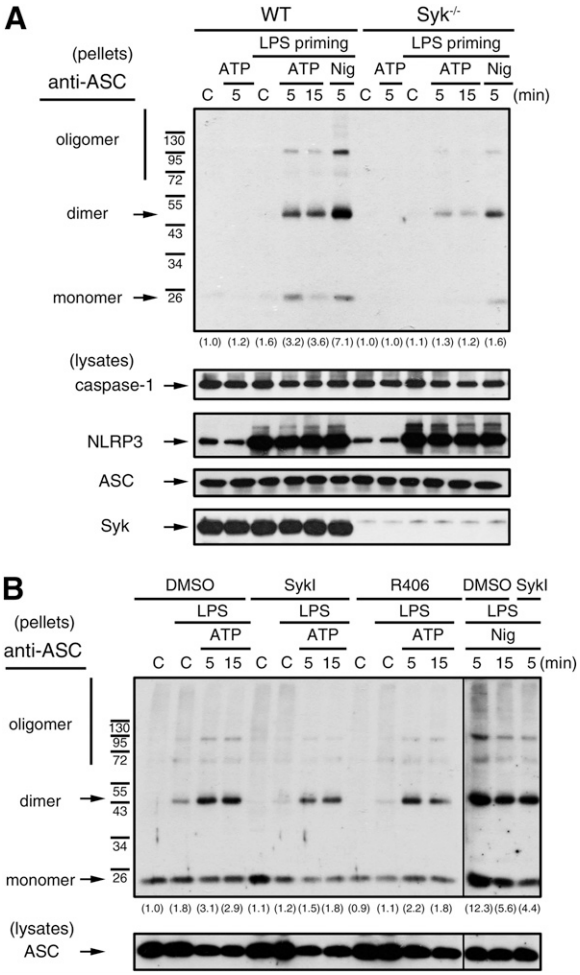

Figure 3. Syk promotes NLRP3 inflammasome-dependent ASC oligomerization. (A) After LPS (1 μg/ml) priming for 6 h, WT and Syk$^{-/-}$ macrophages were treated with ATP (5 mM) or nigericin (10 μM) for the indicated time. (B) WT macrophages were first primed with LPS (1 μg/ml) for 6 h, followed by treatment with SykI (10 μM) or R406 (1 μM) for 1 h. Then, macrophages were stimulated with ATP or nigericin for 5 or 15 min. After treatment, the crude pellets were collected for conduction of the ASC oligomerization assay, as described in Materials and Methods. The numbers in parentheses were the quantification values for ASC dimer and oligomer. Data are means from 3 independent experiments. Some immunoblotting studies were conducted in the total cell lysate samples.

necessary for its association with NLRP3 and ASC (Fig. 4C and D). Nevertheless, no direct interaction was observed between Syk and procaspase-1 in the HEK293T cell system (Fig. 4E). Notably, we found that the interaction between Syk and procaspase-1 can be detected in cells expressing Myc-tagged ASC, and their interaction was enhanced further in the presence of Myc-tagged NLRP3 (Fig. 4F). Collectively, these results indicate that Syk can promote NLRP3 inflammasome complex formation upon NLRP3 stimulation.

Syk phosphorylates ASC at Y146 and Y187 and leads to increased NLRP3 inflammasome formation

After observing the recruitment of Syk to NLRP3 inflammasome following ligand stimulation, we were interested to understand if Syk is an upstream kinase of NLRP3 inflammasome components. To address this issue, rhGST-fusion proteins of procaspase-1, ASC, and NLRP3 were used as substrates for an in vitro Syk kinase assay. The results shown in **Fig. 5A** revealed that the ASC and NLRP3 but not procaspase-1 can be phosphorylated by Syk. Next, to identify which amino acid residues of ASC were phosphorylated by Syk, we used the KinasePhos2.0 database and predicted 2 consensus phosphorylation sites for Syk in hASC, i.e., Y146 and Y187. Subsequently, we generated the mutated ASC constructs (Y146F, Y187F, and Y146/187F) and performed the in vitro kinase assay by use of these ASC mutant proteins as substrates. As a result, we found that the ASC protein of Y146 or Y187 mutation remained susceptible to phosphorylation by Syk, whereas the double mutations of ASC Y146/187F robustly reduced the extent of Syk-dependent phosphorylation (Fig. 5B). To elucidate the contribution of ASC phosphorylation in NLRP3 inflammasome complex formation, we transiently overexpressed Myc-ASC (WT or mutants), Syk, and Flag-procaspase-1 in HEK293T cells. Immunoprecipitation of procaspase-1 by use of Flag antibody confirmed previous data of Fig. 4F and revealed that the presence of WT ASC can induce Syk and procaspase-1 association (Fig. 5C). However, ASC mutants at single tyrosine residue failed to display this effect (Fig. 5C). Conversely, with the use of ASC antibody for immunoprecipitation, we also achieved similar results, i.e., only WT but not mutant ASC can trigger the binding between Syk and procaspase-1 (Fig. 5D). Moreover, procaspase-1 has a higher binding extent to WT ASC than ASC mutants (Fig. 5D). All together, these data indicate that phosphorylation at both tyrosine residues of ASC is a prerequisite for the Syk/procaspase-1 interaction and can also enhance Syk/ASC and ASC/caspase-1 binding. Collectively, these results suggested that phosphorylation of ASC at Y146 and Y187 residues is crucial for Syk to increase the formation of the NLRP3 inflammasome complex and the subsequent caspase-1 activation.

