Title:

NLRP3 upregulation in A549 cells co-cultured with THP-1 macrophages under hypoxia via deregulated TGF-β signaling

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Abstract

NOD-like receptor family, pyrin domain-containing 3 (NLRP3) is one of the key components of the inflammasome. NLRP3 also participates in the regulation of fibrosis independent of the inflammasome. In this study, we analyzed the mechanism of upregulation of NLRP*3* expression in A549 cells co-cultured with THP-1 macrophages under hypoxia. Upregulation of NLRP3 was suppressed after treatment with inhibitors of TGF-β receptor or p38, but not with inhibitors of the IL-1 receptor and SMAD3. The analysis of downstream molecules of TGF-β signaling in A549 cells co-cultured with THP-1 macrophages under hypoxia showed that TGFBR1 was upregulated and SMAD7 was downregulated.

Taken together, these results suggest that the upregulation of NLRP3 in A549 cells is associated with deregulated TGF-β signaling and that the interaction between NLRP3 and TGF-β signaling plays a fundamental role in fibrogenesis.

Abbreviations list

Idiopathic pulmonary fibrosis (IPF)

epithelial- mesenchymal transition (EMT)

interleukin-1β (IL-1β)

transforming growth factor-β1 (TGF-β1)

NOD-like receptor family, pyrin domain-containing 3 (NLRP3)

apoptosis-associated speck-like protein containing a CARD (ASC)

TGF-β receptor type 1 (TGFBR1)

TGF-β receptor type 2 (TGFBR2)

type II alveolar epithelial cells (AEC II)

Keywords

Fibrosis; NLRP3; TGF-β1; Alveolar epithelial cells; Macrophages; Hypoxia

Introduction

NOD-like receptor family, pyrin domain-containing 3 (NLRP3), together with apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, constitutes an inflammasome. Expression of NLRP3 has been detected in various types of cells, including macrophages, fibroblasts, and epithelial cells [1,2]. The NLRP3 inflammasome mediates the maturation of IL-1β and is associated with inflammation and apoptosis [3].

Activation of the NLRP3 inflammasome requires two signals; the first is the priming signal and the second is the activation signal. Priming signals, such as stimulation with LPS, IL-1β, or TNFα, induce NLRP3 and pro-IL-1β expression [1,3]. Stimulation with a low concentration of oxygen also primes the inflammasomes in some types of cells [4,5]. As a second signal, several molecules and

cellular events, such as reactive oxygen species (ROS), K^+ efflux, Ca^{2+} signaling, and mitochondrial dysfunction, can trigger the activation of the NLRP3 inflammasome [1,3,6].

NLRP3 participates in the regulation of fibrosis independent of the inflammasome, via augmentation of TGF-β signaling or enhancement of TGF-β1 expression [7,8,9]. Many reports have shown that TGF-β1 is involved in fibrosis [10,11]. Therefore, the interaction between NLRP3 and TGF-β1 plays a fundamental role in fibrogenesis.

TGF-β signals through transmembrane serine/threonine kinase receptors (TGFBR) activate different intracellular pathways, such as the SMAD pathway, TAK1/MAPK pathway, RhoA/ROCK, or RAS/MEK pathway [12,13]. The activation of TGF-β signaling is modulated not only by transcriptional enhancement of TGF-β1 but also by the upregulation of TGF-β receptors and components of the TGF-β1 signaling pathway [14,15].

Interaction of cells, including macrophages, fibroblasts, and epithelial cells, is important for the stimulation of fibrogenesis. We previously showed that the interaction between human type II alveolar epithelial cells (AEC II, A549 cells) and macrophages derived from human monocytic cells (THP-1 macrophages) under hypoxia contributes to fibrosis via the enhancement of processes, such as TGF-β1-induced epithelial-mesenchymal transition (EMT), in A549 cells. It is noteworthy that the interaction of cells under hypoxia may exacerbate fibrosis [16]. In this study, we examined *NLRP3* expression in A549 cells co-cultured with THP-1 macrophages under hypoxic conditions.

Methods

Cell Culture

A549 and THP-1 cell lines were purchased from the Japanese Cancer Research Bank (JCRB, Tokyo, Japan) and the American Type Culture Collection (ATCC, Rockville, MD), respectively. In this study, we used A549 cell line as a model of AEC II. A549 cells were cultured in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, GE healthcare Lifesciences, Utah, USA), penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA). THP-1 cells were cultured in RPMI1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS, penicillin, and streptomycin. THP-1 cells differentiated to macrophages after addition of 5 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) for 2 days.

