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ORIGINAL ARTICLE

Suppression of Syk activation by resveratrol inhibits MSU crystal-induced inflammation in human monocytes

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Abstract

Monosodium urate (MSU) crystals are an endogenous sterile particulate that has been identified as a potent damage-associated molecular pattern (DAMP). In humans, the induction of IL-1β production through MSU-induced NLRP3 inflammasome activation in monocytes/macrophages is responsible for pathogenesis of gouty arthritis. It was recently reported that in a murine model of this disease, resveratrol decreases MSU-induced recurrent attacks of gouty arthritis. Despite its demonstrated anti-inflammatory effects, the mechanisms underlying resveratrol-mediated repression of IL-1β production in MSU-activated monocytes remain poorly understood. Here, we show that resveratrol suppresses secretion of active IL-1β by human primary monocytes stimulated with MSU crystals through suppression of Syk activation. Metabolic labeling and pull-down assays to investigate de novo protein synthesis clearly demonstrated that intracellular pro-IL-1β synthesis is rapidly repressed in monocytes after resveratrol treatment due to decreased phosphorylation of Syk and p38. Resveratrol also inhibited NLRP3 inflammasome activation in MSU-stimulated monocytes by suppressing oligomerization of ASC. Furthermore, resveratrol exerted a beneficial effect by reducing IL-1β production and inhibiting neutrophil recruitment in a mouse model of MSU-mediated peritonitis. Our findings suggest that resveratrol exerts anti-inflammatory effects via post-translational regulation of IL-1β production and, thus, may prove beneficial for the treatment of MSU crystal-mediated sterile inflammation.

Key message

- Resveratrol has negative effects on pro-IL-1β synthesis through Syk and p38.
- Resveratrol inhibits oligomerization of ASC.
- Resveratrol is beneficial in a mouse model of MSU-induced peritonitis.

Keywords MSU · Resveratrol · IL-1β · NLRP3 inflammasome · Syk · Gouty arthritis

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Introduction

Self-derived immunostimulatory molecules released by injured tissue or necrotic cells, called damage-associated molecular patterns (DAMPs), initiate sterile inflammation which is a protective biological function to ensure host survival [1, 2]. However, DAMP-mediated sterile inflammation also can cause secondary damage to normal tissues through actions of pro-inflammatory cytokines such as IL-1β. In fact, unchecked or prolonged sterile inflammation is implicated in pathogenesis of many inflammatory disorders such as gout, atherosclerosis, and Alzheimer's disease [1].

Sterile particulates including MSU and cholesterol crystals have been identified as potent DAMPs and the activation of the cytosolic NLRP3 inflammasome by these particulates induces robust inflammatory responses via IL-1β production. This excessive and unremitting inflammation causes damage to healthy tissue and underlies the pathogenesis of many crystal-based diseases [3]. In gout, dying host cells release excessive uric acid that combine with highly concentrated sodium to form MSU crystals [4]. It is the precipitation of these MSU crystals in and around joints that then causes inflammatory arthritis [5]. MSU crystals have the ability to induce the release of many cytokines and chemokines from monocytes/macrophages, resulting in recruitment of inflammatory immune cells. Among these inflammatory mediators, IL-1β plays a key role in gouty inflammation [6].

IL-1β is a potent pro-inflammatory cytokine mainly produced by monocytes/macrophages, and dysregulation of IL-1β production causes many systemic inflammatory diseases [7]. The production of bioactive IL-1β is tightly regulated by multiple distinct steps: synthesis of intracellular pro-IL-1β protein, proteolytic cleavage of pro-IL-1β into the active IL-1β by caspase-1, and release of active IL-1β into the extracellular environment [8]. Activation of caspase-1 requires the assembly and activation of the inflammasome, which is a multiprotein complex consisting of nod-like receptors (NLRs) along with apoptosis-associated speck-like protein containing CARD (ASC) and pro-caspase-1 [9]. Upon activation, oligomerized NLRs interact with ASC resulting in the formation of ASC oligomers, which provide a molecular platform for the recruitment of pro-caspase-1. Activation of caspase-1 through autoproteolytic maturation leads to cleavage of pro-IL-1β and secretion of active IL-1β [10]. A recent study showed that MSU-mediated IL-1β and IL-18 maturation and release is dependent on NLRP3, ASC, and caspase-1 [11].

Resveratrol, a naturally occurring polyphenolic phytoalexin, is found in various plants such as red grapes and their derivatives [12]. Resveratrol exerts a broad spectrum of beneficial effects on human health through its anti-oxidant, anti-inflammatory, anti-obesity/diabetic, and anti-carcinogenesis properties [13–15]. Considering that *inflammation* is a *common* denominator in virtually all *diseases*, special attention has been paid to understanding of the molecular mechanisms underlying the anti-inflammatory properties of resveratrol [16, 17]. NF-κB, MAPK, and mTOR have been identified as major signaling pathways, which are modulated by resveratrol in immune cells [18–20]. A recent study suggested that resveratrol inhibits MSU-mediated IL-1β production in a peritoneal mesothelial cell line and also possesses therapeutic potential to inhibit articular inflammation in an MSU-induced gouty arthritis mouse model [21]. However, the precise molecular mechanism by which resveratrol suppresses the acute inflammatory response to MSU crystals remains uncharacterized. Our recent study demonstrated that MSU crystals induce rapid pro-IL-β synthesis in a post-transcriptional manner and that this is an essential step for IL-1β production in human primary monocytes [22]. However, the effect of resveratrol on IL-1β produced by sterile particles such as MSU crystals in human monocytes has not yet been studied.

Here, we provide evidence that MSU-induced IL-1β production is suppressed by resveratrol in human primary monocytes. This suppression is attributed to inhibition of intracellular pro-IL-1β synthesis due to decreased phosphorylation of Syk and p38 MAPK and to reduced secretion of active IL-1β due to decreased NLRP3 inflammasome activation. Moreover, resveratrol exerts a beneficial effect by diminishing the recruitment of neutrophils and reducing IL-1β production in an MSU-mediated peritonitis mouse model. These findings underscore the anti-inflammatory activity of resveratrol, which may be beneficial in the treatment of MSU-mediated sterile inflammation.

Materials and methods

Antibodies and reagents

The following antibodies and reagents were used in present studies: anti-total and phosphorylated proteins such as mTOR, S6K, Syk, NF-κB, p38, 4E-BP1, Mnk1, and eIF4E antibodies, and anti-IL-1β, anti-ASC, anti-α-tubulin, anti-IRAK1, anti-caspase-1, anti-NLRP3, anti-biotin-HRP-conjugated, and anti-streptavidin-HRP antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin, dimethyl sulfoxide, resveratrol, rapamycin, PD98059, SB202190, ATP, and nigericin were purchased from Sigma-Aldrich (St. Louis, MO). Torin 1 was purchased from Merck Millipore (Billerica, MA). R406, LPS, and MSU crystal were purchased from InvivoGen (San Diego, CA). Human M-CSF was purchased from R&D system (Minneapolis, MN).

Cell preparation and culture

The study protocols were reviewed and approved by the IRB of Seoul National University Hospital. Peripheral blood of healthy volunteers was drawn after obtaining the written informed consent. PBMCs were isolated by density gradient centrifugation (Bicoll separating solution; BIOCHROM Inc., Cambridge, UK). Monocytes were positively separated with anti-CD14 magnetic beads (Miltenyi Biotec Inc., Auburn, CA). The cells were pre-incubated with the indicated concentrations of resveratrol or the various kinase inhibitors for 30 min and stimulated with 400 μg/ml MSU for the indicated time. MSU crystals were prepared for in vivo experiments by recrystallization from uric acid as previously described [23]. Endotoxin levels in MSU crystal preparations were less than 0.05 EU/ml. In some experiments, monocytes were differentiated into monocyte-derived macrophages (MDMs) with 50 ng/ml rhM-CSF for 6 days. LPS-treated MDMs were pre-incubated for 30 min with resveratrol, followed by stimulation with MSU crystals for another 4 h.

Detection of newly synthesized global proteins and pro-IL-1β

Newly synthesized proteins were detected using the Click-iT method (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and our previous report [22]. In brief, monocytes were incubated in methionine-free RPMI 1640 for 60 min before labeling with azide-containing methionine analog AHA (L-azidohomoalaine), followed by stimulation with MSU crystal with or without resveratrol or inhibitors. After incubation, whole cell lysate was prepared and the newly synthesized AHA-incorporated proteins were cross-linked to alkyne-derivatized biotin by Click-iT protein reaction buffer kit. Biotin-cross-linked proteins were mixed with streptavidin-agarose bead slurry (Pierce, Rockfod, IL) in a spin column and incubated at RT for 30 min. After spin-down and washing, matrix-bound proteins were eluted into 2× Laemmli buffer by boiling. Total protein inputs and affinity-purified fractions were immunoblotted with streptavidin-HRP for newly synthesized proteins and anti-IL-1β antibody for pro-IL-1β.

