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Pseudomonas aeruginosa-induced IL-1β Production is Inhibited by *Sophora flavescens* via the NF-κB/inflammasome Pathways

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The proinflammatory cytokine interleukin-1β plays an important role in protecting the host against airway infection; however, it can also trigger a massive influx of neutrophils into the airways, causing tissue damage. Anti-inflammatory treatments are particularly in demand for patients suffering from chronic inflammatory diseases. *Sophora flavescens* is a traditional herbal medicine used to reduce inflammation, but no study has examined its ability to block IL-1β production. Here, we show that *S. flavescens* reduced the *Pseudomonas aeruginosa*-induced expression of IL-1β by lung epithelial cells and macrophages. *S. flavescens* was also effective at reducing IL-1β production induced by either *Staphylococcus aureus* or phorbol 12-myristate 13-acetate, indicating that the effect is generalizable to diverse inflammatory stimuli. In addition, *S. flavescens* blocked the phosphorylation of IKKα/β, key upstream kinases involved in the degradation of IκBα, and the cleavage of caspase-1, a key component of the inflammasome. Thus, this study demonstrates that *S. flavescens* exerts its anti-inflammatory effects by blocking *P. aeruginosa*-mediated NF-κB/inflammasome activation and the subsequent production of IL-1β.

Keywords: IL-1β, inflammasome, *Pseudomonas aeruginosa*, *Sophora flavescens*

Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic bacterial pathogen, is a common cause of admission to intensive care units and is among the leading causes of ventilator-associated pneumonia (Chastre and Fagon, 2002; Gaynes and Edwards, 2005). Infection by *P. aeruginosa* frequently aggravates the symptoms of patients with chronic inflammatory diseases, such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), resulting in reduced pulmonary function and increased morbidity and mortality (Banerjee *et al.*, 2004; Hauser *et al.*, 2011). A prominent early host response to pulmonary infection by *P. aeruginosa* is secretion of the proinflammatory cytokine, interleukin-1β, which recruits neutrophils to the airways.

IL-1β is synthesized from a 35-kDa precursor, pro-IL-1β, which lacks biological activity. The active/mature form of IL-1β (17-kDa) is generated upon cleavage of pro-IL-1β by cysteine protease, IL-1β-converting enzyme (ICE or caspase-1) (Dinarello, 1996). This process requires the assembly of a macromolecular inflammasome complex to which pro-caspase-1 is recruited and cleaved in the presence of a protein belonging to the cytosolic Nod-like receptor (NLR) family (Franchi *et al.*, 2009). However, IL-1β can trigger a massive influx of neutrophils into the airways (Skerrett *et al.*, 2007); such excessive inflammatory responses are harmful to the host because they cause tissue damage (Lappalainen *et al.*, 2005). Therefore, anti-inflammatory drugs may slow the progression of airway diseases by interrupting this vicious cycle of infection and inflammation. Promising candidates may be found among traditional herbal medicines that are produced by living organisms (Maplestone *et al.*, 1992).

Sophora flavescens is a traditional herbal medicine used to treat chronic bronchitis, asthma, chronic hepatitis, coronary heart disease, and diarrhea in East Asian countries (Kuroyanagi *et al.*, 1999; Zhou *et al.*, 2009). It was previously reported that *S. flavescens* extract downregulates the production of proinflammatory cytokines by the human mast cell line, HMC-1 (Hong *et al.*, 2009). However, no study has examined *S. flavescens* as a potential blocker of IL-1β production. Here, we demonstrated that *S. flavescens* inhibits the production of IL-1β by blocking activation of the NF-κB/inflammasome pathways.

Materials and Methods

Reagents

Dried extract of *S. flavescens* and phorbol 12-myristate 13-acetate (PMA) were purchased from KPEB (Korea Plant Extract Bank; http://extract.kribb.re.kr) and Sigma-Aldrich (USA), respectively.

Bacterial strains and culture conditions

P. aeruginosa PAO1 wild-type (wt) strain (Holloway, 1955) and *Staphylococcus aureus* wt strain 1621 (Korean Collection for Type Cultures; http://kctc.kribb.re.kr) were used. *P. aeruginosa* was cultured in Luria (L) agar or L broth at 37°C. *S.*

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aureus was cultured in Tryptic Soy Broth (TSB; BD, USA) agar or TSB at 37°C. Bacterial cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4°C after overnight broth culture. Bacterial lysate was generated as described previously (Shin and Ha, 2011).