For co-culture assay, A549 cells and THP-1 macrophages were seeded and cultured on 6-well plates and 6-well Millicell Hanging Cell Culture Inserts (pore size 1.0 μm) (Millipore, Billerica, MA), respectively, for 1 day. Then, Cell Culture Inserts were transferred to the plates containing A549 cells. Cells were incubated with serum free -RPMI 1640 medium under either normoxia (95% air and 5% CO2) or hypoxia (1% O2, 5% CO2 and 94% N2). Hypoxic condition was created using multi gas incubator (APM-30D, ASTEC, Fukuoka, Japan).

For TGF-β type1 receptor inhibition, 10 μM SB431542 (Sigma) was used. For SMAD3 inhibition, 5

μM SIS3 (Cayman Chemical, Ann Arbor, MI) was used. For p38 MAPK inhibition, 20 μM SB203580 (AdipoGen Life Sciences, San Diego, CA) was used. In addition, 20 μg/mL IL-1R antagonist (Merck, Billerica, MA) was also used.

TGF-β1 knockdown

Silencer Select TGFB1 siRNA (s14055, Thermo Fisher Scientific) or Silencer Select negative control siRNA (4390843, Thermo Fisher Scientific) was transfected into A549 cells and THP-1 macrophages using the Nucleofector II device and Cell Line Nucleofector kit V (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The nucleofector program used for A549 cells was X-001 and that used for THP-1 macrophages was Y-001.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from A549 cells using the ReliaPrep RNA Cell Miniprep System (Promega, Fitchburg, WI). Then, cDNA was synthesized using ReverTra Ace (TOYOBO, Osaka, JAPAN), according to the manufacturer's protocol. Real-time qPCR was performed using QuantStudio 12K flex (Life Technologies, Carlsbad, CA) and the THUNDERBIRD probe qPCR MIX (TOYOBO). The probe and primer mixtures were purchased from Life Technologies; *NLRP3* (Hs00918082_m1), *TGFBR1* (Hs00610320_m1), *SMAD7* (Hs00998193_m1), *β-actin* (Hs99999903_m1).

ELISA

Secreted IL-1β and TGF-β1 were quantified using the Human IL-1 beta/IL-1Fs Quantikine HS

ELISA Kit and the Human TGF-beta 1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), respectively, according to manufacturer's instructions.

Westermblotting

A549 cells were lysed with RIPA buffer (Thermo Fisher Scientific) and protein concentration were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). After SDS-PAGE, protein samples were transferred to a PVDF membrane, and then the membrane was incubated with primary antibody for overnight at 4 °C. Primary antibodies we used in this study were following: NLRP3, phospho-Smad3 (Ser423/425), Smad3, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK (Cell Signaling Technology, Danvers, MA), SMAD7, TGFBR1, TGFBR2 (Thermo Fisher Scientific), and β-actin (Sigma). Following incubation with HRP conjugated secondary antibodies, the proteins were detected by ECL Advance Western blotting detection kit (GE health care) and myECLimager (Thermo Fisher Scientific). The signal intensity was analyzed by ImageJ software.

Statistical analysis

The results were analyzed for statistical variance using student's t-test. The p-value of < 0.05 was considered statistically significant. The data were presented as mean ± SD.

Results

NLRP3 expression was upregulated in AEC II by co-culturing AEC II with macrophages under hypoxia

condition.

To examine the influence of the interaction between AEC II and macrophages or the concentration of oxygen on the expression of *NLRP3* in AEC II, we quantified the expression of NLRP3 at mRNA and protein levels in A549 cells cultured with or without THP-1 macrophages under normoxic or hypoxic conditions for 24 hours. The *NLRP3* mRNA level was significantly higher in A549 cells upon co-culture with THP-1 macrophages under hypoxia compared to other conditions, which was confirmed at protein levels (Figure 1A and B). Since the major role of NLRP3 inflammasomes is the maturation of IL-1β, we measured the level of IL-1β in the culture medium using ELISA. The IL-1β level in the culture medium of A549 cells was below the detection limit irrespective of oxygen concentration. On the other hand, the IL-1β level was detectable in the co-culture of A549 cells and THP-1 macrophages under both normoxia and hypoxia; however, the levels did not significantly differ with changes in the oxygen concentration and presence of LPS priming/ATP stimulation (Figure 1C).