Immunoblot analysis

Total cellular proteins were isolated from monocytes or MDMs using lysis buffer (1% SDS, 100 mM Tris, pH 8.0) containing a protease and phosphatase inhibitor cocktail (Pierce, Rockfod, IL). Methanol and chloroform precipitation were used for protein concentration from cultured medium. Total cell lysates and precipitated supernatants were subjected to 7 to 12% SDS-PAGE and immunoblot analysis. Primary antibodies were incubated overnight at 4 °C and incubated with HRP-conjugated rabbit or mouse anti-IgG (Cell Signaling Technology) for 1 h at room temperature. Protein signals were detected by enhanced chemiluminescence (Merck Millipore).

Cytokine arrays

The levels of 40 cytokines in the culture supernatants were analyzed using Human Inflammation Array C3 according to the manufacturer's protocol (RayBiotech, Norcross, GA). The signal intensity for each cytokine-specific antibody spot was detected by chemiluminescence (Millipore) and dot densities were quantified by densitometry. After subtracting the background (negative control signal), mean values of the spotted duplicates were calculated and normalized against mean values of internal positive controls.

ASC oligomerization assay

Monocytes or MDMs were lysed with TBS buffer [50 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 0.5% Triton X-100, protease, and phosphatase inhibitor mixture (Pierce). The lysates were centrifuged at 6000×*g* for 15 min at 4 °C, and the pellets and supernatants were used as the Triton-insoluble fractions and Triton-soluble fractions, respectively. To detect ASC oligomerization, the Triton-insoluble pellets were washed twice with TBS buffer and resuspended in 300 μl PBS buffer. The pellets were cross-linked using 1 mM disuccinimidyl suberate (Pierce) for 30 min at 37 °C, followed by pelleting by centrifugation at 6000×*g* for 15 min. The cross-linked pellets were dissolved in 2× SDS sample buffer, separated using 12% SDS-PAGE and immunoblotted.

Lactate dehydrogenase assay

The release of lactate dehydrogenase into the culture medium was determined by the colorimetric LDH cytotoxicity assay kit according to the manufacturer's instructions (Pierce).

Enzyme-linked immunosorbent assay

The amount of cytokines in culture supernatant was quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (All from eBioscience, San Diego, CA). The measurement of OD (optical density) was performed using the Infinite 200 Pro Multimode microplate reader (Tecan, Seestrasse, Switzerland).

MSU-induced peritonitis

Female C57BL/6 mice (KOATECH, Pyeongtaek, Gyeonggi, South Korea) aged 8 to 12 weeks were used for the experiments. Mice were intraperitoneally administered with resveratrol (25 mg/kg) or vehicle alone. After 30 min, mice were

intraperitoneally injected with 2.5 mg/kg MSU crystals in 400 μl sterile PBS. After 3 h, mice were euthanized by CO_2 administration and peritoneal exudate cells were harvested by lavage with 2 ml PBS. Supernatants and cells were harvested from the lavage fluid and analyzed by ELISA and flow cytometry, respectively. The peritoneal cells were stained for 30 min at 4 °C with antibodies to CD11b, and Ly6G (BD Bioscience, San Jose, CA). Stained cells were acquired by a BD LSRFortessa™ X-20 (BD Bioscience).

Statistical analysis

Two-tailed paired *t* test or unpaired *t* test was done to analyze data using Prism 5 software (GraphPad Software Inc. La Jolla, CA). *p* values of less than 0.05 were considered statistically significant.

Results

Resveratrol suppresses pro-IL-1β production and active IL-1β secretion by human monocytes in response to MSU stimulation

Resveratrol has pleiotropic effects on immune responses [24, 25]. To investigate its effect on the MSU-induced inflammatory response of human monocytes, we screened culture media harvested from MSU-stimulated monocytes using a human cytokine array. Compared to PBS-treated controls, the levels of major inflammatory cytokines such as IL-1β, MIP-1α, TNF-α, and IL-6 were rapidly and predominantly upregulated in MSU-treated human monocytes (bold line boxes in Fig. 1b left and middle). This upregulation was almost completely inhibited by resveratrol (bold line boxes in Fig. 1b middle and right). These findings were also confirmed by ELISA (Fig. 1c, Suppl. Fig. 1). In contrast, IP-10 and TNF-β were not induced by MSU crystals, but were upregulated following treatment with resveratrol (dotted line boxes in Fig. 1b). These findings suggest that resveratrol suppresses the MSU-induced production of pro-inflammatory cytokines in monocytes.

IL-1β has been identified as the major effector cytokine of various crystal-induced inflammatory diseases. Considering resveratrol-mediated downregulation of IL-1β secretion by MSU-activated monocytes in our screening approach (Fig. 1b), we sought to identify the underlying mechanism. Resveratrol significantly diminished the secretion of IL-1β by MSU-activated monocytes in a dose-dependent manner (Fig. 1c). This inhibitory effect was observed as early as 3 h post-stimulation and was maintained at later time points (Fig. 1d). Recently, we reported that MSU crystals induce rapid synthesis of pro-IL-1β via post-transcriptional regulation, which is an essential step for IL-1β production in human monocytes [22]. Therefore, we next examined whether resveratrol interferes with MSU-induced production of pro-IL-1β at an early time point. Consistent with our previous report [22], production of pro-IL-1β protein in primary human monocytes was markedly increased as soon as 1 h post-stimulation with MSU (second lane of Fig. 1e) compared to the control (first lane of Fig. 1e). This rapid production of pro-IL-1β was significantly reduced by resveratrol in a dose-dependent manner (Fig. 1e, f). As a result, the amount of IL-1β secreted into the culture medium for 1 h decreased in a dose-dependent manner (Fig. 1g). The ability to enhance synthesis of pro-IL-1β via translational regulation is not limited to MSU crystals. Thus, we assessed the ability of resveratrol to inhibit induction of pro-IL-1β downstream of calcium pyrophosphate dihydrate (CPPD) stimulation, another type of endogenous crystal involved in the pathogenesis of pseudogout, and obtained a similar result. In contrast to endogenous particulates such as MSU and CPPD crystals, resveratrol had no effect on the LPS-induced production of pro-IL-1β or IL-1β secretion (Fig. 1h, i). Since LPS is not a potent NLRP3 inflammasome activator requiring cleavage of pro-IL-1β, the level of secreted IL-1β in the culture supernatant did not increase as much as in the MSU or CPPD treatment groups even though LPS markedly induced the production of intracellular pro-IL-1β (Fig. 1h, i). These results suggest that resveratrol suppresses the induction of IL-1β secretion by endogenous sterile particulates by inhibiting pro-IL-1β production in human monocytes.

Resveratrol inhibits rapid synthesis of nascent global proteins and pro-IL-1β induced by MSU in human monocytes

It has been demonstrated that resveratrol inhibits protein translation in hepatic cells and ameliorates high glucose-induced protein synthesis in glomerular epithelial cells [26, 27]. Given the critical role of translational regulation in pro-IL-1β production by MSU-stimulated monocytes [22], we next investigated whether resveratrol directly represses early protein synthesis in MSU-stimulated monocytes. To this end, metabolic labeling and a pull-down assay were applied to analyze newly synthesized global and pro-IL-1β proteins after MSU stimulation. Coomassie blue staining revealed that the amount of total proteins was comparable irrespective of resveratrol or PBS treatment (Fig. 2a). However, MSU-induced rapid incorporation of the methionine analog was significantly decreased in a dose-dependent manner by resveratrol treatment of monocytes (Fig. 2b, c), indicating a direct repression of global protein induction by resveratrol. To more specifically examine the effect of resveratrol on pro-IL-1β synthesis, streptavidin-agarose was utilized to pull down biotin-conjugated proteins and the production of pro-IL-1β was evaluated by immunoblotting. As shown in Fig. 2d, e, 10 μM resveratrol repressed MSU-induced synthesis of pro-IL-1β. These data suggest that resveratrol suppresses MSU-induced pro-IL-1β production via inhibition of protein synthesis in human monocytes.