Cell culture

All media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone, USA) and 1% penicillin/streptomycin (Gibco, USA). A549 (human alveolar epithelial) and THP-1 (human macrophage) cells were cultured in RPMI-1640 (Hyclone). RAW 264.7 (mouse macrophage) and RAW-blue (mouse macrophage reporter cells; InvivoGen, USA) cells were maintained in DMEM (containing high glucose, L-glutamine, and sodium pyruvate; HyClone). Cells were seeded in 12-well plates (1.0×10^6 cells/well) and incubated at 37°C in a humidified, 5% CO_2, air-jacketed incubator for 20 h. After washing to remove non-adherent cells, adherent cells were incubated in FBS-free DMEM for 2 h at 37°C and then infected with either *P. aeruginosa* or *S. aureus* at a multiplicity of infection (MOI) of 10 at 37°C for 4 h. The supernatant from RAW-blue cells was obtained by centrifugation at 140 × *g* for 15 min.

Quantitative Real-Time PCR (qRT-PCR) analysis

Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. SYBR Green PCR Master Mix (KAPA Biosystems, USA) was used for the qRT-PCR. cDNA was synthesized from total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Japan). Reactions were amplified and quantified in a CFX96 Real-Time PCR System (Bio-Rad, USA) under the following conditions: stage 1, 50°C for 2 min followed by 95°C for 10 min; stage 2, 95°C for 15 sec followed by 60°C for 1 min. Stage 2 was repeated for 40 cycles. Data were analyzed using the comparative Ct method and the amount of mRNA was normalized against the housekeeping gene, GAPDH. The following primers were used: human IL-1β, 5′-AAACAGA TGAAGTGCTCCTTCCAGG-3′ and 5′-TGGAGAACACC ACTTGTTGCTCCA-3′; human IL-8, 5′-AACATGACTT CCAAGCTGGCC-3′ and 5′-TTATGAATTCTCAGCCCT CTTC-3′; human TNF-α, 5′-CAGAGGGAAGAGTTCCC CAG-3′ and 5′-CCTTGGTCTGGTAGGAGACG-3′; mouse IL-1β, 5′-TGGTGTGTGACGTTCCCATT-3′ and 5′-CAG CACGAGGCTTTTTTGTTG-3′; human GAPDH, 5′-CCC TCCAAAATCAAGTGG-3′ and 5′-CCATCCACAGTCTT CTGG-3′; and mouse GAPDH (5′-TGTGTCCGTCGGGA TCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGAT-3′.

ELISA assay

The amount of IL-1β in the culture supernatants was measured using human IL-1β ELISA kits (Thermo Scientific, USA) according to the manufacturer's instructions.

Immunoblotting analysis

Antibodies specific for IKKα, p-IKKα/β, IκBα, p-IκBα, IL-1β, and Caspase-1 (D7F10) (Cell Signaling Technology, USA) were used in this study. Cells were lysed in lysis buffer [10 mM Tris-HCl; pH 7.4, 150 mM NaCl, 2.5 mM EDTA, and 0.1% (v/v) Triton-X] in distilled water supplemented with PMSF (phenylmethylsulfonyl fluoride; Thermo Scientific) and sodium orthovanadate (Sigma-Aldrich). Samples containing 30 μg of protein were run in 8% SDS-PAGE gels and the separated proteins transferred onto a polyvinylidene difluoride membrane (PVDF; Millipore Corp., USA). Membranes were blocked for 2 h in 5% (w/v) non-fat dried milk in PBS containing 0.1% (v/v) Tween 20 (Sigma-Aldrich) and then probed with a primary antibody. Immunoreactive bands were visualized using a horseradish peroxidase-linked secondary antibody (Cell Signaling Technology) and enhanced chemiluminescence reagents (Intron Biotechnology, Korea). The membrane was then stripped and re-probed with a polyclonal anti-β-actin antibody (Santa Cruz Biotechnology, USA) as a loading control.

Alkaline phosphatase assay

RAW-blue cells, a secreted embryonic alkaline phosphatase (SEAP) reporter system, were incubated with *P. aeruginosa* for the indicated times in the presence or absence of *S. flavescens* extract (50 ng/μl) and the cell supernatant obtained by centrifugation at 140 × *g* for 15 min. SEAP levels (activated by NF-κB/AP-1) were measured by incubating the supernatant with the Quanti-Blue (InvivoGen) substrate for 1 h. Absorption was measured in a SUNRISE microplate reader (Tecan, Switzerland) at 595 nm.