NLRP3 expression is regulated by NF-κB in some cell types. IL-1β is one of the ligands which activate NF-κB signaling [17]. Then, we examined whether secreted IL-1β in co-culture medium is involved in the upregulation of NLRP3 expression in A549 cells under hypoxia using IL-1R antagonists. After the treatment with IL-1R antagonists, no changes of the NLRP3 expression levels in A549 were observed (Figure 1A and B).

NLRP3 expression was reduced by inhibition of TGF-β1

The TGF-β1 is one of the regulators that enhance the NLRP3 expression [7,8,9]. Under either normoxia or hypoxia, TGF-β1 levels were increased when A549 cells were co-cultured with THP-1 macrophages (Figure 2A). However, NLRP3 expression was enhanced in A549 cells only when they were co-cultured with THP-1 macrophages under hypoxia (Figure 1A). We investigated the involvement of TGF-β1 in the upregulation of NLRP3 expression in AEC II induced by co-culturing with macrophages under hypoxia. Thus, we analyzed the expression of NLRP3 using siRNA mediated gene silencing for TGF-β1. The efficiency of siRNA was confirmed by ELISA for TGF-β1 in the medium (Figure 2B). Knockdown of TGF-β1 in both A549 cells and THP-1 macrophages significantly suppressed the upregulation of NLRP3 in A549 cells (Figure 2C and D).

Upregulation of TGFBR1 in AEC II in the co-cultured under hypoxia increases the sensitivity for TGF-β1

Considering these results (Figure 1A and 2), we hypothesized that the sensitivity for TGF-β1 in AEC II is associated with the upregulation of NLRP3 in AEC II. Thus, we examined the expression levels of the TGF-β receptors, TGF-β receptor type 1 (TGFBR1), and TGF-β receptor type 2 (TGFBR2). The TGFBR1 expression in A549 cells was significantly increased only in the co-culture under hypoxia (Figure 3A), which was also confirmed at mRNA levels (Figure 3B). On the other hand, the expression of TGFBR2 was upregulated in all conditions other than in normoxia without

macrophages (Figure 3A). The treatment with SB431542, a TGFBR1 kinase inhibitor, significantly suppressed the enhancement of NLRP3 expression in A549 cells induced by co-culturing with THP-1 macrophages under hypoxia (Figure 3C and D)

Reduction of SMAD7 may be associated with upregulation of TGFBR1 in AEC II in co-culture with macrophages under hypoxia

SMAD7 is a major suppressor protein involved in TGF-β signaling pathway [12]. SMAD7 inhibits TGF-β signaling via several pathways, one of which is that SMAD7 directly binds to the *TGFBR1* promoter and negatively regulates its transcriptional activity [18]. Thus, we next examined the expression levels of SMAD7 in AEC II. Under hypoxic condition, SMAD7 in A549 cells co-cultured with THP-1 macrophages under hypoxia were significantly less than those in A549 cells cultured alone at mRNA and protein levels (Figure 4A, B)

The TGF-β1 /p38 MAPK pathway was involved in the transcription of NLRP3 in AEC II

To reveal the downstream signaling of TGF-β1 involved in the induction of NLRP3 transcription, we treated A549 cells with SIS3, Smad3 phosphorylation inhibitor, and the SB203580 p38 MAPK inhibitor. Treatment with SIS3 had no effect on the upregulation of NLRP3 expression in A549 cells induced upon co-culturing with THP-1 macrophages under hypoxia (Figure 5A and B), whereas the

inhibition of p38 MAPK, significantly suppressed the upregulation of NLRP3 mRNA levels under the same conditions (Figure 5C and D).

Discussion

In this study, we showed that the expression of NLRP3 in A549 cells was increased via the TGF-β1/p38 MAPK pathway when A549 cells and THP-1 macrophages were co-cultured under hypoxia. The upregulation of NLRP3 was associated with the upregulation of TGFBR1, accompanied by the downregulation of SMAD7.

The prominent upregulation of NLRP3 expression in AEC II required both co-culture with macrophages and hypoxic environment. Hypoxia is one of the factors that induces fibrogenesis [22,23]. We previously reported that EMT of A549 cells was significantly promoted when A549 cells were co-cultured with THP-1 macrophages under hypoxia, which reflects interaction of cells under hypoxia contributes to changes of relevant molecules [16]. Previous reports revealed that hypoxia influenced the NLRP3 expression [4,5,24,25]; however, the response to hypoxia differed in different cell types [5,25,26]. Our Preliminary experiments using inhibitors and siRNA for HIF1α provided no significant effects on the NLRP3 expression in A549 cells (data not shown), although more detailed studies are required to confirm the relevance of hypoxia with regulation of NLRP3 expression.