A

	a	b	c	d	e	f	g	h	i	j	k	l
1 2	POS	POS	NEG	NEG	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	GCSF	GM-CSF	ICAM-1 (CD54)	IFN-gamma	I-309 (CCL1)	IL-1 alpha
3 4	IL-1 beta	IL-2	IL-3	IL-4	IL-6	IL-6R	IL-7	IL-8 (CXCL8)	IL-10	IL-11	IL-12 p40	IL-12 p70
5 6	IL-13	IL-15	IL-16	IL-17A	IP-10 (CXCL10)	MCP-1 (CXCL2)	MCP-2 (CXCL8)	M-CSF	MIG (CXCL9)	MIP-1 alpha	MIP-1 beta	MIP-1 delta
7 8	RANTES (CCL5)	TGF beta1	TNF alpha	TNF beta	TNF RI	TNF RII	PDGF-BB	TIMP-2	BLANK	BLANK	NEG	POS

POS: positive conrol spot
NEG: negative conrol spot

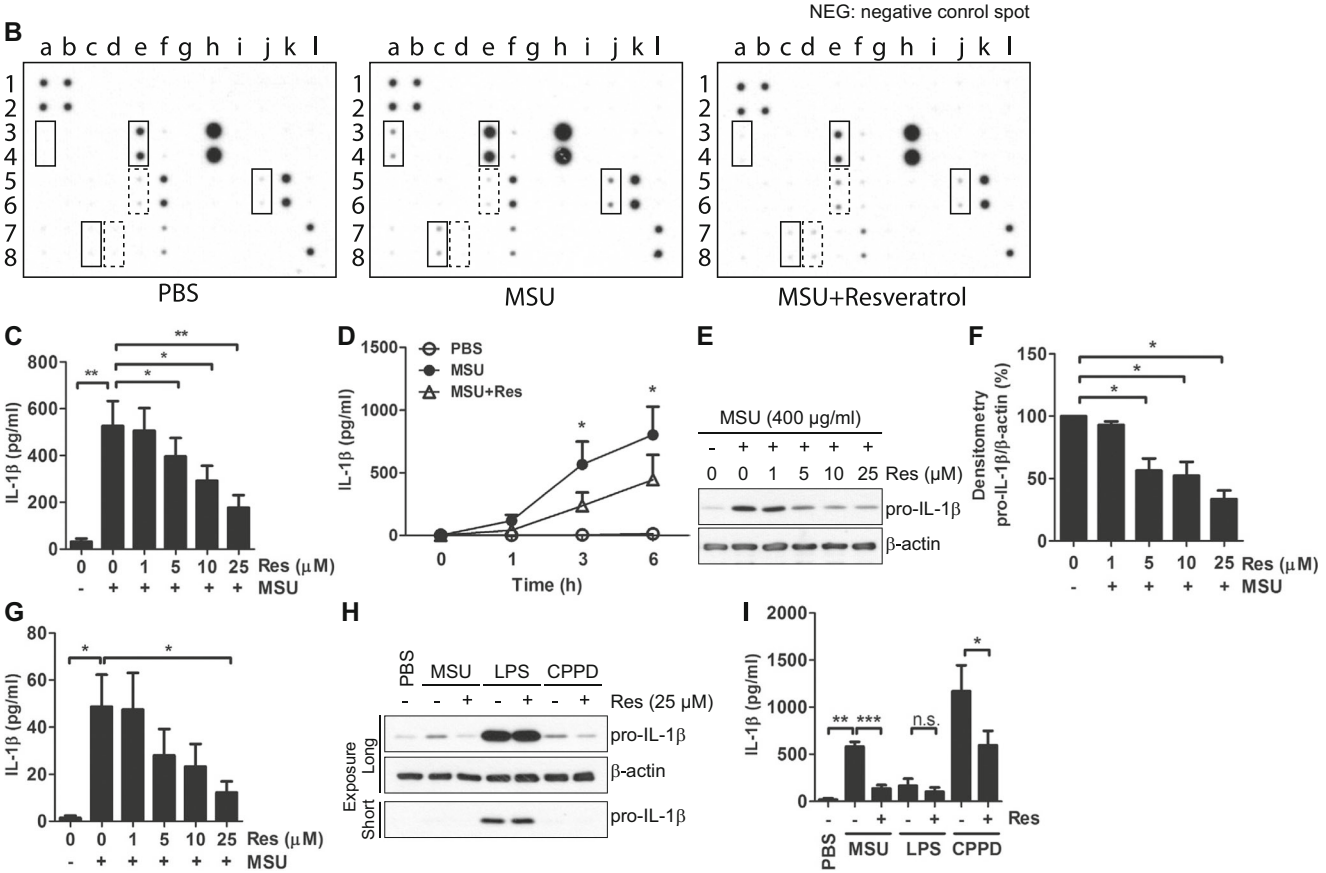

Fig. 1 Resveratrol suppresses MSU-induced production of pro-IL-1β as well as IL-1β secretion in monocytes. **a** Layout of the human cytokine array. **b** Supernatants from purified human monocytes pre-incubated with or without resveratrol (25 μM) for 30 min, followed by stimulation with MSU crystals (400 μg/ml) for another 4 h, were subjected to analysis by cytokine array. The data is representative of two independent experiments with two different donors. The expression of soluble molecules in the bold line boxes is upregulated by MSU treatment and inhibited by resveratrol, whereas molecules in the dotted line boxes are increased by resveratrol treatment. **c** Monocytes were pre-incubated for 30 min with various concentrations of resveratrol and were then treated with MSU for 4 h. The amount of IL-1β in culture supernatants was assessed by ELISA. **d** IL-1β was assessed in culture supernatants of MSU (400 μg/ml)-stimulated monocytes pretreated with resveratrol (25 μg/ml) for the indicated time periods. **e** Intracellular pro-IL-1β protein in cell lysate was analyzed at 1 h post-stimulation. **f** The inhibitory effect of resveratrol on pro-IL-1β production in cell lysate is presented as the fold change compared with the MSU (400 μg/ml) treatment group (second lane of **e**). **g** The amount of IL-1β in the culture supernatants in **e**. **h** Monocytes were stimulated for 4 h with MSU crystals (400 μg/ml), LPS (50 ng/ml), or CPPD crystals (100 μg/ml) with or without resveratrol (25 μM). **i** The amount of IL-1β in the culture supernatants in **h**. The immunoblots are representative of two or three independent experiments with two or three different donors. The graph presents the mean ± SEM of three independent experiments with three different donors. NS not significant. $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$ by paired *t* test

Resveratrol suppresses MSU-induced pro-IL-1β protein synthesis via inhibition of the p38 MAPK signaling pathway

Our previous study revealed that activation of p38 and mTOR pathways in MSU-stimulated monocytes is responsible for rapid synthesis of pro-IL-1β and global proteins, respectively [22]. Thus, to explore the molecular mechanism by which resveratrol suppresses pro-IL-1β and global protein synthesis in MSU-stimulated monocytes, we examined the p38 and mTOR signaling pathways. As seen in Fig. 3a, resveratrol inhibits MSU-