MTT assay

The MTT assay was performed according to the method developed by Mosmann (1983). Briefly, cells were incubated with various concentrations of *S. flavescens* extract (10, 25, 50, and 100 ng/μl) for 5 h and cell viability measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, USA) according to the manufacturer's instructions

Statistical analysis

One-way ANOVA followed by Tukey's post-hoc multiple range test was used for statistical analysis. All analyses were performed using the Instat package (GraphPad, USA). A *P*-value < 0.05 was considered statistically significant. All tests were based on n≥3.

Results

S. flavescens reduces the *P. aeruginosa*-induced expression of IL-1β

To examine whether *S. flavescens* blocks the bacteria-induced expressions of proinflammatory cytokines including *Il1β*, *Il8*, and *Tnfα*, we measured the amount of mRNA in A549 cells exposed to *P. aeruginosa* in the presence or absence of *S. flavescens*. As shown in Fig. 1A, *S. flavescens* reduced the levels of all expressions tested. Since IL-1β is known as a potent mediator of responses to infection and injury (Dinarello, 1998), the reduction of *Il1β* expression was further repli-

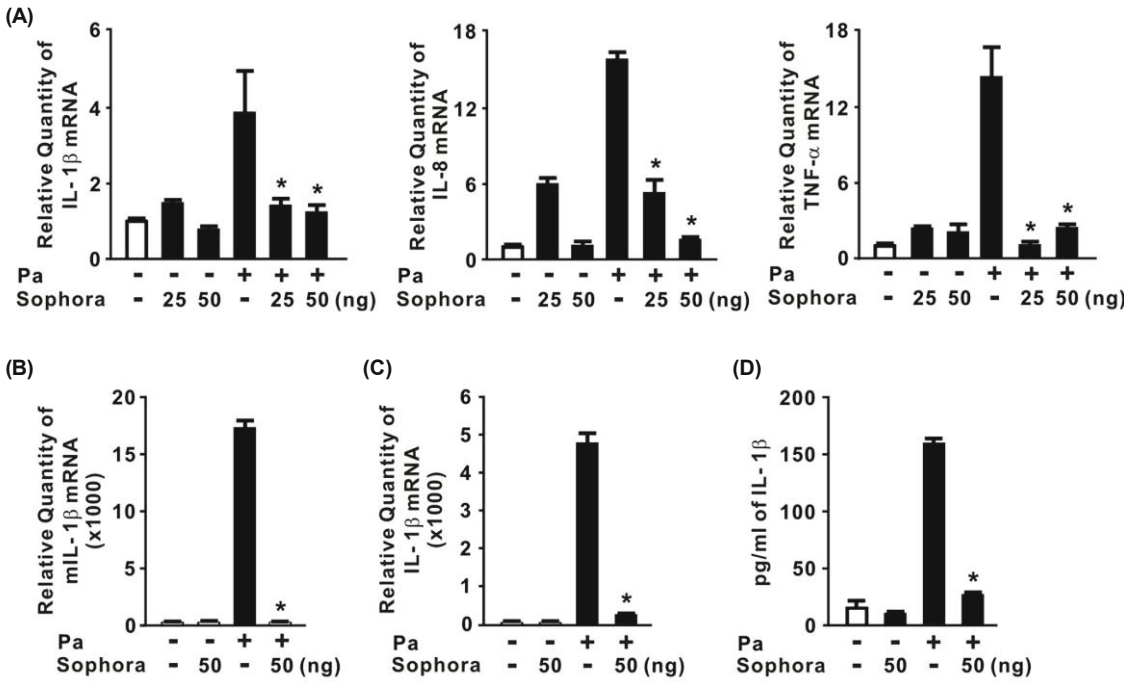

Fig. 1. ***S. flavescens*** **reduces** ***P. aeruginosa*****-induced IL-1β expression.** Cells were treated with *P. aeruginosa* (Pa) at an MOI of 10 for 4 h in the presence (+) or absence (-) of *S. flavescens* (Sophora). (A) *Il1β*, *Il8*, and *Tnfα* mRNA levels were measured in A549 cells by qRT-PCR. *Il1β* mRNA levels were measured in RAW 264.7 (B) and THP-1 (C) cells by qRT-PCR. (D) Levels of IL-1β protein secreted by THP-1 cells were measured by ELISA. Data are expressed as the mean ± SD (n=3). *$p < 0.05$ vs. Pa treatment alone.

cated in RAW 264.7 mouse macrophages and THP-1 human macrophages (Figs. 1B and 1C), and it was confirmed at the protein level in THP-1 cells (Fig. 1D). These data clearly demonstrate that *S. flavescens* reduces the expression of IL-1β mRNA and protein induced by *P. aeruginosa* infection, and that this anti-inflammatory effect of *S. flavescens* may be generalizable to diverse cell types.