Tyrosine residues 146 and 187 on ASC are required for ASC oligomerization

Next, we investigated whether the ASC oligomer formation is regulated by Syk kinase-dependent phosphorylation on ASC. We cotransfected the plasmids encoding the Myc-tagged ASC (WT or mutants), Flag-tagged NLRP3, Flag-tagged procaspase-1, and/or hSyk into the HEK293T cells and performed the ASC oligomerization assay. We observed that a single tyrosine mutation of ASC can reduce the ASC oligomerization, and the double mutation of ASC nearly eliminated the oligomer formation (**Fig. 6A**). Moreover, our data revealed that the Syk-mediated ASC oligomerization can be detected even in the absence of NLRP3, and in fact, NLRP3 existence alone without the presence of Syk only slightly enhanced ASC oligomerization (Fig. 6B). These data indicated that the Syk-mediated phosphorylation of both tyrosine residues in ASC is critical for the formation of ASC oligomers and the subsequent proteolytic processing of procaspase-1 and pro-IL-1β. To elucidate the contribution of ASC phosphorylation in NLRP3 inflammasome-mediated IL-1β secretion, we adapted the reconstitution system by overexpressing pro-IL-1β, procaspase-1, NLRP3, and/or ASC (WT and mutants) in HEK293T cells and measured the production of IL-1β. As shown in Fig. 6C, the presence of WT ASC can increase caspase-1-mediated IL-1β

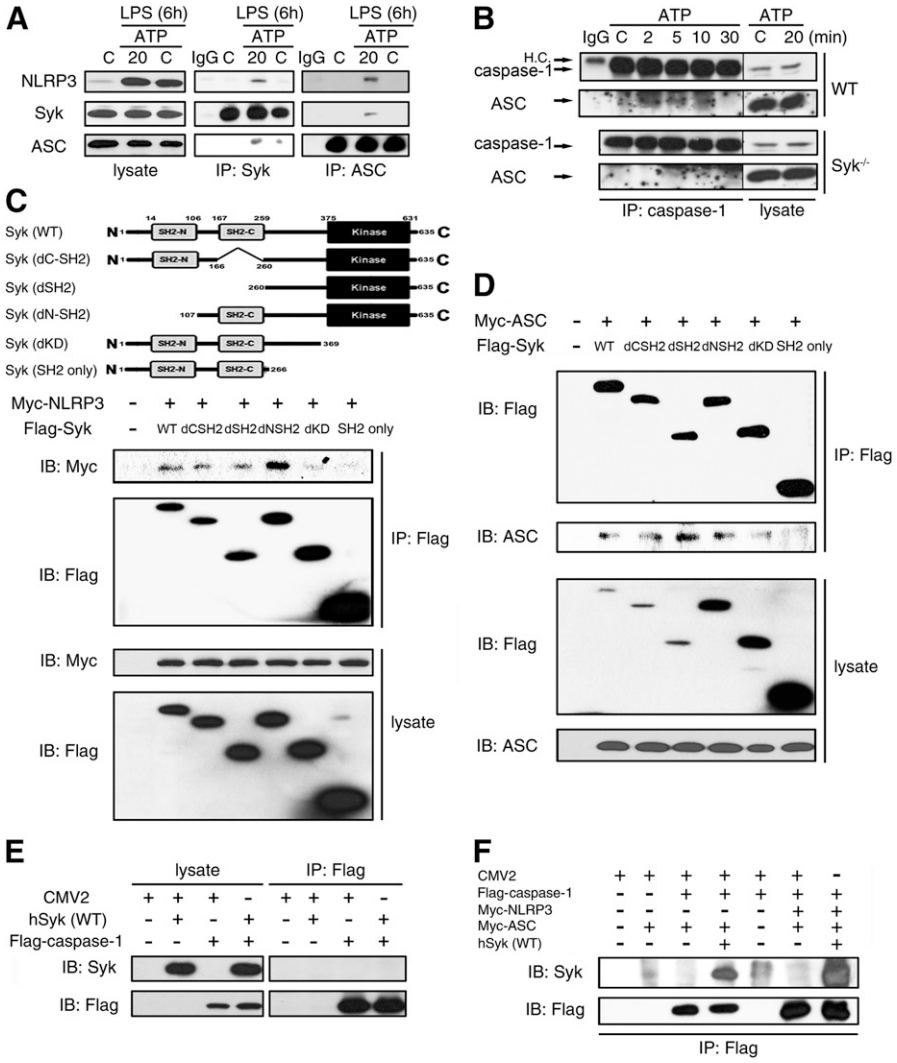

Figure 4. Syk can bind to NLRP3 and ASC and is recruited to the activated NLRP3 inflammasome. (A) After priming with LPS (1 μg/ml) for 6 h, macrophages were treated with ATP (5 mM) for 20 min. Whole-cell lysates were immunoprecipitated (IP) with anti-Syk or anti-ASC antibody, and samples were analyzed by immunoblotting (IB) for antibodies specific against NLRP3, ASC, and Syk. (B) WT and Syk$^{-/-}$ macrophage lysates were immunoprecipitated with anticaspase-1 antibody and then subjected to SDS-PAGE. HEK293T cells were cotransfected with plasmid encoding Myc-tagged NLRP3 (C) or Myc-tagged ASC (D), together with plasmids encoding the indicated FLAG-tagged Syk-deletion mutants. Twenty-four hours later, cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody and were analyzed by Western blotting with antibodies against the FLAG or Myc, as indicated. The schematic diagram of FLAG-tagged Syk constructs includes WT, C-terminal Src homology 2 (SH2) domain deleted (dC-SH2), SH2 domain deleted (dSH2), N-terminal SH2 domain deleted (dN-SH2), KD (dKD), linker B and KD (SH2 only). H.C., heavy chain. (E) HEK293T cells were transiently transfected with control vector (CMV2), hSyk (WT), and plasmids encoding FLAG-tagged caspase-1. Cell lysates were used to perform the immunoprecipitation with anti-FLAG antibody and then analyzed by Western blotting with antibodies against FLAG or Syk. (F) HEK293T cells were transfected with control vector (CMV2), hFlag-tagged caspase-1, plasmids (1 μg) encoding the Myc-tagged hNLRP3 and hASC, and hSyk (WT). After 24 h incubation, whole-cell lysates were subjected to immunoprecipitation by anti-Flag antibody and then immunoblotting with antibodies against Flag, Myc, or Syk, as indicated. All Western blots are representatives of 3 independent experiments.