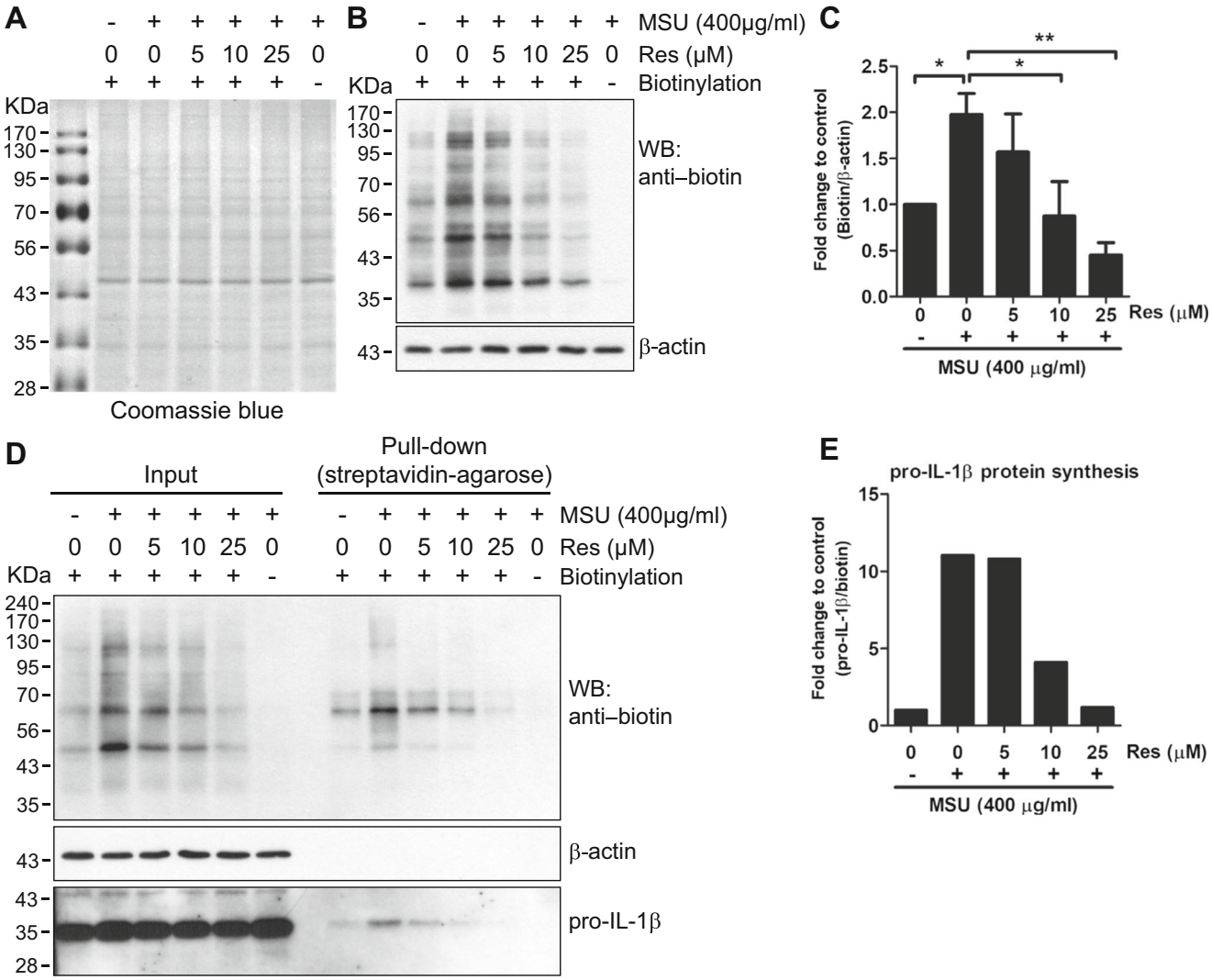

Fig. 2 Resveratrol inhibits MSU-induced pro-IL-1β and global protein synthesis in monocytes. Monocytes were pre-incubated for 1 h in methionine-free RPMI medium and then treated with resveratrol. After 30 min, the cells were metabolically labeled with the azide-containing methionine analog AHA, followed by stimulation with MSU crystals for 1 h. After incubation, newly synthesized proteins were detected using the Click-iT method as described in the "Materials and methods." **a** Total protein amount was determined by Coomassie blue staining. **b** Newly synthesized proteins containing biotin-conjugated AHA were detected with streptavidin-HRP. **c** The effect of resveratrol on the synthesis of nascent global proteins is presented as the fold change compared with vehicle control (first lane). The protein levels were normalized to β-actin. Data is representative of three independent experiments with three different donors (**a**–**c**). **d** Biotin-conjugated newly synthesized proteins (Input) were collected using streptavidin-agarose (Pull-down) and the indicated proteins were immunoblotted with streptavidin-HRP (upper) or anti-IL-1β antibody (lower). **e** The effect of resveratrol on the synthesis of pro-IL-1β is presented as the fold change compared with vehicle control (first lane). The protein levels were normalized to total biotinylated protein (pull-down). Data is representative of two independent experiments with two different donors (**d**, **e**). $*p < 0.05$ and $**p < 0.01$ by paired *t* test

induced sequential phosphorylation of p38/Mnk1/eIF4E as well as the mTOR/S6K/4E-BP1 signaling cascade. These results indicate that the inhibitory effect of resveratrol on MSU-induced pro-IL-1β and global protein synthesis is mediated by repression of phosphorylation of p38 MAPK and mTOR, respectively. In spite of obvious MSU-mediated signaling activation, WB analysis also showed an increased phosphorylation signal in control PBS conditions (Fig. 3a). Human monocytes are highly sensitive to many environmental stimuli such as TLR agonists, DAMP, and even attachment. It was reported that adhesion of human monocytes to culture dish as well as extracellular matrix component such as fibronectin and collagen induced cell activation, representing the synthesis of large amounts of IL-1β mRNA synthesis [22, 28]. Although we minimized attachment to its surface, we could not completely inhibit attachment of monocytes to the bottom of tube during the incubation but markedly reduced. However, it should be noted that MSU stimulation significantly upregulates phosphorylation signals compared with PBS control (Fig. 3a). To better understand the inhibitory effect of resveratrol on protein

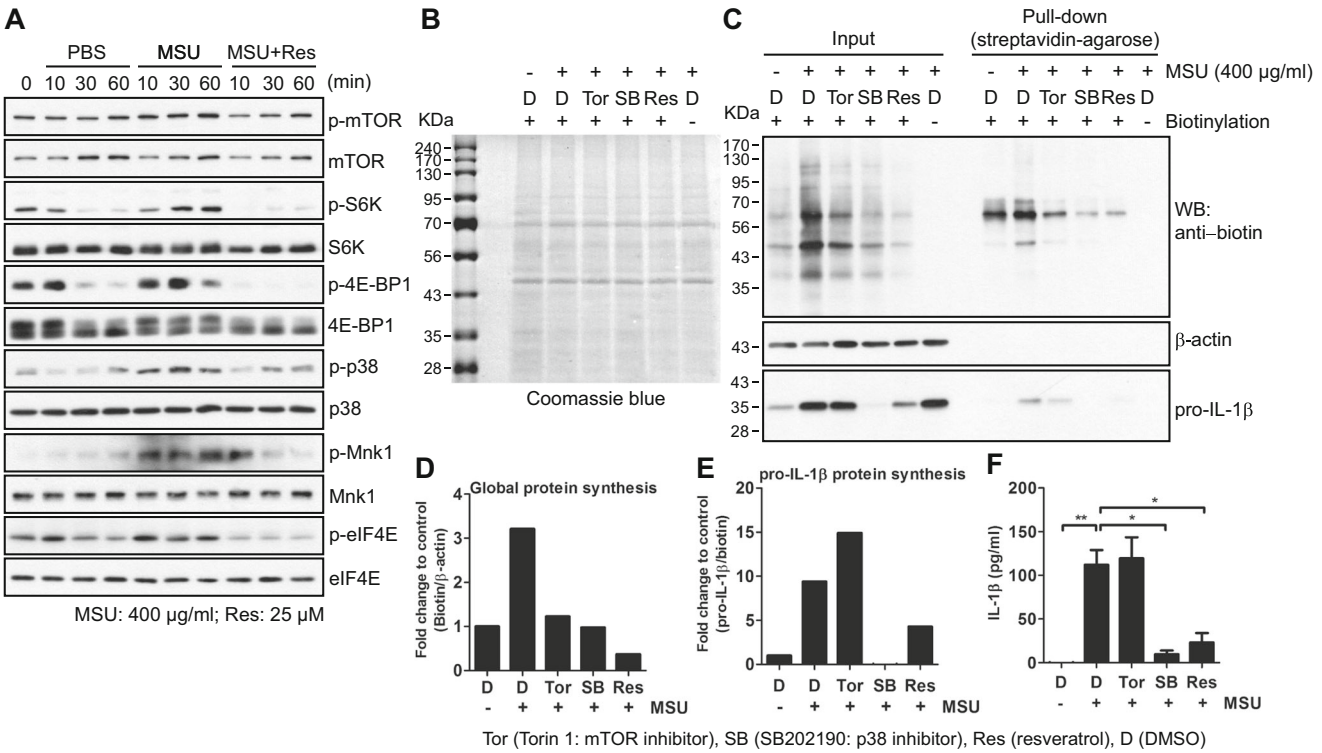

Fig. 3 Resveratrol suppresses MSU-induced global protein expression and pro-IL-1β through inhibition of mTOR and the p38 MAPK signaling pathway in monocytes. **a** Phosphorylation of the indicated signaling molecules in monocytes was analyzed by immunoblotting. **b**, **c** Monocytes were pre-treated for 30 min with Torin 1 (Tor, 50 nM), SB20210 (SB, 5 μM), resveratrol (Res, 25 μM), or DMSO (D), followed by stimulation with MSU crystals for 60 min. **b** Coomassie blue staining. **c** Newly synthesized total proteins and pro-IL-1β were immunoblotted with streptavidin-HRP (upper) or anti-IL-1β antibody (lower) as described in Fig. 2d. **d**, **e** The immunoblots were quantified by densitometry to obtain the fold change in nascent global protein synthesis (**d**) and pro-IL-1β protein synthesis (**e**) compared with vehicle control (first lane). Global protein and pro-IL-1β protein expression were normalized to β-actin (Input) and total biotinylated protein (Pull-down), respectively. Data is representative of two independent experiments with two different donors. **f** The amount of IL-1β in the culture supernatants from **b** were quantified by ELISA ($n = 3$). *$p < 0.05$ and **$p < 0.01$ by paired t test

synthesis, the effects of resveratrol were compared with those of mTOR and p38 MAPK inhibitors on MSU-induced global and pro-IL-1β protein synthesis using Click-iT analysis. The levels of total proteins were similar among different treatment groups (Fig. 3b). MSU-induced global protein synthesis was almost completely reduced by Torin 1 (Tor: mTOR inhibitor), SB202190 (SB: p38 MAPK inhibitor), and resveratrol, whereas pro-IL-1β synthesis was reduced by SB202190 and resveratrol (Fig. 3)c–e. Although Torin 1 appeared to reduce slightly the synthesis of pro-IL-1β protein (Fig. 3c), this is probably due to markedly reduced global protein synthesis. Therefore, pro-IL-1β in Torin 1-treated samples seems to be somewhat increased among the newly synthesized proteins (Fig. 3e). Torin 1 is a more potent inhibitor of global protein synthesis than rapamycin, an mTORC1 inhibitor, in MSU crystal-stimulated human monocytes (data not shown). Moreover, MSU-induced IL-1β secretion was also inhibited by SB202190 and resveratrol, but not by Torin 1 (Fig. 3f), paralleling the results seen for pro-IL-1β synthesis. These results indicate that reduced p38 MAPK activation is associated with resveratrol-mediated inhibition of MSU-induced pro-IL-1β synthesis in monocytes.