S. flavescens reduces IL-1β expression induced by diverse inflammatory stimuli

We next examined the effect of *S. flavescens* on *Il1β* expression induced by diverse inflammatory stimuli. As shown in Fig. 2A, *S. flavescens* reduced the expression of *Il1β* induced by *S. aureus*, a Gram-positive bacterial pathogen frequently found in the human respiratory tract. Furthermore, *S. flavescens* clearly reduced the levels of *Il1β* expression mediated by PMA, a potent chemical stimulator of inflammatory responses (Fig. 2B). Taken together, these data suggest that the anti-inflammatory effect of *S. flavescens* may be generalizable to diverse inflammatory stimuli.

S. flavescens suppresses *P. aeruginosa*-mediated IKKα/β activation

A regulatory region within the human IL-1β gene contains an NF-κB-like binding site (Auron and Webb, 1994). To determine whether *S. flavescens* suppresses *P. aeruginosa*-mediated NF-κB activation, we assayed NF-κB activity in RAW-blue cells over time in the presence or absence of *S. flavescens*. Figure 3A shows that *S. flavescens* significantly suppressed *P. aeruginosa*-mediated NF-κB activation. To further confirm this result, we next examined whether *S. flavescens* inhibited the phosphorylation of IKKα/β and both the phosphorylation and degradation of IκBα in A549 and THP-1 cells treated with *P. aeruginosa* lysate. Immunoblotting analysis revealed that *S. flavescens* clearly inhibited IKKα/β activation in A549 and THP-1 cells, thereby block-

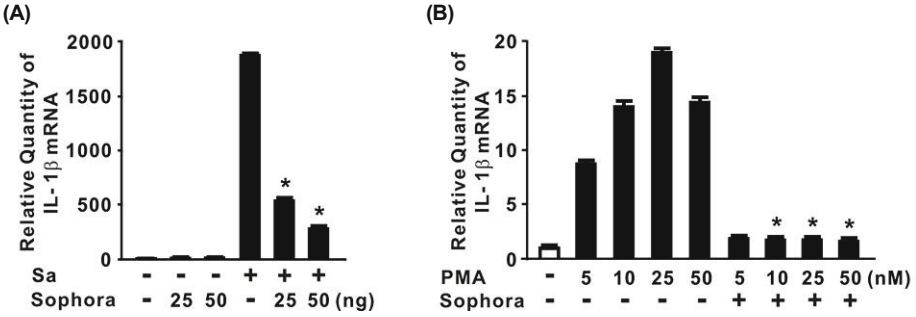

Fig. 2. ***S. flavescens*** **reduces the expression of IL-1β induced by diverse inflammatory stimuli.** A549 cells were treated with either *S. aureus* (Sa; A) at an MOI of 10 for 4 h or with the indicated doses of PMA (B) in the presence (+) or absence (-) of *S. flavescens* (Sophora) and the levels of *Il1β* mRNA were measured by qRT-PCR. Data are expressed as the mean ± SD (n=3). *$p < 0.05$ vs. Sa treatment alone (A) or PMA treatment alone (B).