production, whereas ASC mutants had much weaker effects than WT form. As expected, NLRP3 expression can enhance ASC/caspase-1-mediated IL-1β secretion. Nevertheless, under such a condition, Syk can induce a much greater effect on caspase-1 activation compared with the condition without NLRP3 (Fig. 6C). Collectively, these results indicate that the phosphorylation of ASC on Y146 and Y187 is involved in the promotion of ASC oligomerization for the subsequent induction of caspase-1 activation, and the level of Syk-dependent caspase-1 activation, resulting from ASC phosphorylation, is relying on the status of full NLRP3 complex.

Syk regulates IL-1β secretion through affecting the gene expression of NLRP3 and pro-IL-1β

After observing the crucial role of Syk in mediating NLRP3 inflammasome formation and caspase-1 activation, we further examined the real outcome of IL-1β production in response to NLRP3 stimuli in LPS-primed WT and Syk$^{-/-}$ macrophages. The reason we conducted this experiment is because in our previous study, we showed the negative role of Syk in LPS-induced NF-κB signaling and in turn, the proinflammatory mediator expression. As expression of NLRP3 and pro-IL-1β are also under the control of the NF-κB pathway, Syk might also influence their levels and then affect IL-1β production indirectly. As shown in **Fig. 7A**, we found that the amount of mature IL-1β secretion upon treatment of each NLRP3 stimulus (ATP, nigericin, MSU, or Alum) in LPS-primed macrophages was greater in Syk-null macrophages, which seems to be opposite of our previous findings showing that Syk is a positive regulator of caspase-1 activation. Furthermore, we excluded that the increased IL-1β secretion under Syk$^{-/-}$ is associated with cell death. When performing MTT and crystal violet assays, we did not see a significant difference of cell viability in WT and Syk$^{-/-}$ macrophages under NLRP3 ligand stimulation (Supplemental Fig. 3).

To investigate further the mechanisms underlying the paradoxical data of IL-1β secretion in LPS-primed Syk$^{-/-}$ macrophages, we first detected the expression of pro-IL-1β. We observed that the protein (Fig. 7B) and gene expressions (Fig. 7D, left) of pro-IL-1β following LPS treatment were higher in Syk$^{-/-}$ macrophages than in WT macrophages. Actually, the basal pro-IL-1β

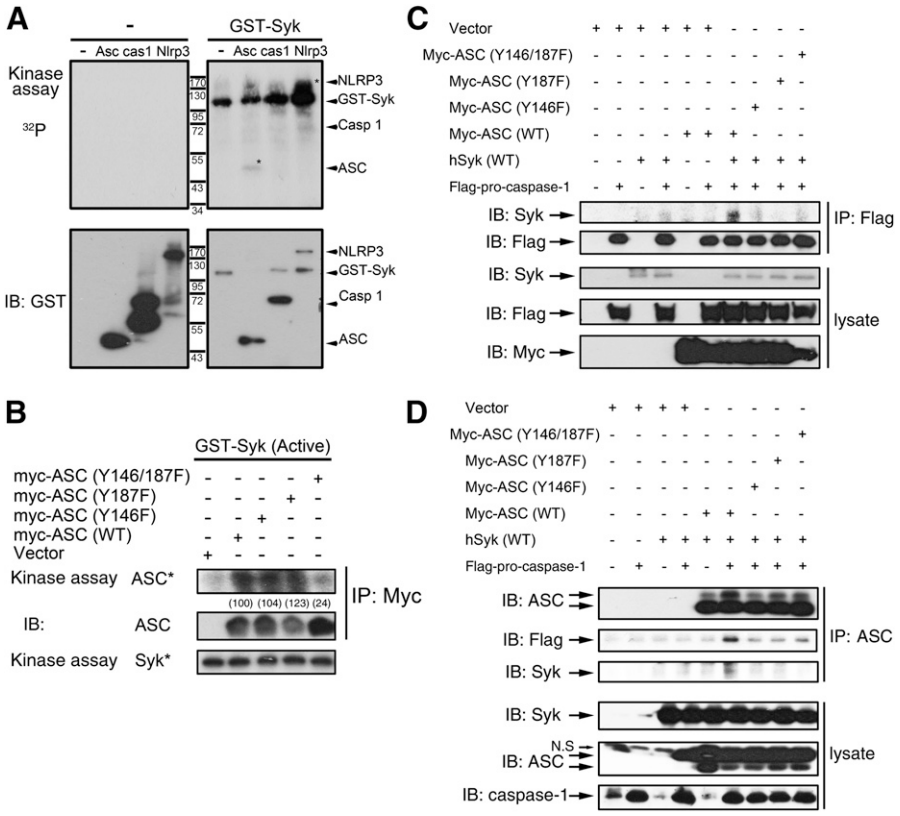

Figure 5. Tyrosine residues Y146 and Y187 of ASC are phosphorylation sites for Syk. (A) GST-fusion proteins of active Syk (100 ng), ASC (200 ng), caspase-1 (200 ng), and NLRP3 (200 ng) were added, as indicated for the in vitro kinase assay. Samples were analyzed by running SDS-PAGE, followed by autoradiography or immunoblotting with GST antibody. (B) HEK293T cells were transfected with control vector or plasmids encoding ASC (Y146/187F), ASC (Y187F), ASC (Y146F), or ASC (WT). Twenty-four hours later, cell lysates were immunoprecipitated with an anti-Myc antibody. The immunoprecipitated products were incubated with GST-Syk (100 ng) for the in vitro kinase assay. *, protein phosphorylation. (C and D) HEK293T cells were transfected with control vector or plasmids (1 μg) encoding hMyc-tagged ASC mutants or WT, hSyk (WT), and/or hFlag-tagged pro-caspase-1, as indicated. After 24 h incubation, cell lysates were immunoprecipitated with anti-Flag (C) or anti-ASC antibody (D), followed by immunoblotting for the indicated antibodies. Data are representative of 3 independent experiments. N.S, Non-specific.