Syk mediates the suppression of MSU-induced pro-IL-1β synthesis by resveratrol in human monocytes

The finding that both mTOR and p38 MAPK activities are suppressed by resveratrol suggests that an upstream regulator of these two signaling pathways might mediate these effects. Several mechanisms by which cells recognize MSU have been suggested including through binding to cell-surface receptors in macrophages, such as CD14, TLR2, and TLR4, followed by initiation of inflammatory signaling [29]. More recent studies have shown that MSU can bind to plasma membrane lipids in dendritic cells (DCs) resulting in TLR-independent Syk activation [30]. To identify the upstream signaling molecule that regulates both mTOR and p38 MAPK activities in MSU-stimulated human monocytes, we first investigated TLR4-mediated signaling events in response to MSU crystals. An immunoblotting assay demonstrated that LPS induces an ubiquitin-dependent band shift of interleukin-1 receptor-associated kinase 1 (IRAK1) and phosphorylation of p65, but fails to induce phosphorylation of Syk to the same degree as MSU crystal stimulation [31] (Fig. 4a).

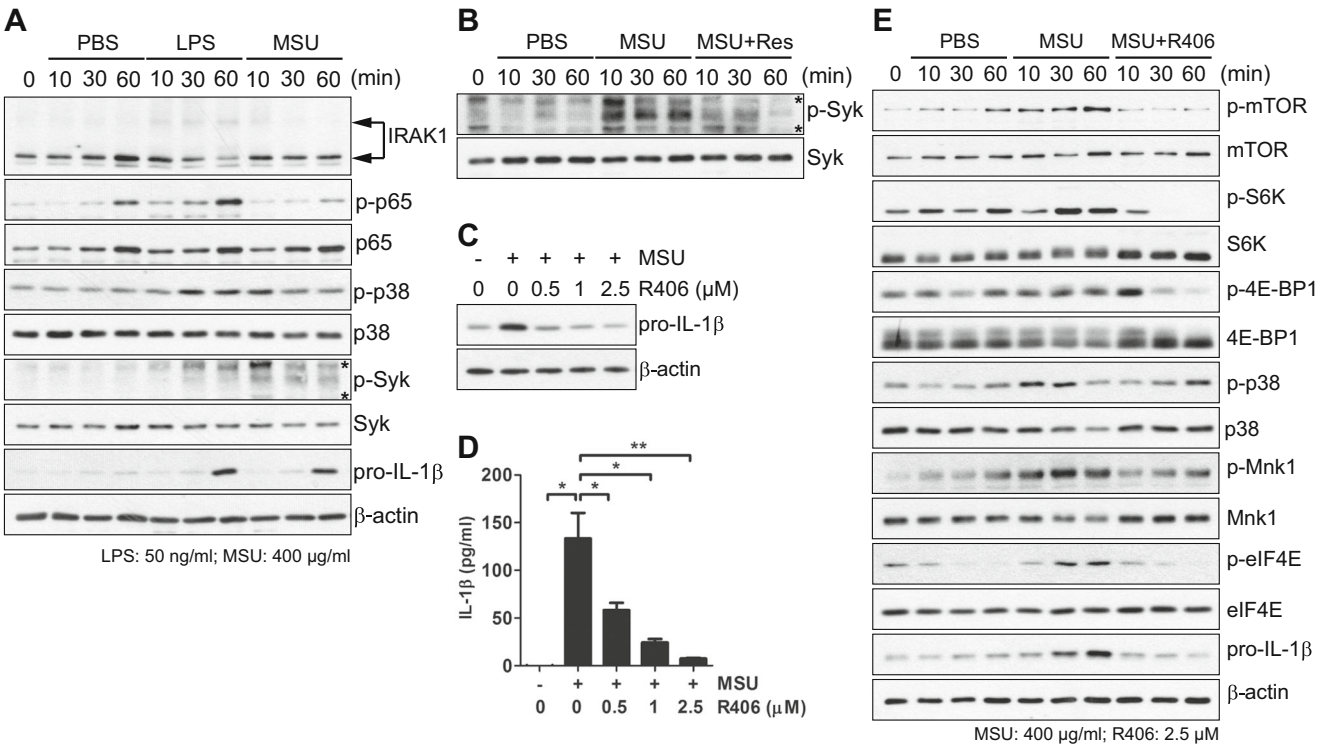

Fig. 4 Resveratrol suppresses MSU-mediated Syk activity, which is required for activation of mTOR and p38 signaling pathways in activated monocytes. **a** Monocytes were stimulated with LPS (50 ng/ml), MSU crystals (400 μg/ml), or PBS. Immunoblotting was performed for the indicated signaling proteins. **b** Phosphorylation of Syk was assessed by immunoblotting. Data is representative of two independent experiments with two different donors (**a**, **b**). **c** Monocytes were stimulated with MSU crystals with the indicated concentrations of Syk inhibitor (R406) for 1 h. Cell lysates were immunoblotted for pro-IL-1β. Data is representative of three independent experiments with three different donors. **d** The amount of IL-1β in the culture supernatants in **c** was quantified by ELISA ($n = 4$). $^{*}p < 0.05$ and $^{**}p < 0.01$ by paired t test. **e** Monocytes were stimulated with MSU crystals with or without 2.5 μM R406. Phosphorylation of the indicated signaling molecules was analyzed by immunoblotting. Data is representative of two independent experiments with two different donors. Asterisks indicate non-specific bands (**a**, **b**)

In contrast, MSU crystals induced phosphorylation of Syk but failed to activate IRAK1 or p65 in primary monocytes (Fig. 4a). As shown previously, both MSU crystals and LPS induced phosphorylation of p38 MAPK and pro-IL-1β production in human monocytes (Fig. 4a). These data suggest that MSU-mediated activation of monocytes is mainly associated with TLR-independent recognition of MSU. We next investigated whether resveratrol influences the phosphorylation of Syk upon MSU-crystal stimulation and found that phosphorylation of Syk was clearly suppressed by resveratrol in a time-dependent manner (Fig. 4b). To further examine the modulatory role of Syk in pro-IL-1β production in MSU-simulated monocytes, cells were pretreated with R406, a selective pharmacological inhibitor of Syk. The production of cytoplasmic pro-IL-1β and release of IL-1β into culture media was markedly diminished by R406 in a dose-dependent manner (Fig. 4c, d). These results prompted us to investigate whether inhibition of Syk affects the MSU-induced activation of the mTOR and p38 signaling pathways in human monocytes. Inhibition of Syk with R406 noticeably repressed activity of the mTOR/S6K/4E-BP1 and p38/Mnk1/eIF4E pathways in response to MSU crystal stimulation of monocytes (Fig. 4e).

Finally, we investigated whether the inhibition of Syk phosphorylation led to the suppression of pro-IL-1β synthesis in MSU-stimulated human monocytes. Both global protein and pro-IL-1β synthesis were significantly suppressed in a dose-dependent manner by treatment with the Syk inhibitor (Fig. 5a, b), implicating Syk as an upstream regulator of these signaling pathways. Furthermore, these data suggest that the inhibitory effect of resveratrol on MSU-induced pro-IL-1β synthesis in human monocytes is mainly mediated by suppression of Syk phosphorylation.

MSU-induced NLRP3 inflammasome activation is repressed by resveratrol in human monocytes and macrophages

Maturation and release of IL-1β from monocytes/macrophages in response to MSU crystals is dependent on the NLRP3 inflammasome [11]. Therefore, we next sought to investigate the effect of resveratrol on the activation of the NLRP3 inflammasome in these cells. Proteolytic processing and secretion of caspase-1 and IL-1β into the culture supernatants in response to MSU crystals was inhibited in a dose-dependent

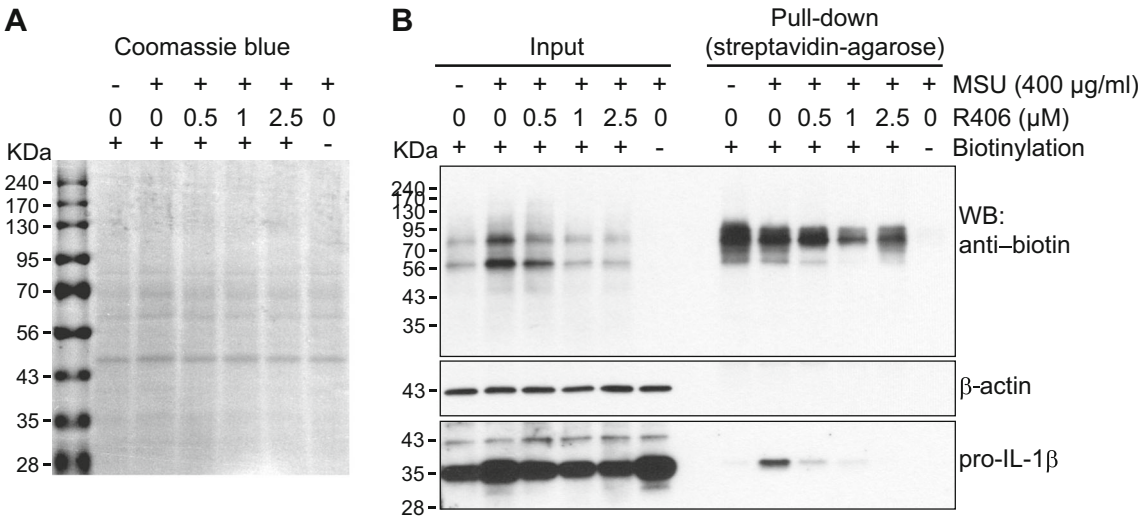

Fig. 5 MSU-stimulated global protein and pro-IL-1β synthesis are dependent on Syk activity. Monocytes were stimulated for 1 h with MSU crystals along with the indicated concentration of R406. **a** Coomassie blue staining. **b** Newly synthesized total proteins and pro-IL-1β were immunoblotted with streptavidin-HRP (upper) or anti-IL-1β antibody (lower) as described in Fig. 2d. Global protein and pro-IL-1β production were normalized to β-actin (Input) and total biotinylated protein (Pull-down), respectively. Data is representative of two independent experiments with two different donors