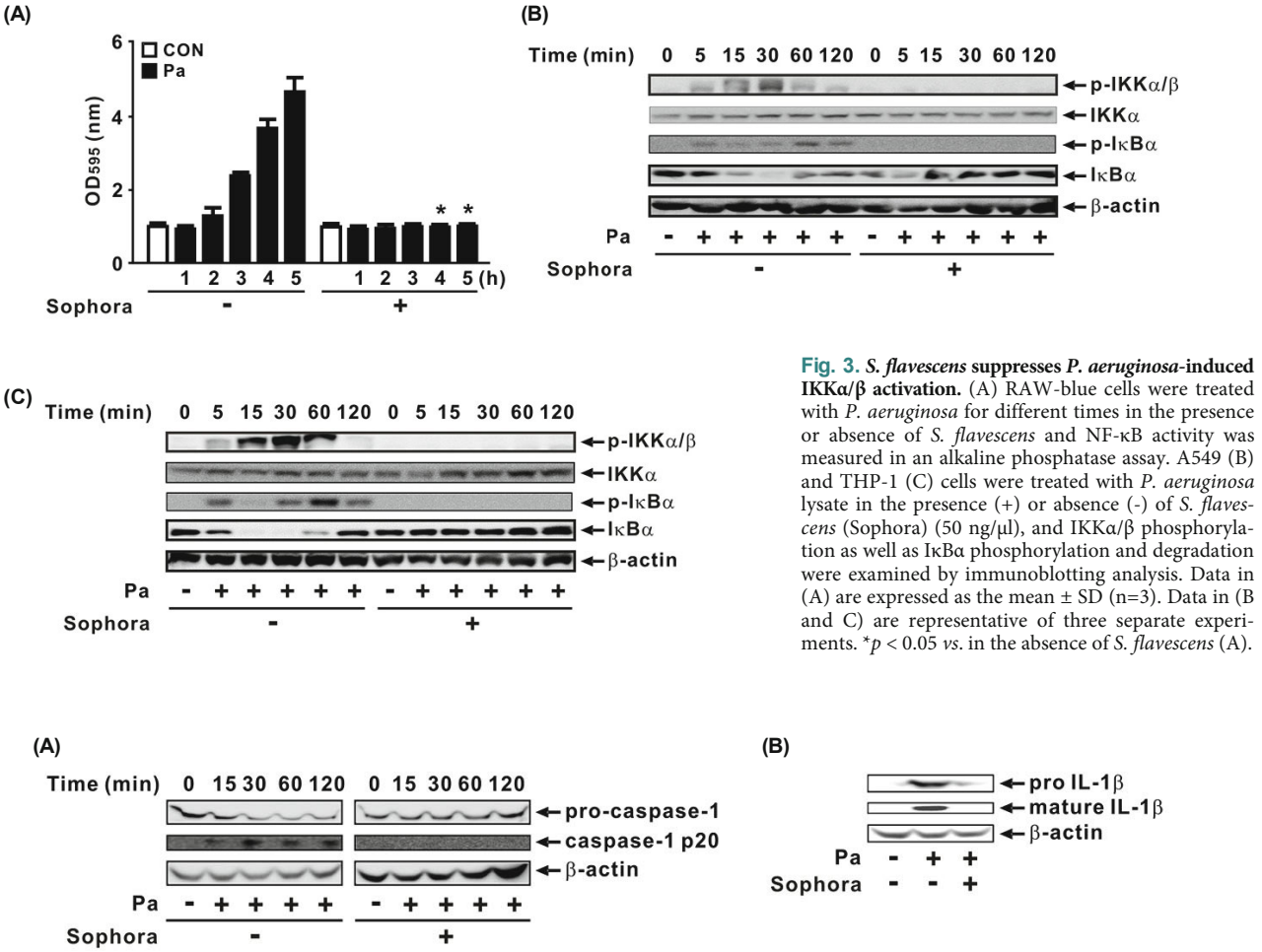

Fig. 3. *S. flavescens* suppresses *P. aeruginosa*-induced IKKα/β activation. (A) RAW-blue cells were treated with *P. aeruginosa* for different times in the presence or absence of *S. flavescens* and NF-κB activity was measured in an alkaline phosphatase assay. A549 (B) and THP-1 (C) cells were treated with *P. aeruginosa* lysate in the presence (+) or absence (-) of *S. flavescens* (Sophora) (50 ng/μl), and IKKα/β phosphorylation as well as IκBα phosphorylation and degradation were examined by immunoblotting analysis. Data in (A) are expressed as the mean ± SD (n=3). Data in (B and C) are representative of three separate experiments. *$p < 0.05$ *vs.* in the absence of *S. flavescens* (A).

Fig. 4. *S. flavescens* inhibits *P. aeruginosa*-induced cleavage of caspase-1. *P. aeruginosa*-induced caspase-1 activation (A) and IL-1β production (B) were assessed in THP-1 cells in the presence (+) or absence (-) of *S. flavescens* (Sophora) (50 ng/μl). Data are representative of three separate experiments.

ing IκBα phosphorylation and degradation (Figs. 3B and 3C). Taken together, these results suggest that *S. flavescens* suppresses the *P. aeruginosa*-mediated activation of NF-κB by inhibiting IKKα/β.