mRNA level was also increased slightly, by 1.65-fold, in Syk-null macrophages (Fig. 7D, right). Next, we determined the expression of the main components of NLRP3 inflammasome under LPS treatment in WT and Syk$^{-/-}$ macrophages. We found that the protein levels of ASC and procaspase-1 remained unaltered in the WT and Syk-null macrophages upon LPS stimulation (Fig. 7C, left). However, for NLRP3, we found that its basal and LPS-induced protein levels were higher in Syk$^{-/-}$ macrophages (Fig. 7C, right). In agreement with this finding, the NLRP3 mRNA at basal condition (Fig. 7D, right) or under induction by LPS (Fig. 7D, middle) was also increased in Syk$^{-/-}$ macrophages. All of these results indicate that Syk can negatively regulate the expressions of pro-IL-1β and NLRP3, in agreement with our previous findings highlighting Syk as a negative regulator of TLR4-mediated TRAF6 signaling and production of proinflammatory cytokines [25].

DISCUSSION

The roles of Syk in immune responses have been investigated extensively over the past decade, and its role in the activation of the NLRP3 inflammasome has attracted considerable attention recently. Numerous studies have indicated that kinases, such as protein kinase R [37], AMP-activated protein kinase [7], Lyn, and Syk [16, 29, 38, 39], are involved in NLRP3 inflammasome activation. However, the molecular mechanisms of NLRP3 inflammasome activation remain unclear, and many questions are unanswered. In this study, we demonstrate a novel and common intracellular molecular event that is induced by various stimuli for NLRP3 and conclude that Syk positively triggers caspase-1 activation to perform critical cellular functions. The Syk-stimulated caspase-1 activation through NLRP3 inflammasome is executed by triggering ASC phosphorylation at the Tyr146 and Tyr187 residues, enhancing ASC oligomerization and increasing formation of the NLRP3 inflammasome complex.

In this study, we observed the distinct effects of Syk$^{-/-}$ on caspase-1 activation and mature IL-1β secretion induced by various NLRP3 stimuli. Mature IL-1β secretion was higher in Syk$^{-/-}$ macrophages (Fig. 7A); however, the caspase-1 activation in the Syk$^{-/-}$ macrophages was lower than that in the WT macrophages (Fig. 2). To resolve this inconsistency, we provided evidence to show that Syk can negatively regulate LPS-induced pro-IL-1β and NLRP3 expression (i.e., Step 1 signal for mature IL-1β production; Fig. 7B–D), whereas it positively regulates NLRP3 inflammasome (i.e., Step 2 signal for mature IL-1β production; Fig. 2). We assert that the increased pro-IL-1β and NLRP3 expression in the LPS-treated Syk$^{-/-}$ macrophages is resulting from the enhancement of TAK1, IKK, and JNK signaling pathways, as we predicted, based on our previous study for other LPS-induced proinflammatory mediators [25]. Thus, it is suggested that the increased pro-IL-1β and NLRP3 protein expression in Syk$^{-/-}$ macrophages accounts for the higher secretion of mature IL-1β, even under the condition of reduced caspase-1 activity.

Another possibility for the increased IL-1β secretion in the Syk$^{-/-}$ macrophages is that other protease-processing pathways beyond

