

## RESEARCH ARTICLE

# Carbon monoxide decreases interleukin-1 $\beta$ levels in the lung through the induction of pyrin

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Carbon monoxide (CO) can act as an anti-inflammatory effector in mouse models of lung injury and disease, through the downregulation of pro-inflammatory cytokines production, though the underlying mechanisms remain unclear. The nucleotide-binding oligomerization domain-, leucine-rich region-, and pyrin domain-containing-3 (NLRP3) inflammasome is a protein complex that regulates the maturation and secretion of pro-inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ). In this report, we show that the CO-releasing molecule (CORM-2) can stimulate the expression of pyrin, a negative regulator of the NLRP3 inflammasome. CORM-2 increased the transcription of pyrin in the human leukemic cell line (THP-1) in the absence and presence of lipopolysaccharide (LPS). In THP-1 cells, CORM-2 treatment dose-dependently reduced the activation of caspase-1 and the secretion of IL-1 $\beta$ , and increased the levels of IL-10, in response to LPS and adenosine 5'-triphosphate (ATP), an NLRP3 inflammasome activation model. Genetic interference of IL-10 by small interfering RNA (siRNA) reduced the effectiveness of CORM-2 in inhibiting IL-1 $\beta$  production and in inducing pyrin expression. Genetic interference of pyrin by siRNA increased IL-1 $\beta$  production in response to LPS and ATP, and reversed CORM-2-dependent inhibition of caspase-1 activation. CO inhalation (250 ppm) *in vivo* increased the expression of pyrin and IL-10 in lung and spleen, and decreased the levels of IL-1 $\beta$  induced by LPS. Consistent with the induction of pyrin and IL-10, and the downregulation of lung IL-1 $\beta$  production, CO provided protection in a model of acute lung injury induced by intranasal LPS administration. These results provide a novel mechanism underlying the anti-inflammatory effects of CO, involving the IL-10-dependent upregulation of pyrin expression.

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## INTRODUCTION

Acute lung injury (ALI) and acute respiratory distress syndrome are severe critical illnesses that are associated with the accumulation of neutrophils in the lung and the excess production of inflammatory mediators, such as complement activation products, cytokines, chemokines, proteases, and oxidants.<sup>1</sup> During ALI, the level of interleukin-1 $\beta$  (IL-1 $\beta$ ), a product of inflammasome activation secreted by the infiltrated macrophages and neutrophils, was increased in bronchoalveolar lavage (BAL) fluid.<sup>2</sup> Inflammasomes are cytosolic multi-protein complexes that exert a major function in innate immune responses by regulating the cleavage and proteolytic

processing of caspase-1. Activated caspase-1 in turn catalyzes the maturation and secretion of pro-inflammatory cytokines (e.g., IL-1 $\beta$  and IL-18). Among the known inflammasomes, the nucleotide-binding oligomerization domain-, leucine-rich region-, and pyrin domain-containing-3 (NLRP3) inflammasome is involved in the pathogenesis of several acute or chronic inflammatory diseases. NLRP3 forms a complex consisting of pro-caspase-1, and the adaptor protein apoptosis-associated speck-like protein containing caspase activation and recruitment domain (ASC). The NLRP3 inflammasome senses soluble pathogen and danger-associated molecular patterns as well as biological crystals.<sup>3</sup> The priming and activation of the

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NLRP3 inflammasome both require stimulation by various endogenous factors and exogenous factors.<sup>3–5</sup> IL-1 $\beta$  is synthesized in its inactive pro-form (pro-IL-1 $\beta$ ), which is cleaved by caspase-1 to produce the active mature form of IL-1 $\beta$  that is then secreted.<sup>6</sup> Caspase-1 is also synthesized as an inactive 45-kDa protein (pro-caspase-1) that is proteolytically processed after assembly of the inflammasome in response to activating stimuli such as muramyl dipeptide, uric acid crystals, asbestos, silica, or alum adjuvant.<sup>7–10</sup> Stimulation of cells with lipopolysaccharide (LPS), which activates Toll-like receptor-4, in combination with the second signal adenosine 5'-triphosphate (ATP), which triggers K<sup>+</sup> efflux, represents a classical model of NLRP3 inflammasome activation *in vitro*.<sup>2,7,11</sup>