TGF-β1 regulated the expression of NLRP3 in AEC II co-cultured with macrophages under hypoxia.

The inhibition of TGF-β1, TGFBR1 kinase or p38 MAPK was seen to suppress the upregulation of NLRP3 expression. TGF-β1 is a major cytokine that plays important roles in several conditions, including inflammation and fibrosis [10,11]. However, the regulators for NLRP3 expression have not been fully elucidated yet [1,3]. NF-κB is usually activated by the stimulation with Toll-like receptors, IL-1 receptors and TNF-α receptors. Meanwhile, NF-κB is also activated by TGFβ-1 through TAK1 [27]. p38 MAPK is a downstream factor of TAK1 and known to activate some transcription factors including NF-κB. Thus, we utilized p38 MAPK inhibitor to examine the relevance of TGFβ-1/TAK1/p38 MAPK pathway with the upregulation of NLRP3 in the co-culture and hypoxic condition. The present results suggest that TGF-β1 regulates the expression of NLRP3 through the activation of p38.

Upregulation of TGFBR1 may lead to the upregulation of NLRP3 in AEC II. We showed that the concentration of TGF-β1 increased when A549 cells were co-cultured with THP-1 macrophages under hypoxia is not significantly deferent from that under normoxia [Figure 2A]; however, NLRP3 was upregulated in co-culture under hypoxic condition and the upregulation of NLRP3 was significantly suppressed by siRNA for TGF-β1 from A549 cells and macrophages [Figure 2C, D]. These results suggested that the sensitivity of AEC II for TGF-β1 is increased under such conditions. Thus, we examined the expression level of TGFBRs and demonstrated that TGFBR1 was significantly increased in A549 cells cocultured with THP-1 macrophages under hypoxia. Upregulation of TGF-β receptors

has been reported in cervical cancer and non-small-cell lung cancer. Overexpression of TGFBR1 has been shown to contribute to lung tumorigenesis via the increased phosphorylation of its downstream targets, such as PAK1, MEK1, ERK, and Snail, irrespective of the amounts of its ligands [14,15].

Downregulation of SMAD7 might be involved in the upregulation of NLRP3 expression via the upregulation of TGFBR1. SMAD7 expression was shown to be suppressed when A549 cells were cultured with THP-1 macrophages. SMAD7 has been reported to negatively regulate the TGF-β pathway via the downregulation of TGFBR1 transcription and the promotion of the degradation of TGFBR1 [18,19]. The decrease in SMAD7 expression is associated with an increase in TGF-β1 signaling under various conditions, such as tubulointerstitial fibrosis [20,21].

Conclusions

Our study demonstrated that the expression of NLRP3 may be upregulated upon an increase in TGF-β1 signaling via the upregulation of TGFBR1, which was associated with the suppression of SMAD7, in a co-culture of AEC II and macrophages under hypoxia.

Author contribution

S.F. and K.M. designed and performed research, analyzed data, and wrote the paper; M.S., T.U., and T.H. analyzed data. All authors approved the final manuscript.

Declarations of interest

The authors have no conflicts of interest to declare.

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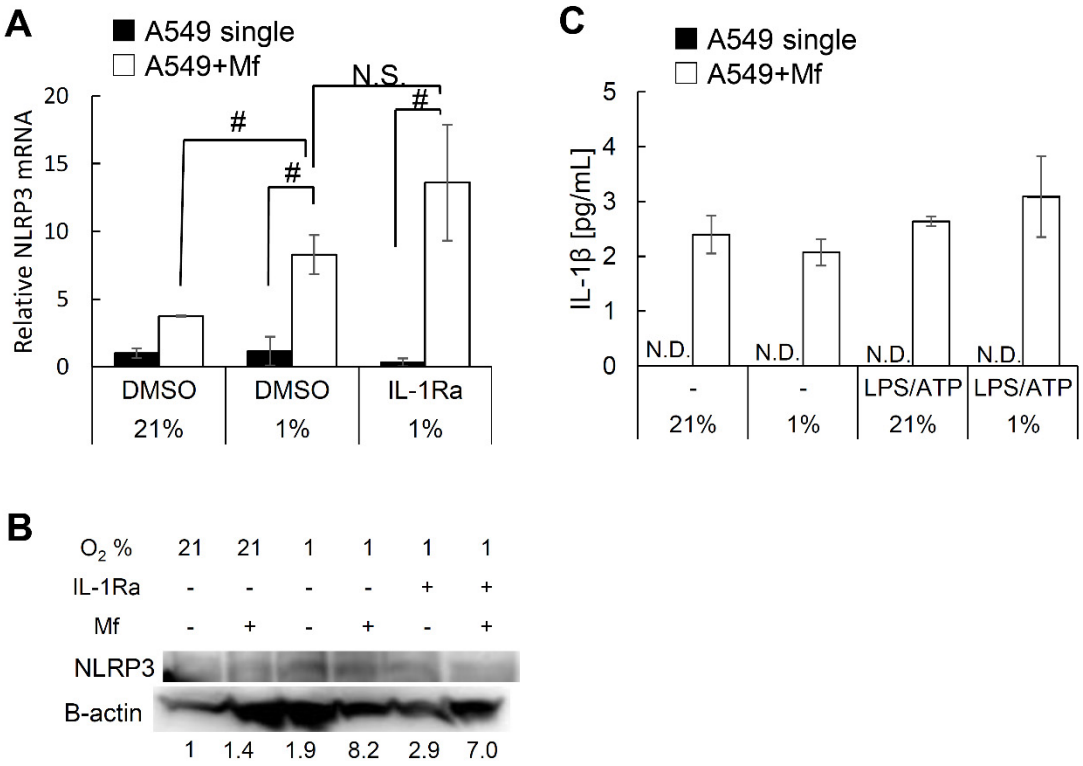
Figure