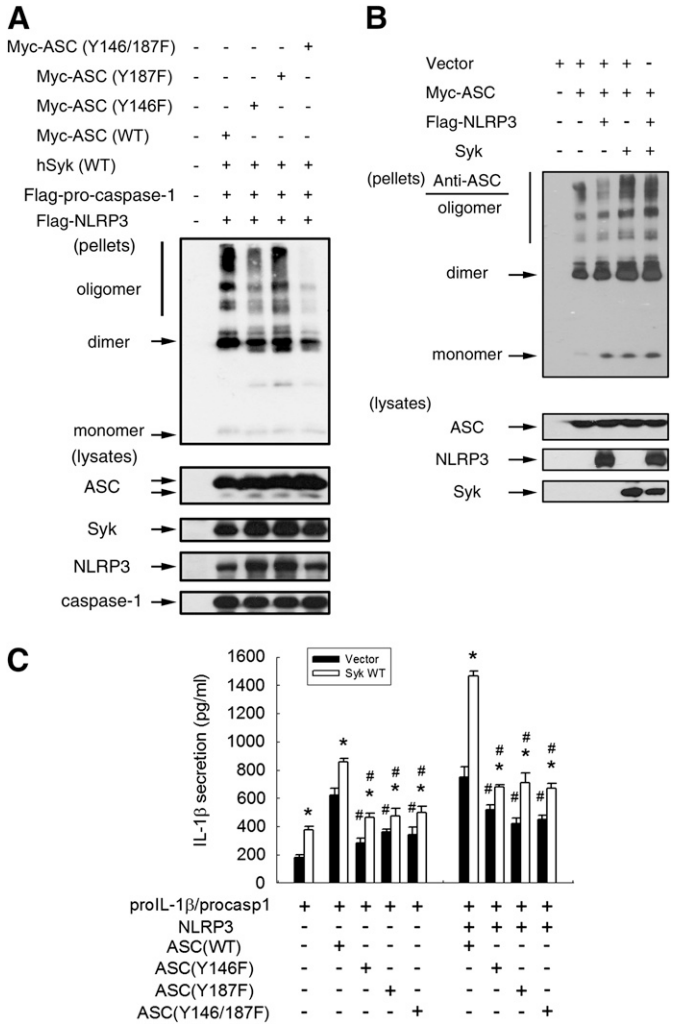

Figure 6. The Y146 and Y187 of ASC are required for Syk-dependent ASC oligomerization and IL-1β secretion. (A and B) HEK293T cells were transfected with control vector or plasmids (1 μg) encoding Syk, Flag-tagged NLRP3, Flag-tagged procaspase-1, and Myc-tagged ASC (WT or mutants), as indicated. Twenty-four hours later, we harvested cells and performed ASC oligomerization assay. The expressions of caspase-1, NLRP3, Syk, and ASC in whole-cell lysates were used as internal controls. (C) HEK293T cells were transfected with control vector, hSyk (WT; 100 ng), mouse pro-IL-1β (150 ng), hFlag-tagged procaspase-1 (10 ng), hFlag-tagged NLRP3 (100 ng), and/or hMyc-tagged ASC mutants (100 ng), as indicated. After 6 h incubation, cells were fed with fresh complete DMEM and incubated for another 24 h. Then, the media were collected to determine the mouse IL-1β secretion. Data are means ± SEM from 3 independent experiments. $*P < 0.05$ when comparing vector and Syk (WT) groups; $\#P < 0.05$ when comparing WT and mutant constructs of ASC in corresponding vector or Syk expression group.

caspase-1 might be involved. Activated caspase-1 is a short half-life enzyme [40], and other proteases (e.g., matrix metalloproteinases, proteinase-3, elastase, and chymase) can cleave pro-IL-1β and pro-IL-18 to become mature cytokines, either intracellularly or extracellularly; moreover, these proteases can be released from short-lived and damaged cells [41]. In this study, we did not examine whether these protease activities might be different between WT and Syk$^{-/-}$ macrophages; thus, we cannot exclude any possible involvement of other Syk-regulated molecular events that might control pro-IL-1β processing and IL-1β secretion.

Shio et al. [16] reported that activated Syk can weakly associate with NLRP3 and ASC following Hemozoin stimulation. Besides Hemozoin, we found that Syk is a common signaling molecule of NLRP3 inflammasome under various stimuli (Fig. 1A). After stimulating macrophages with NLRP3 stimuli, Syk can be recruited to NLRP3 inflammasome (Fig. 4A and B). In this study, we confirm the associations among Syk, ASC, and NLRP3 in ATP- and Alum-stimulated macrophages. In addition, we showed further the interaction between Syk and procaspase-1 under ATP and Alum stimulation (Fig. 4A and Supplemental Fig. 2A). Notably, we found that Syk is able to enhance the ASC/caspase-1 complex formation under ATP and Alum stimulation (Fig. 4B and Supplemental Fig. 2B). These findings explain why ATP- and Alum-induced caspase-1 activities are lower in Syk$^{-/-}$ macrophages. With the use of ectopic expression and a reconstituted model in the HEK293T cells, we assert that Syk uses the kinase domain to associate with ASC and NLRP3 directly (Fig. 4C and D). Notably, even though no direct interaction was observed between Syk and procaspase-1 in the HEK293T cell overexpression system (Fig. 4E), ASC and NLRP3 possibly could serve as bridges to induce the association of Syk and procaspase-1 in macrophages (Fig. 4F). Taken together, our data demonstrate a novel protein kinase component in the NLRP3 inflammasome, which exerts its kinase-dependent function for caspase-1 activation.

Numerous studies have shown that posttranslational modifications (e.g., phosphorylation, acetylation, and ubiquitination) are critical steps in the regulation of innate and adaptive immunity [10, 42–44]. However, so far, few studies reported the post-translational modifications on NLRs and inflammasome components [35, 45–47]. In this study, we unravel the phosphorylation function of Syk in regulating the NLRP3 inflammasome assembly and caspase-1 activation. Here, we identify ASC as a new substrate of Syk. Two tyrosine residues (Y146 and Y187) on hASC are Syk phosphorylation targets (Fig. 5B), which are both required for mediating the phosphorylation-dependent enhancement of ASC oligomerization and caspase-1 activation. Mutations of both sites reduce ASC association with Syk and procaspase-1 (Fig. 5D), Syk-mediated ASC oligomerization (Fig. 6A), and caspase-1 activity (Fig. 6C). As both tyrosine resides are localized at the CARD domain of ASC, which is well characterized for association with procaspase-1, we speculate that such mutation might also change the ASC protein conformation, ASC/procaspase-1 interaction, and in turn, caspase-1 activation. The speculation is based on the unexpected finding that caspase-1 activity in the reconstituted model without NLRP3 expression can be decreased by ASC mutants (Fig. 6C). Moreover, Hara et al. [28] suggested that other phosphorylation resides of mouse ASC, including S58, T125, T151, T152, S153, and Y144 (equivalent to hASC Y146), might being able to regulate IL-1β secretion in response to NLRP3 activation. Collectively, the phosphorylation of ASC on serine, threonine, and/or tyrosine residues is a new identified mechanism to control NLRP3 inflammasome-mediated caspase-1 activation. Besides ASC, we suggest that NLRP3 is a putative substrate of Syk (Fig. 5A) and the detailed phosphorylation site(s) and related functional outcome require further research.