manner by resveratrol (Fig. 6a). Similar to MSU crystal-stimulated monocytes in Fig. 1i, resveratrol also significantly reduced the secretion of IL-1β after stimulation with ATP or nigericin, a potent NLRP3 inflammasome inducer, in the absence or presence of LPS (Suppl. Fig. 2), confirming its inhibitory role for NLRP3 inflammasome activation. In addition, resveratrol potently suppressed pyroptosis, a pro-inflammatory form of cell death, by caspase-1 following NLRP3 inflammasome activation (Suppl. Fig. 3A). Given that ASC oligomerization is an essential step in activation of the NLRP3 inflammasome as it leads to the recruitment of pro-caspase-1 to promote efficient caspase-1 processing [10], we examined whether reduced caspase-1 activation by resveratrol is associated with suppression of ASC oligomerization. Intact inflammasome complexes were isolated by Triton-insoluble fractionation and chemical cross-linking and their components were analyzed by immunoblotting. The results revealed that MSU-induced ASC oligomerization is reduced by resveratrol in a dose-dependent manner (Fig. 6b). To confirm the inhibitory effect of resveratrol on NLRP3 inflammasome activation, we investigated the effect of resveratrol on NLRP3 inflammasome activation following MSU crystal stimulation of LPS-primed human monocyte-derived macrophages (HMDMs). We found that resveratrol reduced MSU-induced secretion of IL-1β from HMDMs primed with LPS (Fig. 6c) and suppressed proteolytic processing and secretion of caspase-1 (p20) and IL-1β (p17) into the culture medium (Fig. 6d) in a dose-dependent manner. Furthermore, MSU-induced ASC oligomerization and the release of LDH into culture supernatants during pyroptosis were reduced by resveratrol treatment of HMDMs, similar to the results seen in human monocytes (Fig. 6e, Suppl. Fig. 3B). These results demonstrate that resveratrol has the ability to suppress NLRP3 inflammasome activation as well as inhibit pro-IL-1β synthesis downstream of MSU crystal stimulation of human monocytes and macrophages.

Resveratrol inhibits neutrophil recruitment and reduces IL-1β production in an MSU-mediated peritonitis mouse model

To determine the biological relevance of resveratrol-mediated suppression of IL-1β, we adopted a murine model of MSU-mediated peritonitis, a disease model utilized for studying the consequence of NLRP3 activation [32]. C57BL/6 mice were intraperitoneally pretreated with resveratrol (25 mg/kg) 0.5 h prior to intraperitoneal injection of MSU crystals. MSU stimulation for 3 h greatly increased the peritoneal level of IL-1β and the frequency of $CD11b^{+}Ly6G^{+}$ neutrophils in the peritoneal cavity. Pretreatment with resveratrol or R406 (Syk inhibitor) significantly reduced the amounts of peritoneal IL-1β, but not TNF-α (data not shown), and diminished the frequency of recruited $CD11b^{+}Ly6G^{+}$ neutrophils when compared to the vehicle control (Fig. 7a, b). Our findings suggest that the resveratrol-mediated suppression of Syk signaling reduces IL-1β production and the recruitment of neutrophils to the peritoneal cavity, thus mitigating the inflammatory symptoms in this in vivo model.

Discussion

Resveratrol has pleiotropic beneficial effects on the immune system, including anti-inflammatory, anti-oxidant, and

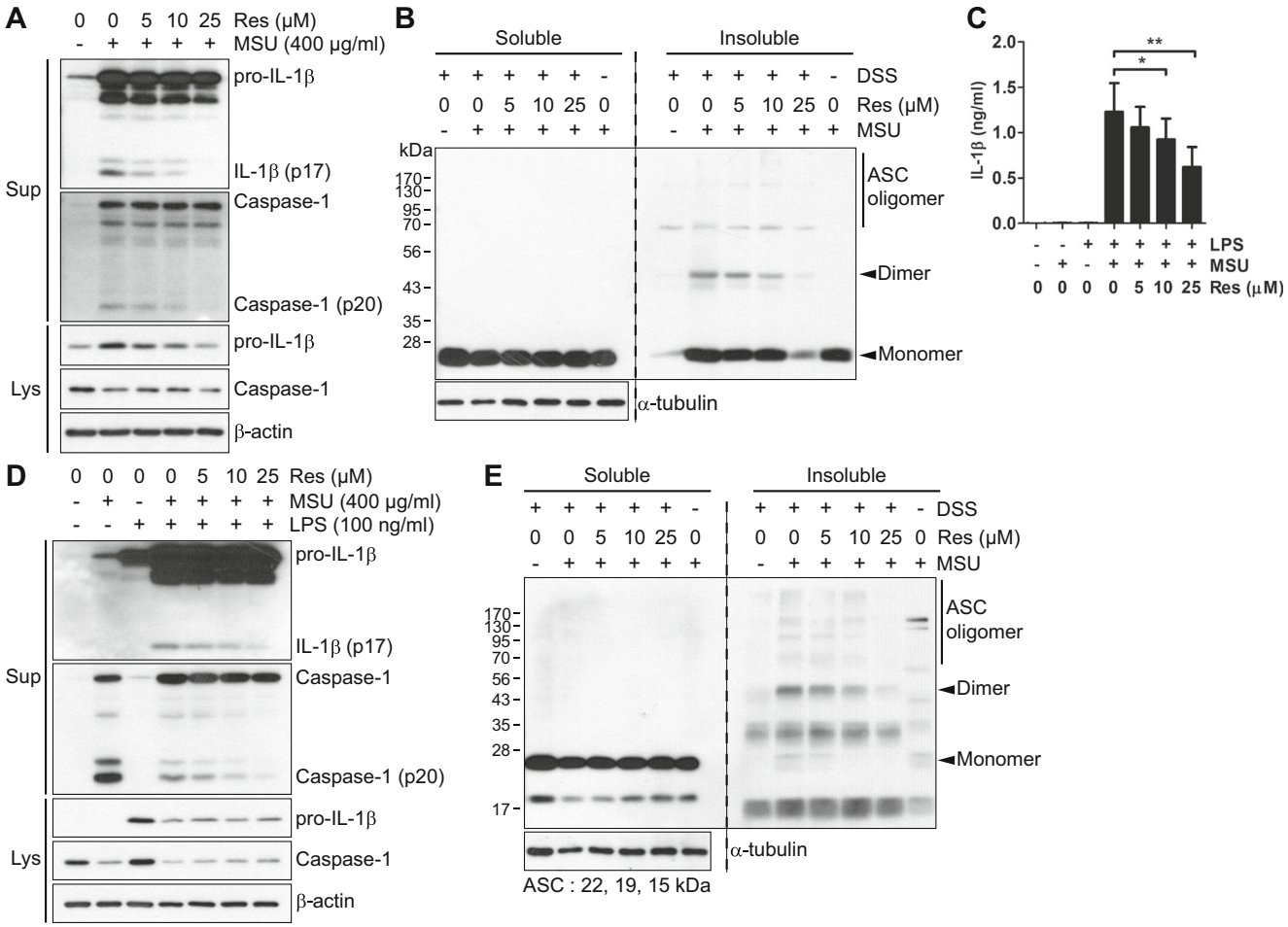

Fig. 6 Resveratrol attenuates MSU-mediated NLRP3 inflammasome activation in monocytes/macrophages. **a**, **b** Monocytes were stimulated for 4 h with MSU crystals with or without resveratrol. **a** Cell lysates (Lys) and culture supernatants (Sup) were immunoblotted for caspase-1 and IL-1β processing. **b** An ASC oligomerization assay was performed as described in the "Materials and methods." **c**–**e** Human monocyte-derived macrophages were primed with LPS (100 ng/ml) overnight and were then stimulated with MSU crystal with or without resveratrol for 4 h. **c** The amount of IL-1β in the culture supernatants was quantified by ELISA ($n = 4$). **d** Caspase-1 and IL-1β processing in cell lysates (Lys) and culture supernatants (Sup) was determined by immunoblotting. **e** Biochemical analysis of ASC oligomerization. Blots (**b**, **d**, **e**) are representative of two independent experiments with two different donors. $^{*}p < 0.05$ and $^{**}p < 0.01$ by paired *t* test

immunomodulatory properties, and as a result is reported to have a wide range of potential targets in various pathological conditions [24, 33]. Although its clinical benefits have not yet been confirmed in human trials, accumulating evidence from ex vivo studies suggest resveratrol ameliorates inflammatory responses and it has shown therapeutic effects in preclinical mouse models of autoimmune diseases [34, 35]. Moreover, it was recently reported that resveratrol mitigates articular inflammation in a murine model of MSU-induced gouty arthritis, suggesting its therapeutic potential in sterile particulate-mediated acute inflammation [21]. However, the precise molecular mechanisms of action of this compound remain unknown. Therefore, we investigated the anti-inflamatory effects of resveratrol on MSU-induced acute inflammation in vitro and in vivo, and found that resveratrol remarkably reduced the production of pro-inflammatory cytokines including IL-1β, a crucial mediator of sterile inflammatory diseases. Analysis of nascent protein synthesis revealed that resveratrol manifestly represses the translationally regulated rapid synthesis of pro-IL-1β by decreasing phosphorylation of Syk. Furthermore, NLRP3 inflammasome activation is inhibited by resveratrol via suppression of ASC oligomerization. Thus, resveratrol is involved in controlling both the *priming step* for production of pro-IL-1 β and the *activating step* for cleavage of pro-IL-1β, which is critical for secretion of bioactive IL-1β into the extracellular environment (Fig. 8). Moreover, treatment with resveratrol caused a significant reduction of neutrophil recruitment into the peritoneal cavity in a murine model of MSU-mediated acute peritonitis, a disease model utilized for studying the consequence of NLRP3 activation.