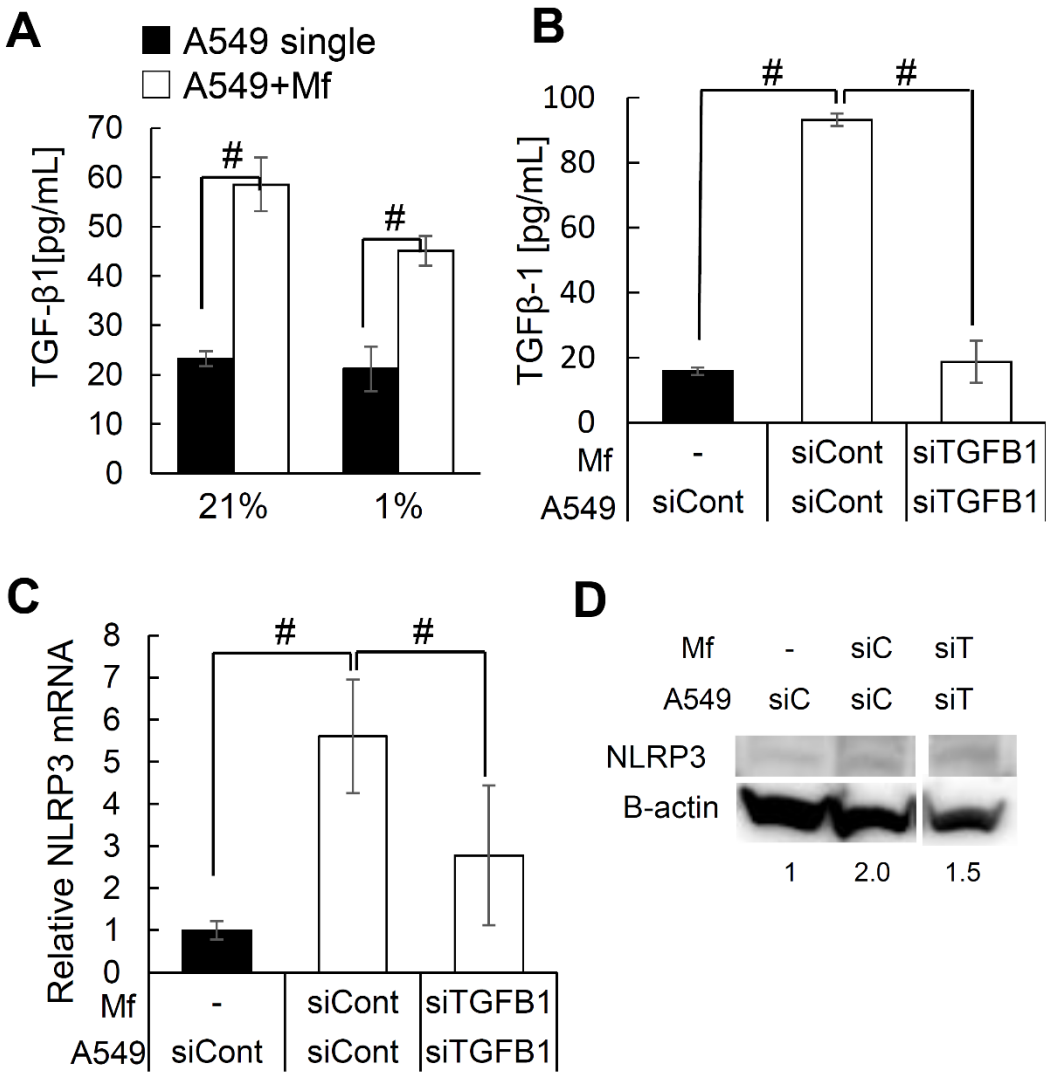
Figure 1



NLRP3 expression was upregulated in A549 cells when co-cultured with THP-1 macrophages under hypoxia conditions, in an IL-1β-independent manner.

A549 cells co-cultured with or without THP-1 macrophages were treated with either DMSO or IL-1 receptor antagonists (IL-1Ra) under either 21% or 1% O_2 for 24 hours. *NLRP3* mRNA were measured by qPCR (A). NLRP3 protein levels were analyzed