Pyrin is a multi-domain protein of 781 amino acids that is an important regulator of the inflammasome.<sup>12</sup> Initially recognized as a protein encoded by the MEFV gene, pyrin can be detected in patients with familial Mediterranean fever, a recessively inherited systemic autoinflammatory disease.<sup>13,14</sup> Pyrin is a cytoplasmic protein that consists of five domains: pyrin, b-Zip, B-Box, coiled-coiled, and PRY/SPRY (B30.2)<sup>15</sup> which is expressed in granulocytes, monocytes, and synovial fibroblasts and tightly regulated by cytokines.<sup>16</sup> IL-10, an anti-inflammatory cytokine, can induce pyrin expression in macrophages.<sup>17</sup> The IL-10-dependent induction of pyrin may serve as a negative regulator for caspase-1 activity and therefore inhibit inflammation.<sup>12,18</sup>

Carbon monoxide (CO) recognized as a vasodilatory agent, at low concentrations (50–500 parts per million [ppm]) exerts anti-inflammatory and anti-apoptotic effects during hyperoxia-induced lung injury,<sup>19</sup> sepsis,<sup>20</sup> ischemia/reperfusion injury,<sup>21</sup> and organ transplantation.<sup>22</sup>

In particular, experimental studies have shown that non-toxic, low doses of CO provide anti-inflammatory and cytoprotective effects in specific models of pulmonary disease.<sup>23–25</sup> CO is endogenously produced during heme metabolism by the enzyme heme oxygenase-1 (HO-1), which represents one of the major mammalian stress proteins.<sup>26</sup>

Treatment with low-dose inhaled CO at 250 ppm inhibits gene expression of the pro-inflammatory cytokines IL-1 $\beta$ , tumor-necrosis factor (TNF- $\alpha$ ), IL-6,<sup>27,28</sup> and induces the anti-inflammatory cytokine IL-10 without significantly altering hemodynamic parameters and oxygen carrying capacity.<sup>29,30</sup>

The mechanisms underlying the protective effects of CO in lung injury remain incompletely understood. We found that CO increases the expression of pyrin in mouse lung and spleen. To demonstrate the role of CO-induced pyrin in inflammasome formation *in vivo*, LPS was injected intranasally in the mouse followed by CO inhalation. Inhalation of CO attenuated LPS-induced IL-1 $\beta$  production *via* the increase of pyrin expression. We demonstrate that the anti-inflammatory effect of CO *in vitro* was abrogated by genetic knockdown of pyrin or its regulatory cytokine IL-10. Taken together, we suggest a novel mechanism for the anti-inflammatory effects of CO, involving the IL-10-dependent upregulation of pyrin expression.

## MATERIALS AND METHODS

### Chemicals and reagents

Tricarbonyldichlororuthenium (II) dimer (CORM-2), dichlororuthenium (II) dimer (RuCl<sub>2</sub>), actinomycin D (Act D), cycloheximide (CHX), and bacterial LPS (from *Escherichia coli* 055:B5) were purchased from Sigma-Aldrich (St Louis, MO, USA). ATP was purchased from Roche (Indianapolis, IN, USA). Recombinant human IL-10 was purchased from Peprotech (Rocky Hill, NJ, USA). Antibodies against caspase-1, pyrin, ASC, and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibody against IL-1 $\beta$  was purchased from Cell Signaling (Danvers, MA, USA). Antibody against NLRP3 was purchased from IMGENEX (San Diego, CA, USA), and IL-10 was purchased from Novus Biologicals (Littleton, CO, USA). Scrambled small interfering RNA (siRNA), pyrin siRNA, and IL-10 siRNA were purchased from Santa Cruz Biotechnology. All other chemicals were obtained from Sigma-Aldrich.