One of the well-defined pharmacological effects of resveratrol is the modulation of cancer cell growth through inhibition of global protein synthesis via mTOR-dependent and

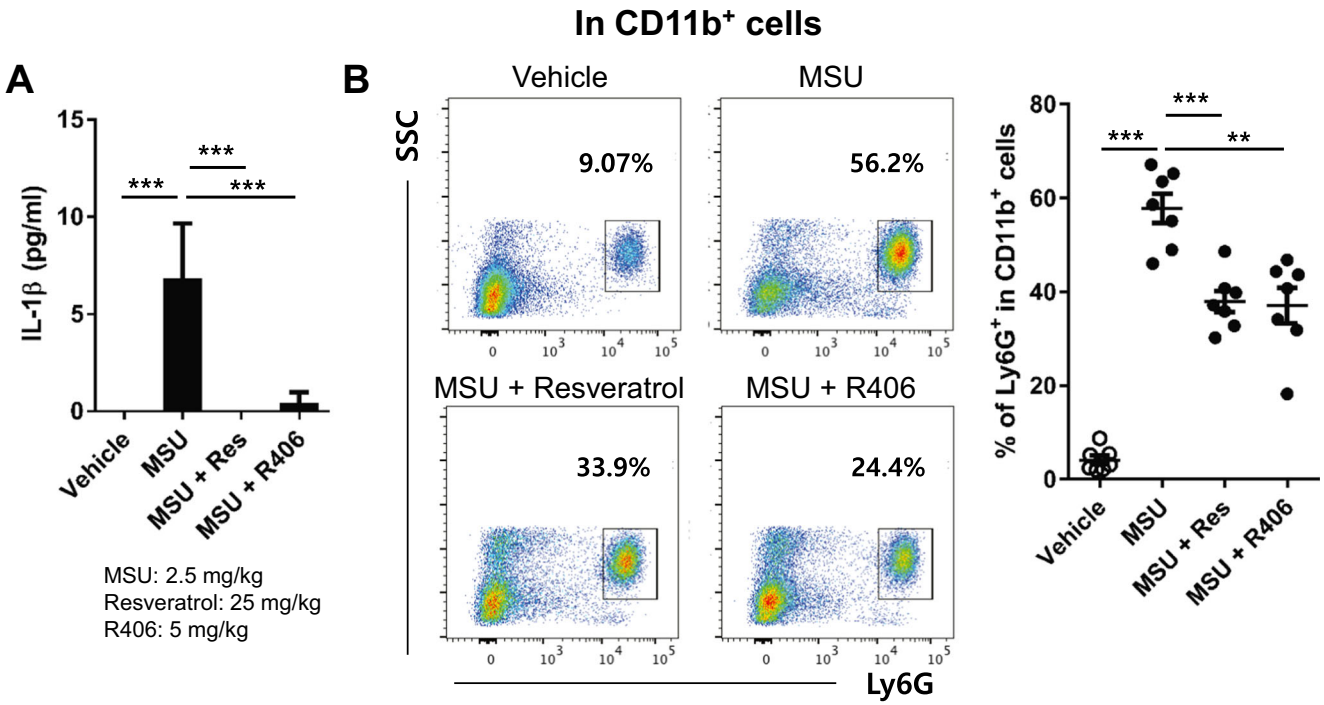

Fig. 7 Resveratrol attenuates MSU-mediated peritonitis by reducing IL-1β production and inhibiting neutrophil recruitment in a mouse model. C57BL/6 mice were intraperitoneally pretreated with resveratrol (25 mg/kg) or R405 (5 mg/kg) for 0.5 h prior to intraperitoneal injection of MSU crystals (2.5 mg/kg) or PBS alone (**a**). The amounts of IL-1β in the lavage fluid were quantified by ELISA. The data is representative of three independent experiments with at least five mice in each treatment group (**b**). The frequency of the $CD11b^{+}Ly6G^{+}$ neutrophils retrieved from the peritoneal lavage fluid was analyzed using flow cytometry (left). The data is representative of two independent experiments with at least five mice in each treatment group. The graph presents the mean ± SEM and each symbol represents an individual mouse (right). $^{**}p < 0.01$ and $^{***}p < 0.005$ by paired *t* test

mTOR-independent signaling pathways [26, 36]. Since MSU crystals rapidly enhance global protein synthesis, including pro-IL-1β in monocytes/macrophages [22, 37], it would appear reasonable to examine the effect of resveratrol on protein synthesis in MSU-activated monocytes. To this end, we found that resveratrol inhibits MSU-mediated global and pro-IL-1β protein synthesis via inhibition of mTOR and p38 MAPK signaling pathways (Figs. 1, 2, and 3). Moreover, these inhibitory effects of resveratrol on MSU-induced rapid protein synthesis may affect production of other inflammatory cytokines (Fig. 1), suggesting a novel anti-inflammatory function of resveratrol through suppression of sterile particulate-induced inflammatory protein synthesis.

Although resveratrol is able to directly interact with multiple molecular targets of signaling pathways [38], the findings that the relatively unrelated mTOR and p38 MAPK activities are concurrently suppressed by resveratrol raise the possibility of involvement of an upstream regulator of both signaling pathways. In addition to conventional phagocytosis of MSU crystals, several different, but not mutually exclusive, mechanisms have been suggested for cellular recognition of MSU crystals, likely depending on how and where the crystals form [39]. For example, CD14, TLR2, and TLR4 on murine macrophages were identified as receptors which can bind to MSU crystals and initiate inflammatory responses via p38 phosphorylation and NF-κB signaling [29, 40]. $CD14^{-/-}$ macrophages show attenuated phosphorylation of p38 and reduce IL-1β release in response to MSU due to decreased pro-IL-1β protein expression and inflammasome activity. In contrast, other studies reported that MSU-mediated DC activation does not require MyD88 or TRIF, major players in TLR-mediated signaling, and cellular recognition of MSU crystals has been shown to occur independently of TLRs [6, 30]. A more recent study revealed that MSU crystals can directly engage cholesterol components in cellular membranes of murine DCs in a receptor-independent manner. This event leads to lipid sorting, followed by the recruitment of Syk to membrane-associated immunoreceptor tyrosine-based activation motif (ITAM), which in turn phosphorylates PI3K, an upstream regulator of mTOR [30]. Syk, along with Zap70, is a member of the Syk family of non-receptor cytoplasmic tyrosine kinases and is broadly expressed in the hematopoietic system, including in monocytes/macrophages, and is involved in a variety of signal transduction pathways [41]. Here, we found that Syk is phosphorylated following stimulation with MSU crystals but not with LPS, whereas activation of IRAK1, a major component of TLR/IL-1R-mediated signaling, was induced only by LPS. This suggests that cellular recognition of MSU crystals is mainly mediated by TLR-independent mechanisms in human primary monocytes. Of note, resveratrol represses MSU