by western blotting. The values showed at the bottom were the signal intensities analyzed by ImageJ software and normalized to β-actin expression (B). Secreted IL-1β levels were measured using ELISA (B). Data are shown as mean ± SD. N = 3. $^{\#}p < 0.05$. Mf, THP-1 macrophages; N.S., not significant; N.D., not detected.

Figure 2

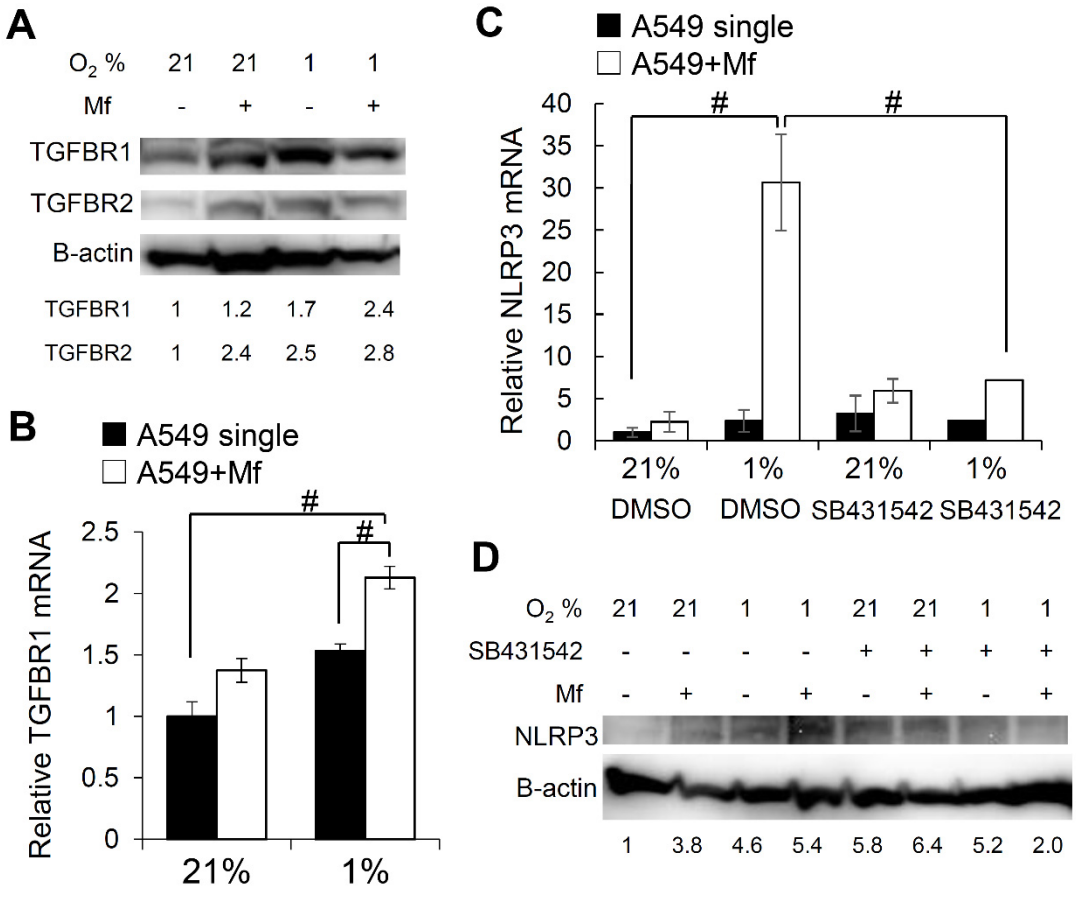


NLRP3 expression was reduced by inhibition of TGF-β signaling.