### Cell culture

The human monocytic leukemia cell line, THP-1, was purchased from the Korean Cell Line Bank (Seoul, Korea). The cultures were maintained at 37 °C in humidified incubators containing an atmosphere of 5% CO<sub>2</sub>/95% air. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL<sup>-1</sup> penicillin, and 100  $\mu$ g mL<sup>-1</sup> streptomycin. *In vitro* CO gas exposures were performed as previously described.<sup>20</sup>

### Animals

Seven-week-old wild-type male C57BL/6 were purchased from ORIENT (Pusan, Korea). Animal experiments were approved by the Animal Care Committee of the University of Ulsan. The mice were maintained under specific pathogen-free conditions at 18–24 °C and 40–70% humidity, with a 12-h light/12-h dark cycle and given access to food and drinking water *ad libitum*. C57BL/6 mice were exposed to room air or CO gas (Core Gas Ulsan, Korea). CO was also administered as 250 ppm mixed with room air. Mice were placed in the CO exposure chamber (LB Science, Daejeon, Korea) at room temperature for 4 h per day for 5 days. The CO levels in the chamber were continuously measured using a CO analyzer (Tongoy Control Technology, Beijing, China) in order to maintain the CO concentration at 250 ppm at all times. On day 6, mice were treated with 10 mg kg<sup>-1</sup> of LPS by intraperitoneal (i.p.) injection or intranasal administration of LPS (2.5 mg kg<sup>-1</sup>). The same amount of phosphate-buffered saline (PBS) solution was administered to the control group. Mice were sacrificed under anesthesia at 24 h after LPS injection. Samples of spleen, lung, and blood were collected for analysis.

### Cytokine analysis

Serum, tissue homogenate, and cell culture supernatant samples were analyzed for cytokine levels using commercially available DuoSet ELISA kits, according to the manufacturer's

instructions. Human IL-1 $\beta$  (BioLegend, San Diego, CA, USA), human IL-10 (BioLegend), human TNF- $\alpha$  (BD Biosciences, San Jose, CA, USA), mouse IL-1 $\beta$  (R&D Systems, Minneapolis, MN, USA), and mouse IL-10 (BioLegend).

### Transfection

The siRNA duplexes corresponding to human pyrin and IL-10 were purchased from Santa Cruz Biotechnology. Transient transfection of siRNA was carried out using Metafectene Transfection Reagent (Biontix Laboratories GmbH, Martinsried, Germany). In brief, THP-1 cells were cultured in 12-well plates at 70%–80% confluence and transfected with siRNA (100 nM) by Metafectene Transfection Reagent according to the manufacturer's instructions. The transfection efficiency was determined by real-time PCR. Scrambled siRNA (Santa Cruz Biotechnology) was used as a control.

### RNA extraction and real-time PCR

Total RNA were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. First-strand cDNA synthesis was performed with 2  $\mu$ g of total RNA using M-MLV reverse transcriptase, RNase inhibitor, 10 mM dNTP mixture, M-MLV reverse transcription buffer, and oligo(dT) adaptor as a primer (Promega, Madison, WI, USA). Total RNA and oligo (dT) 15 primer were incubated at 70 °C for 5 min. All other components were added and the mixture was incubated at 42 °C for 1 h, and then terminated at 95 °C for 5 min. Real-time PCR was carried out using SYBR Green PCR reagents (Qiagen, Valencia, CA, USA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The expression of GAPDH was used as an internal control. The forward and reverse primers used for PCR were as follows: Human pyrin: forward primer 5'-TCA TTT TCC CTC AGA ACC CC-3' reverse primer 5'-CAA TCC AGT CTG CTT GCG TT-3'. Human IL-10: forward primer 5'-CTT GCT GGA GGA CTT TAA GGG TT-3' reverse primer 5'-GGA GTT CAC ATG CGC CTT G-3'. Human GAPDH: forward primer 5'-CAA TGA CCC CTT CAT TGA CCT C-3' reverse primer 5'-AGC ATC GCC CCA CTT GAT T-3'.