S. flavescens inhibits *P. aeruginosa*-mediated cleavage of caspase-1

Since activation of caspase-1 is essential for IL-1β processing, we investigated the effect of *S. flavescens* on *P. aeruginosa*-mediated caspase-1 cleavage by performing immunoblotting analysis to detect pro-caspase-1 and caspase-1 p20 (cleaved form). As shown in Fig. 4A, treatment with *S. flavescens* blocked *P. aeruginosa*-mediated activation of caspase-1 in THP-1 cells. In line with this, we next examined whether *S. flavescens* inhibited the production of mature IL-1β. We found that no mature IL-1β was produced in the presence of *S. flavescens* (Fig. 4B). To rule out the possibility that the anti-inflammatory effect of *S. flavescens* may actually be due to cytotoxic effects, we examined the toxicity of *S. flavescens* in MTT assays based on A549 lung epithelial cells and RAW 264.7 macrophages. As shown in Fig. 5, *S. flavescens* was not cytotoxic to these cells when used at 10, 25, or 50 ng/μl (the doses used for the experiments in this study). Therefore, the decrease in IL-1β expression observed at these doses is unlikely to be due to cytotoxic effects. Taken together, these data suggest that *S. flavescens* blocks IL-1β production by suppressing *P. aeruginosa*-mediated activation of caspase-1.

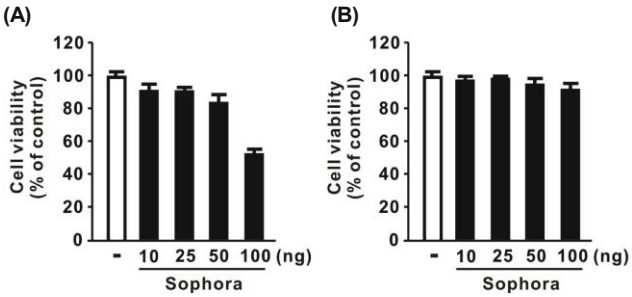

Fig. 5. The anti-inflammatory effect of *S. flavescens* is not mediated by cellular cytotoxicity. A549 (A) and RAW264.7 (B) cells were treated with nothing (-) or different doses of *S. flavescens* (Sophora) for 5 h and cell viability was measured using an MTT assay. Data are expressed as the mean ± SD (n=3).

Discussion

Upon airway infection, inflammation is mediated by the release of a range of host mediators to facilitate pathogen clearance (Koyama *et al.*, 1991). The proinflammatory cytokine, IL-1β, is a potent mediator of responses to infection and injury (Dinarello, 1998). However, even a minute amount of IL-1β *in vivo* can trigger a massive influx of neutrophils into the airways and induce the production of other cytokines, which in turn mediate other inflammatory immune responses. Such excessive activation of inflammatory responses is harmful to the host as it damages tissues and causes lung injury (Lappalainen *et al.*, 2005). Bacterial infections can be treated effectively with antibiotics, and co-treatment with anti-inflammatory medications effectively slows the progression of lung disease by interrupting the vicious cycle of infection and inflammation. Possible therapeutic interventions may be based on neutralizing cytokines and chemokines using specific antibodies, or on synthetic chemical compounds that act as receptor antagonists, or on compounds that inhibit the intracellular signaling cascades that result in cytokine production. However, due to the possible side effects (including hepatotoxicity and cardiovascular toxicity) of these treatments, alternative anti-inflammatory agents are desirable. Traditional herbal medicines may be a promising solution because these are chemical compounds or substances produced by living organisms that normally have few side effects (Maplestone *et al.*, 1992).