The levels of secreted TGF-β1 were measured after 24 hours of A549 cell culture with or without THP-1 macrophages under either 21% or 1% O_2 (A). A549 cells and THP-1 macrophages were transfected with control siRNA (siCont) or *TGFB1* siRNA (siTGFB1) and co-cultured under hypoxia for 24 hours. Secreted TGF-β1 levels were measured using ELISA (B). *NLRP3* mRNA in A549 cells

were measured by qPCR (C). NLRP3 protein levels were analyzed by western blotting. The values showed at the bottom were the signal intensities analyzed by ImageJ software and normalized to β-actin expression (D). Data are shown as mean ± SD. N = 3. $^{\#}p < 0.05$. Mf, THP-1 macrophages.

Figure 3

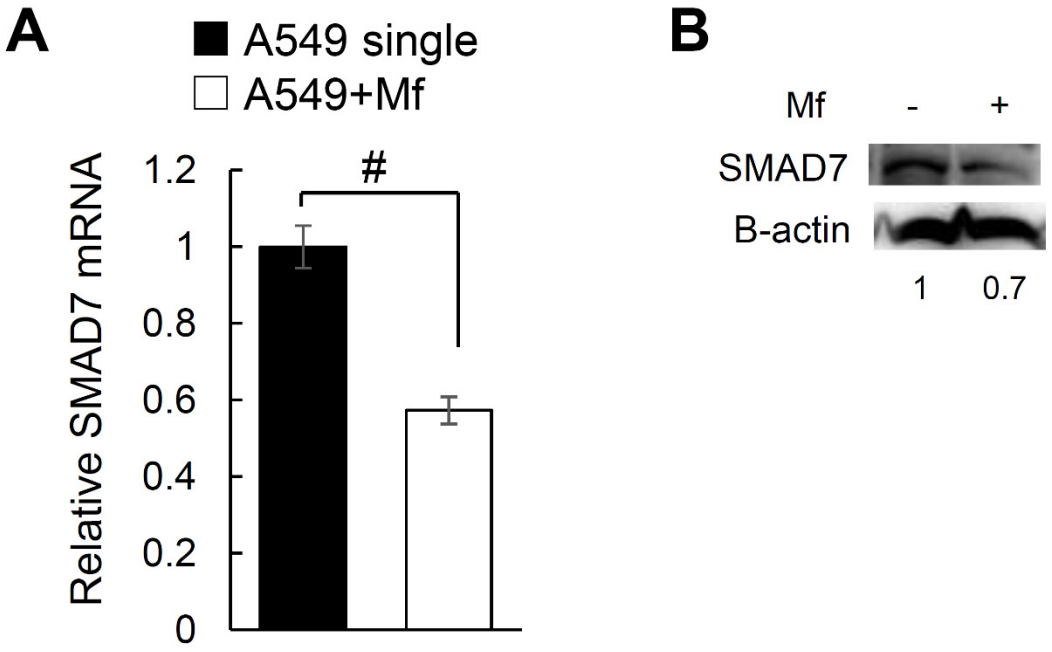


Upregulation of TGFBR1 in A549 cells leads to the upregulation of NLRP3 expression during co-culture with THP-1 macrophages under hypoxia.

A549 cells were co-cultured with or without THP-1 macrophages under either 21% or 1% O_2 for 24

hours. TGFBR1 and TGFBR2 protein levels were analyzed by western blotting (A). *TGFBR1* mRNA levels in A549 cells were measured by qPCR (B). A549 cells with or without THP-1 macrophages were treated with DMSO or SB431542 for 24 hours under normoxia or hypoxia, and then, *NLRP3* mRNA levels in A549 cells were quantified by qPCR (C). NLRP3 protein levels were analyzed through western blotting. The values showed at the bottom were the signal intensities analyzed by ImageJ software and normalized to β-actin expression (D). Data are shown as mean ± SD. N = 3. $^{\#}p < 0.05$. Mf, THP-1 macrophages.