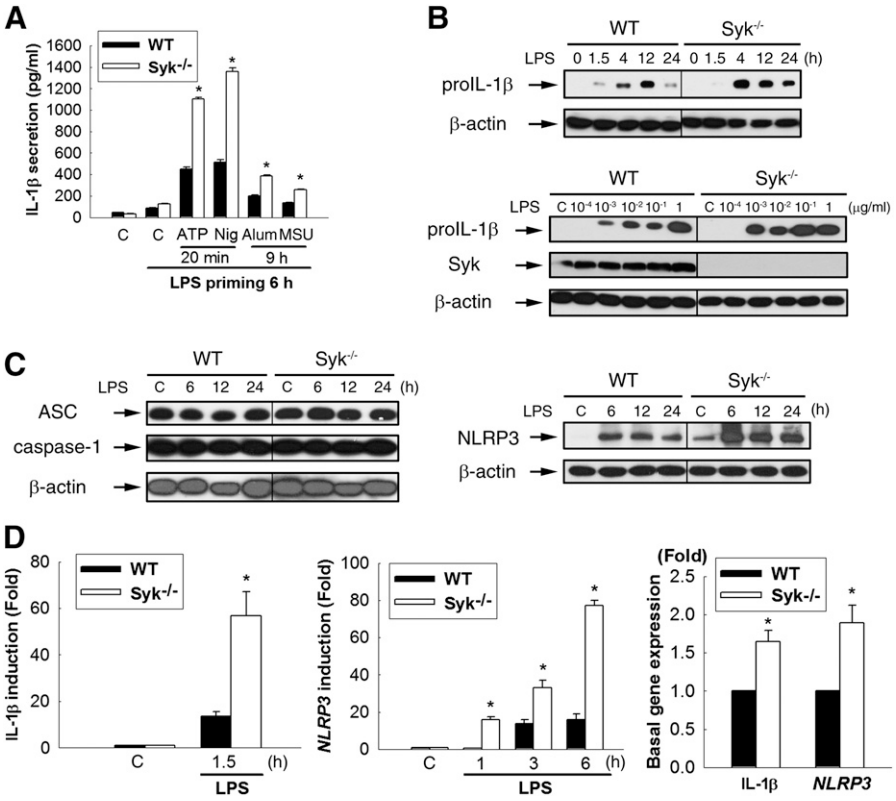

Figure 7. Syk negatively regulates gene expressions of NLRP3 and pro-IL-1β. (A) WT and Syk$^{-/-}$ macrophages priming with LPS (1 μg/ml) for 6 h were stimulated with ATP (5 mM) or nigericin (10 μM) for 20 min, Alum adjuvant (150 μg/ml), or MSU (100 μg/ml) for 9 h. After incubation, the media were collected to determine the concentrations of IL-1β. (B) WT and Syk$^{-/-}$ macrophages were treated with LPS (1 μg/ml) for the time indicated (upper) or were treated with the indicated concentrations of LPS for 24 h (lower). After treatment, whole-cell lysates were analyzed by immunoblotting with antibodies specific for pro-IL-1β and Syk. (C and D) After treating LPS (1 μg/ml) for indicated time in WT and Syk$^{-/-}$ macrophages, whole-cell lysates were collected for immunoblotting (C), and the total RNA was extracted and analyzed by real-time quantitative PCR to determine the induction of the indicated mRNA expression (D). (D) Gene expressions of IL-1β (left) and NLRP3 (middle) were normalized to β-actin mRNA and expressed relative to those of untreated control. We also compared the basal gene expressions of NLRP3 and IL-1β under an unstimulated condition in WT and Syk$^{-/-}$ macrophages (right). (A and D) Data are means ± SEM from 3 independent experiments. *$P < 0.05$ when comparing WT and Syk$^{-/-}$ macrophages.

While preparing this manuscript, Hara et al. [28] and He et al. [31] reported related findings about the role of Syk in NLRP3 inflammasome. As with our data, Hara et al. [28] also observed less caspase-1 activity in Syk$^{-/-}$ peritoneal macrophages. In their case, pro-IL-18 processing was used as the readout. Moreover, the phosphorylation of mouse ASC through Syk and JNK was found crucial for the formation of ASC oligomerization and caspase-1 activation under nigericin and poly(deoxyadenylic:deoxythymidylic) acid stimulation. In addition, the Tyr144 of mouse ASC is suggested to be a putative phosphorylation site for Syk. However, in that study, the biochemical phosphorylation and modification-associated functions were not characterized. Unlike Hara and coworkers' [28] and our data, the study from He et al. [31] excluded the role of Syk in the Step 2 signal (caspase-1 activation) for regulating NLRP3 inflammasomes in BMDMs. The reason for such discrepancy might be a result of the different animal strain use, as they used 129S mice, and we use C57BL/6.

Syk has numerous crucial biologic functions in many cell types and plays a key role in many upstream kinase signaling cascades (e.g., BCR, FcγR, CLRs, TLRs, and IL-1R) that are related to innate and adaptive immunity [11, 22, 25, 26]. Our study highlights the crucial regulatory role of Syk in promoting the NLRP3 inflammasome assembly and subsequent caspase-1 activation in macrophages. Numerous diseases are mediated by excessive caspase-1 activation and overproduction of IL-1β [17]. Thus, many pharmaceutical companies are studying drugs to target caspase-1, IL-1β, and IL-1R. The findings of this study provide evidence that Syk is a novel target for developing drugs to reduce inflammation-related diseases.

AUTHORSHIP

Y.-C.L. designed and performed the experiments, analyzed the data, and prepared the manuscript. D.-Y.H. performed the experiments and generated constructs. J.-S.W. generated constructs. Y.-L.L. and S-L.H. helped in discussion. K.-C. H. helped in discussion and prepared the manuscript. W.-W.L. designed the experiments and prepared the manuscript.