We had previously identified *S. flavescens* when screening for natural products showing an anti-inflammatory effect anddecided to examine its effect on *P. aeruginosa*-mediated IL-1β production. Macrophages are the primary source of IL-1β, although many other cells, including bronchial and alveolar epithelial cells, also produce IL-1β (Dinarello, 1996; Yang *et al.*, 1999). These cells play a critical role in the pathophysiology of CF and COPD. *P. aeruginosa* is recognized as a relevant pathogen in both these conditions, and airway infection by this pathogen contributes to the airway inflammation that is a hallmark of these diseases; such infections cause acute exacerbations, which are associated with substantial morbidity and mortality (Banerjee *et al.*, 2004; Hauser *et al.*, 2011). *S. flavescens* inhibited *P. aeruginosa*-mediated IL-1β production by lung epithelial cells and macrophages. We also examined the effect of *S. flavescens* on the expression of *Il8* and *Tnfα*, two additional important proinflammatory cytokines, and the inhibitory effect was still observed (Fig. 1A). Moreover, *S. flavescens* was also effective at reducing inflammatory responses induced by *S. aureus* and PMA. Since *S. flavescens* reduces the production of IL-1β by diverse cell types exposed to *P. aeruginosa* and diverse stimuli, this natural herbal medicine may have clinical application for the treatment of inflammatory conditions. For this purpose, further examination of responsible components in the extracts would be interesting. Previously, it was demonstrated that some flavonoids, including trifolirhizin, isolated from *S. flavescens* extracts, have anti-inflammatory and antiproliferative activities (Zhou *et al.*, 2009; Quang *et al.*, 2013), and these might be the molecules responsible for the reduction of IL-1β production.

PMA is a specific activator of NF-κB, which is a key transcriptional factor involved in the expression of IL-1β (Kim *et al.*, 2012). Since we previously reported that *P. aeruginosa* activates NF-κB signaling (Shin and Ha, 2011), we were interested in finding out whether *S. flavescens* suppresses NF-κB signaling. Indeed, *S. flavescens* suppresses the *P. aeruginosa*-mediated activation of NF-κB. Previously, it was reported that *S. flavescens* suppresses the nuclear translocation of NF-κB by inhibiting the phosphorylation and degradation of IκBα, which is a critical step for NF-κB activation and NF-κB-mediated signaling pathways (Hong *et al.*, 2009). However, any further interaction between *S. flavescens* and NF-κB has not been reported to date. The phosphorylation and degradation of IκBα was also observed in this study as shown in Fig. 3B and 3C. Since IκBα phosphorylation and degradation is regulated by IKKα/β, we examined and found that IKKα/β phosphorylation in epithelial cells and macrophages was also blocked in the presence of *S. flavescens*, suggesting that *S. flavescens* inhibits the expression of *Il1β* mediated by NF-κB signaling by blocking the activation of IKKα/β. However, it is unclear whether *S. flavescens* directly suppresses the phosphorylation of IKKα/β. In the NF-κB signaling pathways, upstream kinases such as NF-κB-inducing kinase (NIK), MEK kinase 1 (MEKK1), NF-κB-activating kinase (NAK) can activate IKK in response to diverse inflammatory stimuli (Tak and Firestein, 2001). Therefore, it is possible that *S. flavescens* may block the activities of those upstream kinases.

The mature form of IL-1β is generated by cleavage of pro-IL-1β by caspase-1, which is activated by the inflammasome (Sahoo *et al.*, 2011; Contassot *et al.*, 2012). Since we previously demonstrated that *P. aeruginosa* stimulates caspase-1 cleavage (Kim *et al.*, 2014), we examined whether *S. flavescens* blocks *P. aeruginosa*-mediated caspase-1 activation. Indeed, *S. flavescens* inhibited the *P. aeruginosa*-mediated cleavage of caspase-1. In line with this, no mature IL-1β was produced in the presence of *S. flavescens*. The results of an MTT assay ruled out the possibility that these effects were due to direct toxicity of *S. flavescens* against the cells. *S. flavescens* was toxic to epithelial cells at high doses (100 ng/μl), but this dose far exceeded the doses used in the current experiments.

S. flavescens is traditionally used as a medicine to reduce inflammation, but no study has evaluated its ability to block IL-1β production. In this study, *S. flavescens* inhibited the expression and production of IL-1β by blocking activation of the NF-κB/inflammasome pathways. Since patients with septic shock, rheumatoid arthritis, tumors, autoimmune disorders, Alzheimer's disease, and chronic inflammatory diseases secrete large amounts of IL-1β (Lanzrein *et al.*, 1998), agents that reduce the production and activity of this proinflammatory cytokine are likely to have clinical applications. For this purpose, future studies should examine which components in the extracts are responsible for the anti-inflammatory properties described herein. Also, further studies are needed to better understand the mechanism by which *S. flavescens* inhibits inflammasome formation and the subsequent cleavage of caspase-1.

Acknowledgements

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