Figure 4

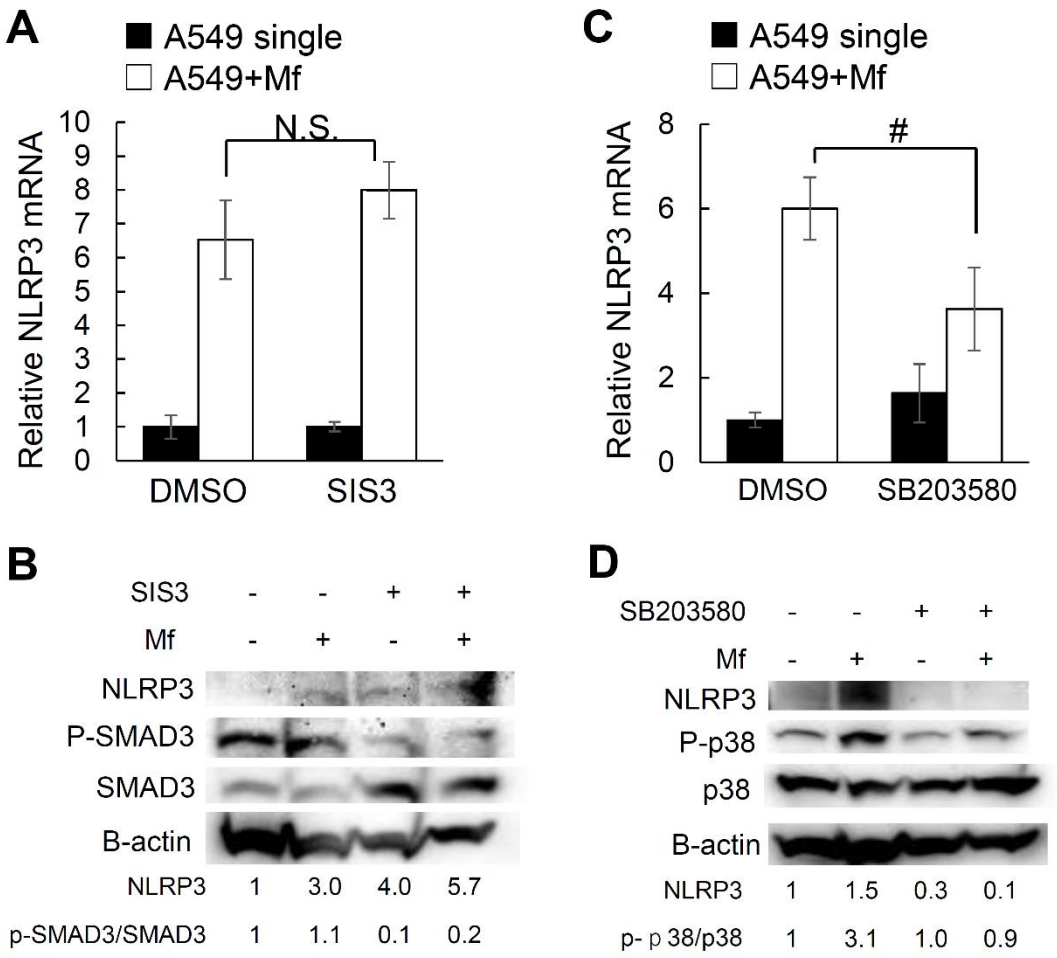


Reduction of SMAD7 may be associated with upregulation of TGFBR1 in A549 cells during co-culture with THP-1 macrophages under hypoxia.

SMAD7 mRNA levels in A549 cells were measured through qPCR after 3 hours of culture with or

without THP-1 macrophages under 1% O_2 (A). SMAD7 protein levels in A549 cells were analyzed through western blotting after 12 hours of culture with or without THP-1 macrophages under 1% O_2. The signal intensity was analyzed by ImageJ software and normalized to β-actin expression. The values showed at the bottom were the signal intensities analyzed by ImageJ software and normalized to β-actin expression (B). Data are shown as mean ± SD. N = 3. $^{\#}p < 0.05$. Mf, THP-1 macrophages.

Figure 5



TGF-β1 /p38 MAPK pathways were involved in the upregulation of NLRP3 in A549 cells.

A549 cells were cultured with or without THP-1 macrophages with DMSO or SIS3 treatments under 1% O_2 for 24 hours. *NLRP3* mRNA levels in A549 cells were measured through qPCR (A). NLRP3 protein levels were analyzed by western blotting (B). A549 cells were cultured with or without THP-1 macrophages with DMSO or SB203580 treatment under 1% O_2 for 24 hours. *NLRP3* mRNA levels in A549 cells were measured by qPCR (C). NLRP3 protein levels were analyzed by western blotting. The values showed at the bottom were the signal intensities analyzed by ImageJ software and normalized to β-actin expression (D). Data are shown as mean ± SD. N = 3. $^{\#}p < 0.05$. N.S., not significant; Mf, THP-1 macrophages.