ACKNOWLEDGMENTS

The authors thank Dr. John Reed (Sanford-Burnham Institute for Medical Research, La Jolla, CA, USA) for providing the expression plasmids of hASC, human procaspase-1, hNLRP3, and mouse Flag-tagged pro-IL-1β. This work was supported by grants from the National Science Council, Taiwan (NSC 102-2325-B-002-033 and NSC 101-2314-B-002-121-MY3); Institute of Biomedical Science, Academia Sinica, Taiwan (IBMS-CRC99-P04); National Health Research Institute, Taiwan (NHRI-EX101-10052SI); and Taipei Medical University (TMU100-AE1-B24).

DISCLOSURES

The authors declare no financial or commercial conflict of interest.

REFERENCES

1. Yu, M., Levine, S. J. (2011) Toll-like receptor, RIG-I-like receptors and the NLRP3 inflammasome: key modulators of innate immune responses to double-stranded RNA viruses. *Cytokine Growth Factor Rev.* **22,** 63–72.
2. Jin, C., Flavell, R. A. (2010) Molecular mechanism of NLRP3 inflammasome activation. *J. Clin. Immunol.* **30,** 628–631.

3. Eisenbarth, S. C., Colegio, O. R., O'Connor, W., Sutterwala, F. S., Flavell, R. A. (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* **453,** 1122–1126.
4. Goldmann, T., Tay, T. L., Prinz, M. (2013) Love and death: microglia, NLRP3 and the Alzheimer's brain. *Cell Res.* **23,** 595–596.
5. Mariathasan, S., Weiss, D. S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W. P., Weinrauch, Y., Monack, D. M., Dixit, V. M. (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440,** 228–232.
6. Martinon, F., Pétrilli, V., Mayor, A., Tardivel, A., Tschopp, J. (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440,** 237–241.
7. Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T., Brickey, W. J., Ting, J. P. (2011) Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* **12,** 408–415.
8. Martinon, F., Burns, K., Tschopp, J. (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* **10,** 417–426.
9. Davis, B. K., Wen, H., Ting, J. P. (2011) The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu. Rev. Immunol.* **29,** 707–735.
10. Huang, W. C., Hung, M. C. (2013) Beyond NF-κB activation: nuclear functions of IκB kinase α. *J. Biomed. Sci.* **20,** 3.
11. Yamada, T., Fujieda, S., Yanagi, S., Yamamura, H., Inatome, R., Yamamoto, H., Igawa, H., Saito, H. (2001) IL-1 induced chemokine production through the association of Syk with TNF receptor-associated factor-6 in nasal fibroblast lines. *J. Immunol.* **167,** 283–288.
12. Bauernfeind, F., Ablasser, A., Bartok, E., Kim, S., Schmid-Burgk, J., Cavlar, T., Hornung, V. (2011) Inflammasomes: current understanding and open questions. *Cell. Mol. Life Sci.* **68,** 765–783.
13. Ouyang, X., Ghani, A., Mehal, W. Z. (2013) Inflammasome biology in fibrogenesis. *Biochim. Biophys. Acta* **1832,** 979–988.
14. Van de Veerdonk, F. L., Netea, M. G., Dinarello, C. A., Joosten, L. A. (2011) Inflammasome activation and IL-1β and IL-18 processing during infection. *Trends Immunol.* **32,** 110–116.
15. Proell, M., Gerlic, M., Mace, P. D., Reed, J. C., Riedl, S. J. (2013) The CARD plays a critical role in ASC foci formation and inflammasome signalling. *Biochem. J.* **449,** 613–621.
16. Shio, M. T., Eisenbarth, S. C., Savaria, M., Vinet, A. F., Bellemare, M. J., Harder, K. W., Sutterwala, F. S., Bohle, D. S., Descoteaux, A., Flavell, R. A., Olivier, M. (2009) Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. *PLoS Pathog.* **5,** e1000559.
17. Strowig, T., Henao-Mejia, J., Elinav, E., Flavell, R. (2012) Inflammasomes in health and disease. *Nature* **481,** 278–286.
18. Mariathasan, S., Monack, D. M. (2007) Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat. Rev. Immunol.* **7,** 31–40.
19. Mariathasan, S., Newton, K., Monack, D. M., Vucic, D., French, D. M., Lee, W. P., Roose-Girma, M., Erickson, S., Dixit, V. M. (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* **430,** 213–218.
20. Guarda, G., Zenger, M., Yazdi, A. S., Schroder, K., Ferrero, I., Menu, P., Tardivel, A., Mattmann, C., Tschopp, J. (2011) Differential expression of NLRP3 among hematopoietic cells. *J. Immunol.* **186,** 2529–2534.
21. Mócsai, A., Ruland, J., Tybulewicz, V. L. (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* **10,** 387–402.
22. Turner, M., Schweighoffer, E., Colucci, F., Di Santo, J. P., Tybulewicz, V. L. (2000) Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunol. Today* **21,** 148–154.
23. Lee, H. M., Yuk, J. M., Kim, K. H., Jang, J., Kang, G., Park, J. B., Son, J. W., Jo, E. K. (2012) *Mycobacterium abscessus* activates the NLRP3 inflammasome via Dectin-1-Syk and p62/SQSTM1. *Immunol. Cell Biol.* **90,** 601–610.
24. Lin, Y. C., Huang, D. Y., Chu, C. L., Lin, W. W. (2010) Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of Toll-like receptors-mediated JNK signaling pathway. *Mol. Immunol.* **47,** 1569–1578.