### Western immunoblotting

Western immunoblot procedures were performed essentially as previously described.<sup>31</sup> In brief, amples containing 20  $\mu$ g protein were separated on 7% or 13% SDS-PAGE followed by transfer to polyvinylidene difluoride membranes (Thermo Scientific, Rockford, IL, USA). Following incubations with primary antibodies and horseradish peroxidase-conjugated secondary antibodies, immunocomplexes were detected using ECL Plus Western Blotting Substrate (Thermo Scientific).  $\beta$ -actin is used to normalize and account for loading variability. The relative signal intensity of bands was determined and standardized using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Evaluation of lung injury severity

Lung tissues were fixed in 10% neutral-buffered formalin solution and then embedded in paraffin. Formalin-fixed

and paraffin-embedded tissues were cut into 5 mm thick sections. Lung tissue sections were stained with hematoxylin and eosin (H&E) to visualize the damage. The lung injury scores (LIS), quantified the severity of lung injury, we determined by the inspection of H&E-stained whole lung sections, as previously described.<sup>32</sup> Briefly, LIS was assessed based on four aspects (alveolar capillary congestion, hemorrhage, infiltration or aggregation of neutrophils in the air space or the vessel wall, and thickness of the alveolar wall/hyaline membrane formation). Each of the four components was categorized from 0 to 4, where a higher number is more severe. Two separate investigators evaluated and scored in a blinded manner.

### Immunoprecipitation

For immunoprecipitations, protein G-coupled magnetic beads (Dynabeads Protein G; Invitrogen) were used according to the manufacturer's instructions. About 50  $\mu$ L of dynabeads protein G was mixed with anti-pyrin or anti-ASC antibodies. The cell lysates were added to the beads-antibody complex and incubated with rotation for 2 h at 4 °C. The supernatant was removed by placing the tube on the magnet and the beads were washed three times with PBS. Proteins in the immunoprecipitates were detected with anti-pro-caspase-1 and anti-NLRP3 antibodies by western blotting.

### Statistical analysis

For statistical comparisons, all values are expressed as mean  $\pm$  SEM and *p*-values were determined using Student's *t*-test.