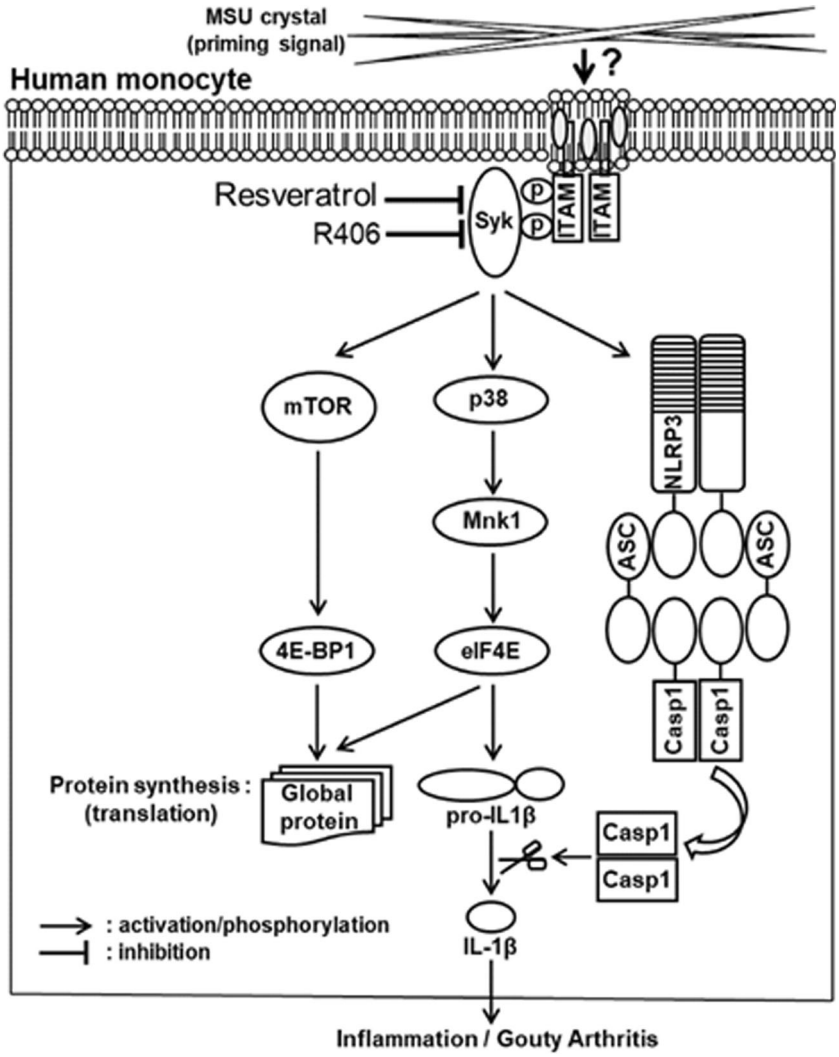

Fig. 8 Proposed mechanism underlying the anti-inflammatory effects of resveratrol on MSU crystal-induced inflammation in human monocytes. Direct or indirect interaction of the MSU crystals with the cholesterol in the lipid rafts induces ITAM-dependent activation of Syk in human monocyte. Syk activates p38 MAPK and mTOR signaling cascade leading to the synthesis of pro-IL-1β and global proteins and to NLRP3 inflammasome activation. The combined effects result in processing and secretion of mature IL-1β, which promotes local inflammation, associated gouty arthritis. Pre-treatment of human monocytes with resveratrol inhibits MSU crystal-induced pro-IL-1β synthesis and NLRP3 inflammasome-dependent processing of pro-IL-1β through suppression of Syk activation

crystal-mediated phosphorylation of Syk (Fig. 4b), which is a very early event following cellular recognition of MSU crystals, and this may explain why the activity of multiple signaling molecules was found to be suppressed by resveratrol (Fig. 3).

A pharmacological inhibitor of Syk, R406, was found to diminish MSU-stimulated pro-IL-1β and mature IL-1β production in a dose-dependent manner via inhibition of the mTOR and p38 MAPK signaling pathways in human monocytes (Fig. 4c–e). This result corroborated our findings that resveratrol-mediated Syk inhibition is responsible for down-regulation of pro-IL-1β production in response to MSU stimulation. Upon LPS stimulation, human monocytes also increase pro-IL-1β production and induce p38 phosphorylation. However, neither event was affected by R406 (Suppl. Fig. 4). These findings demonstrate that cellular recognition of MSU crystals by membranes of human monocytes is a major triggering event for gouty inflammation and that Syk plays an essential role as an upstream signaling initiator of mTOR and p38 MAPK pathways, which are involved with global and pro-IL-1β protein synthesis, respectively. As expected, R406 overtly inhibited the induction of both global and pro-IL-1β protein syntheses following MSU crystal stimulation (Fig. 5).

Recent studies reported that Syk contributes to NLRP3 inflammasome-mediated caspase-1 activation through phosphorylation and increased oligomerization of the ASC adaptor [32, 42]. Consistent with previous findings, we found that R406 inhibited proteolytic processing and secretion of caspase-1 and IL-1β into the culture supernatants in response to MSU crystals in a dose-dependent manner by repressing ASC oligomerization and pyroptosis (Suppl. Fig. 5A-C). Given the inhibitory role of resveratrol on MSU-mediated Syk activation (Fig. 4b), it is possible that the diminished IL-1β production caused by resveratrol treatment may be attributed to inhibition of NLRP3 inflammasome activation in MSU-stimulated monocytes. In our study, we observed that MSU-mediated proteolytic processing and secretion of caspase-1 and IL-1β, ASC oligomerization, and pyroptosis were all partially, but dose-dependently, decreased by

resveratrol in MDMs as well as monocytes (Fig. 6). Although the mechanism by which Syk regulates ASC oligomerization is still unclear, phosphorylation of ASC at Y146 and Y187 is known to be required for Syk-dependent ASC oligomerization and subsequent inflammasome activity [42]. Thus, resveratrol may be involved with these Syk-mediated molecular events.

While it remains unclear how resveratrol regulates Syk phosphorylation and whether Syk is a molecular target of resveratrol, piceatannol (3, 4′, 3′, 5-trans-trihydroxystilbene), a hydroxylated analog of resveratrol, is a well-known *Syk*-selective tyrosine kinase inhibitor [43], which acts by repressing autophosphorylation of Syk. Through docking studies and computer-aided molecular dynamic simulation studies, a recent study also demonstrated that Vam3, a dimeric derivative of resveratrol, interacts with Syk hydrophobically and exerts its anti-inflammatory effects through the Syk pathway [44]. In this study, resveratrol was also shown to dock into the ATP-binding pocket of Syk, implying an interaction between these molecules, although more interactions were observed between Vam3 and Syk than resveratrol [44]. Further investigation will be required to prove whether Syk is a direct target of resveratrol. Another possibility for its inhibitory effect on Syk activity is the interaction of resveratrol with membranes and subsequent structural modifications of lipid domains [45]. Shi and colleagues suggested a possible model of the TLR-independent Syk activation in MSU-activated DCs. In this model, lipid sorting occurs as a consequence of the electrostatic interaction of cholesterol-rich lipid rafts with MSU, which results in the organization of the membrane in such a way to activate downstream signaling events through ITAM-containing receptors [30]. This clustering triggers ITAM phosphorylation and provides docking sites for kinases (e.g., Syk) resulting in downstream signals leading to enhanced phagocytosis. Thus, resveratrol may affect a lipid-sorting event at the cell surface thereby interfering with ITAM clustering and Syk activation. In fact, the accumulation of resveratrol in lipid rafts followed by uptake via raft-mediated endocytosis has been reported [46, 47]. Therefore, further studies will be required to define the detailed molecular mechanisms underlying resveratrol's effects on lipid sorting-mediated Syk activation by MSU crystal stimulation of monocytes. A recent study suggests that DCs deficient for the Src kinases $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ show no binding to alum, which shares a common recognition mechanism with MSU, and exhibit impaired Syk-mediated phagocytic activity [48]. However, we cannot rule out the possibility that unidentified receptor is involved in the recognition of MSU in monocytes/macrophages.

Consistent with previous reports using $Syk^{-/-}$ mice [32], inflammatory responses were significantly alleviated following treatment of mice with R406 in our experiments (Fig. 7), indicating that MSU-induced peritonitis is dependent on Syk activation in vivo. Of note, mice pre-treated with resveratrol exhibit significantly decreased IL-1β secretion and decreased neutrophil recruitment into the peritoneal cavity in response to MSU crystals (Fig. 7). Therefore, in addition to the anti-inflammatory action of resveratrol in PAMP-triggered innate immunity, our study demonstrates that resveratrol also can act as an anti-inflammatory agent during the sterile inflammatory response caused by the recognition of irritant particles such as MSU crystals. In this context, it should be noted that cholesterol crystals, which are endogenous sterile crystals, also activate Syk in human macrophages and DCs via lipid raft formation and Syk inhibition is known to significantly reduce the production of IL-1β and TNF-α in these cells [49].

In summary, this study provides novel insight into the mode of action of resveratrol by investigating its anti-inflammatory effects on MSU-induced IL-1β production in vitro and in vivo. Our results show that resveratrol suppresses MSU-induced pro-IL-1β synthesis and inhibits NLRP3 inflammasome activation, demonstrating its effects on both the activation and priming steps of the IL-1β response. Furthermore, these results suggest that resveratrol possesses anti-inflammatory activities that may prove beneficial for the treatment of MSU-mediated sterile inflammation.

Author contributions Y-H.C.: designed the study, performed most of the experiments, data collection and analysis, and drafted manuscript. H.Y.K., B.R.Y., and Y.J.K.: performed the experiments, and data collection and analysis. W-W.L.: conceived the study, participated in its design and coordination, performed data analysis, and writing of manuscript

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Compliance with ethical standards

The study protocols were reviewed and approved by the IRB of Seoul National University Hospital. Peripheral blood of healthy volunteers was drawn after obtaining the written informed consent.

Conflict of interest The authors declare that they have no conflicts of interest.

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