25. Lin, Y. C., Huang, D. Y., Chu, C. L., Lin, Y. L., Lin, W. W. (2013) The tyrosine kinase Syk differentially regulates Toll-like receptor signaling downstream of the adaptor molecules TRAF6 and TRAF3. *Sci. Signal.* **6,** ra71.
26. Miller, Y. I., Choi, S. H., Wiesner, P., Bae, Y. S. (2012) The SYK side of TLR4: signalling mechanisms in response to LPS and minimally oxidized LDL. *Br. J. Pharmacol.* **167,** 990–999.
27. Poeck, H., Ruland, J. (2010) SYK kinase signaling and the NLRP3 inflammasome in antifungal immunity. *J. Mol. Med.* **88,** 745–752.
28. Hara, H., Tsuchiya, K., Kawamura, I., Fang, R., Hernandez-Cuellar, E., Shen, Y., Mizuguchi, J., Schweighoffer, E., Tybulewicz, V., Mitsuyama, M. (2013) Phosphorylation of the adaptor ASC acts as a molecular switch that controls the formation of speck-like aggregates and inflammasome activity. *Nat. Immunol.* **14,** 1247–1255.
29. Gross, O., Poeck, H., Bscheider, M., Dostert, C., Hannesschläger, N., Endres, S., Hartmann, G., Tardivel, A., Schweighoffer, E., Tybulewicz, V., Mocsai, A., Tschopp, J., Ruland, J. (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* **459,** 433–436.
30. Desaulniers, P., Fernandes, M., Gilbert, C., Bourgoin, S. G., Naccache, P. H. (2001) Crystal-induced neutrophil activation. VII. Involvement of Syk in the responses to monosodium urate crystals. *J. Leukoc. Biol.* **70,** 659–668.
31. He, Y., Varadarajan, S., Muñoz-Planillo, R., Burberry, A., Nakamura, Y., Núñez, G. (2014) 3,4-Methylenedioxy-β-nitrostyrene inhibits NLRP3 inflammasome activation by blocking assembly of the inflammasome. *J. Biol. Chem.* **289,** 1142–1150.
32. Hamerman, J. A., Tchao, N. K., Lowell, C. A., Lanier, L. L. (2005) Enhanced Toll-like receptor responses in the absence of signaling adaptor DAP12. *Nat. Immunol.* **6,** 579–586.
33. Wu, N. L., Lee, T. A., Tsai, T. L., Lin, W. W. (2011) TRAIL-induced keratinocyte differentiation requires caspase activation and p63 expression. *J. Invest. Dermatol.* **131,** 874–883.
34. Liao, Y. H., Lin, Y. C., Tsao, S. T., Lin, Y. C., Yang, A. J., Huang, C. T., Huang, K. C., Lin, W. W. (2013) HMG-CoA reductase inhibitors activate caspase-1 in human monocytes depending on ATP release and P2X7 activation. *J. Leukoc. Biol.* **93,** 289–299.
35. Py, B. F., Kim, M. S., Vakifahmetoglu-Norberg, H., Yuan, J. (2013) Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. *Mol. Cell* **49,** 331–338.
36. Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B. G., Fitzgerald, K. A., Hornung, V., Latz, E. (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J. Immunol.* **183,** 787–791.
37. Lu, B., Nakamura, T., Inouye, K., Li, J., Tang, Y., Lundbäck, P., Valdes-Ferrer, S. I., Olofsson, P. S., Kalb, T., Roth, J., Zou, Y., Erlandsson-Harris, H., Yang, H., Ting, J. P., Wang, H., Andersson, U., Antoine, D. J., Chavan, S. S., Hotamisligil, G. S., Tracey, K. J. (2012) Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* **488,** 670–674.
38. Kankkunen, P., Teirilä, L., Rintahaka, J., Alenius, H., Wolff, H., Matikainen, S. (2010) (1,3)-beta-Glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J. Immunol.* **184,** 6335–6342.
39. Palomäki, J., Välimäki, E., Sund, J., Vippola, M., Clausen, P. A., Jensen, K. A., Savolainen, K., Matikainen, S., Alenius, H. (2011) Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism. *ACS Nano* **5,** 6861–6870.
40. Walsh, J. G., Logue, S. E., Lüthi, A. U., Martin, S. J. (2011) Caspase-1 promiscuity is counterbalanced by rapid inactivation of processed enzyme. *J. Biol. Chem.* **286,** 32513–32524.
41. Stehlik, C. (2009) Multiple interleukin-1beta-converting enzymes contribute to inflammatory arthritis. *Arthritis Rheum.* **60,** 3524–3530.
42. Tigno-Aranjuez, J. T., Abbott, D. W. (2012) Ubiquitination and phosphorylation in the regulation of NOD2 signaling and NOD2-mediated disease. *Biochim. Biophys. Acta* **1823,** 2022–2028.
43. Jiang, X., Chen, Z. J. (2012) The role of ubiquitylation in immune defence and pathogen evasion. *Nat. Rev. Immunol.* **12,** 35–48.
44. Malynn, B. A., Ma, A. (2010) Ubiquitin makes its mark on immune regulation. *Immunity* **33,** 843–852.
45. Juliana, C., Fernandes-Alnemri, T., Kang, S., Farias, A., Qin, F., Alnemri, E. S. (2012) Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J. Biol. Chem.* **287,** 36617–36622.
46. Rodgers, M. A., Bowman, J. W., Fujita, H., Orazio, N., Shi, M., Liang, Q., Amatya, R., Kelly, T. J., Iwai, K., Ting, J., Jung, J. U. (2014) The linear ubiquitin assembly complex (LUBAC) is essential for NLRP3 inflammasome activation. *J. Exp. Med.* **211,** 1333–1347.
47. Van Opdenbosch, N., Gurung, P., Vande Walle, L., Fossoul, A., Kanneganti, T. D., Lamkanfi, M. (2014) Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation. *Nat. Commun.* **5,** 3209.

KEY WORDS:
ATP · IL-1β · NLR · tyrosine kinase