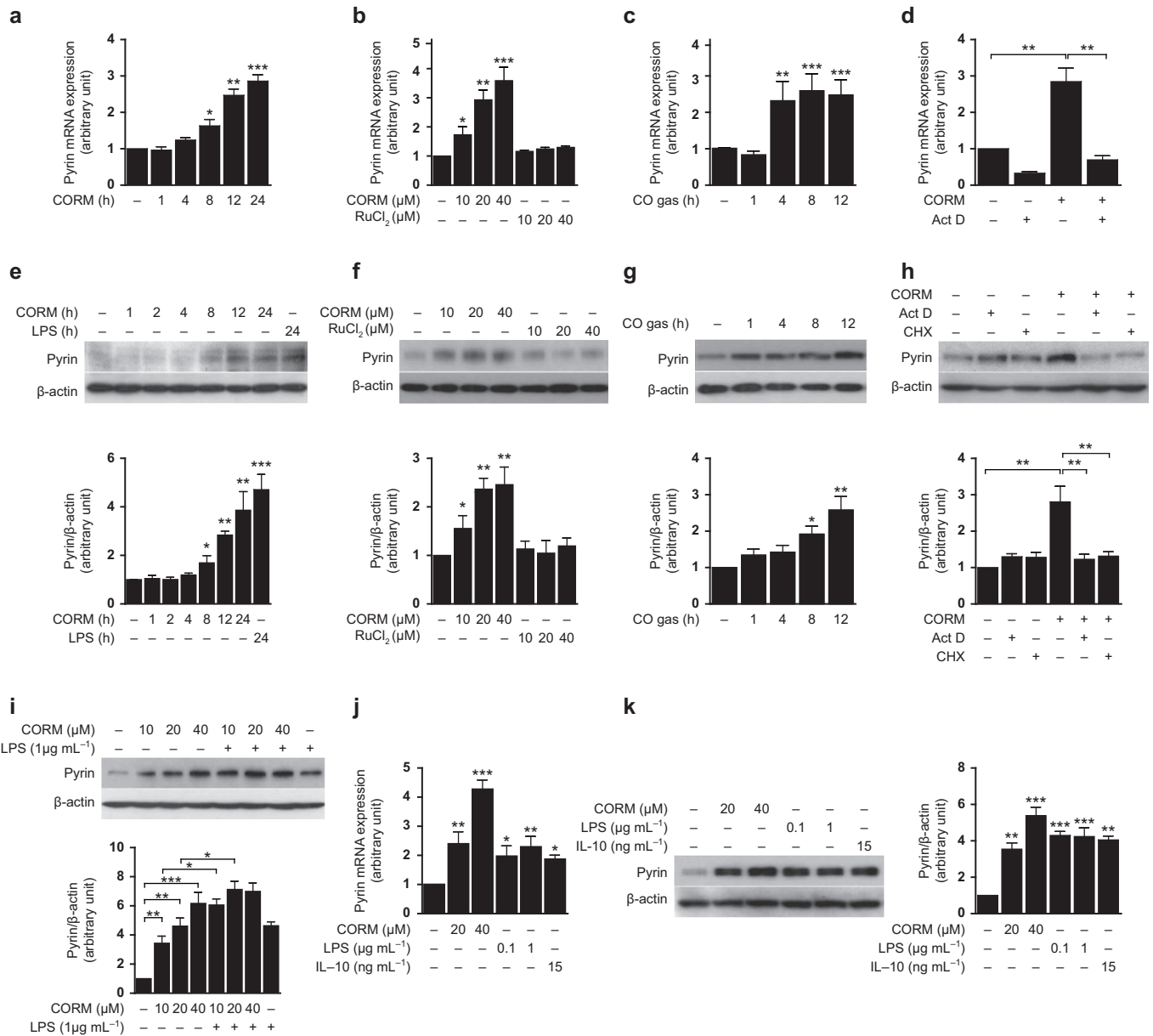
## RESULTS

### Carbon monoxide induces pyrin mRNA and protein expression

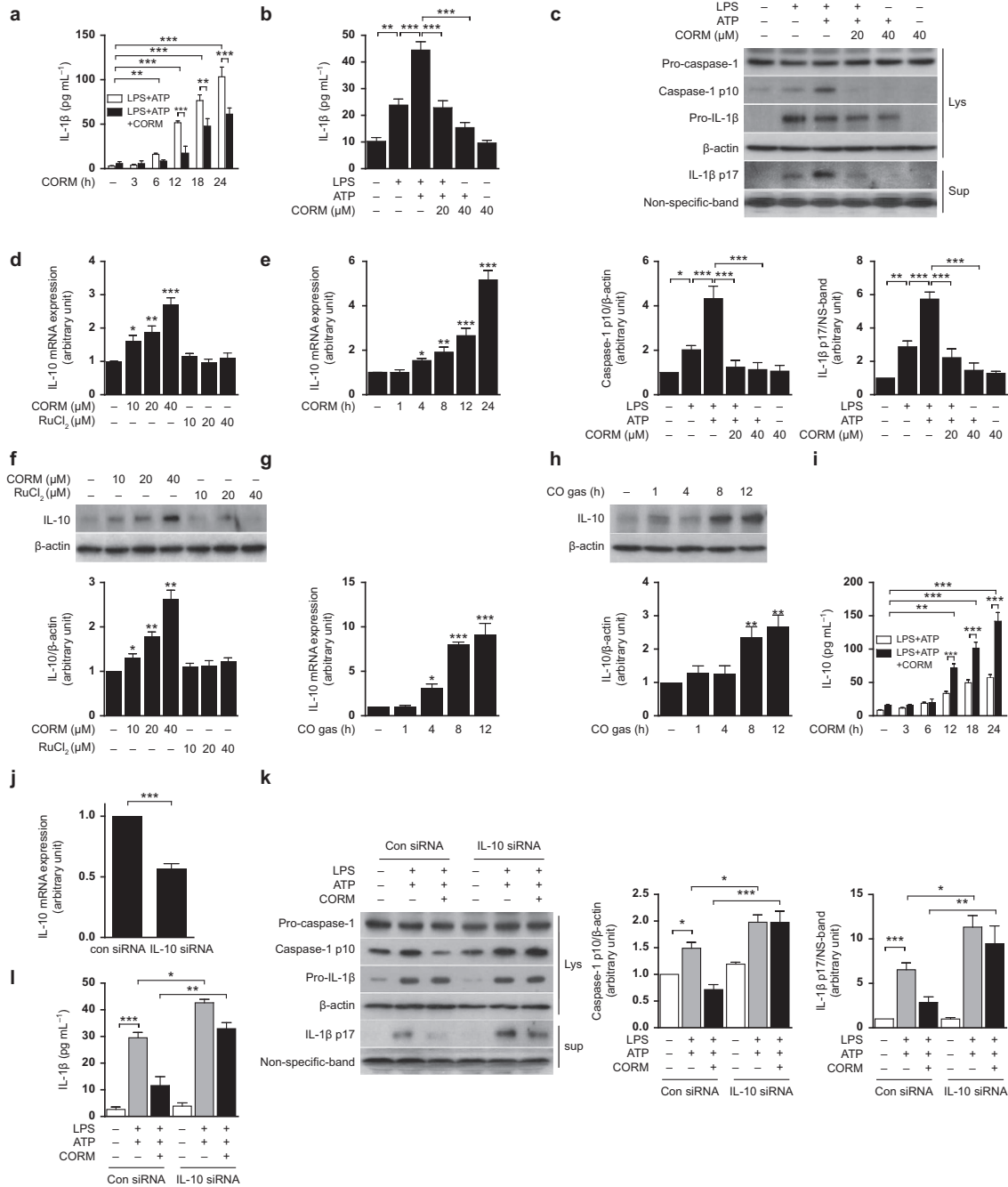
Pyrin can suppress IL-1 $\beta$  production through the inhibition of caspase-1 activation.<sup>33</sup> CO can abrogate the production of pro-inflammatory cytokines induced by LPS including IL-1 $\beta$  and TNF- $\alpha$ .<sup>27,29</sup> Therefore, in this study, we tested the hypothesis that the regulation of pyrin can serve as an underlying mechanism for CO-dependent suppression of IL-1 $\beta$ . To investigate the effects of CO on pyrin expression, the human leukemic cell line (THP-1) was treated with the CO-releasing molecule (CORM-2) at various concentrations and time intervals. Treatment with CORM-2 induced the expression of pyrin mRNA in a time- and dose-dependent manner. After CORM-2 (20  $\mu$ M) treatment, the levels of pyrin mRNA and protein significantly increased at 8 h and continued to increase up to 24 h (Figure 1a and e). Increased mRNA and protein levels of pyrin were observed at doses starting at 10  $\mu$ M in cells treated with CORM-2 for 12 h (Figure 1b and f). We also measured the levels of pyrin mRNA and protein after exposure to CO gas (250 ppm) *in vitro*. CO gas also increased pyrin expression in a time-dependent manner (Figure 1c and g). To explore whether *de novo* transcription was required for the induction of pyrin by CO, THP-1 cells were treated with CORM-2 in the absence or presence of the transcriptional inhibitor, Act D. CORM-2-induced pyrin expression was abrogated by Act D (Figure 1d). Similar time dependency of pyrin protein

expression was observed in response to CORM-2 treatment, beginning at 8 h and continuing to increase to a maximum at 24 h. The response at 24 h was similar to that induced by treatment with  $1 \mu\text{g mL}^{-1}$  LPS (Figure 1e). Subsequently, we confirmed whether *de novo* protein synthesis was required. Treatment of cells with CHX, a protein synthesis inhibitor, completely antagonized the induction of pyrin expression by CO (Figure 2h). Because pyrin expression was induced by LPS, we

also investigated whether CORM-2 treatment could affect the LPS-induced pyrin expression. The induction of pyrin expression by LPS alone was further increased by combination treatment with CORM-2 at doses of 10 and  $20 \mu\text{M}$ . At  $40 \mu\text{M}$  CORM-2, the response was maximal in the absence or presence of LPS (Figure 1i). The levels of pyrin mRNA and protein in response to  $20 \mu\text{M}$  CORM-2 were comparable to that of the induction achieved by other known pyrin-inducing substances,



**Figure 1** (a, b, e, f) THP-1 cells were treated with  $20 \mu\text{M}$  CORM-2 for indicated times and various concentrations of CORM-2 or  $\text{RuCl}_2$  for 12 h. (c and g) THP-1 cells were exposed with CO gas (250 ppm) for indicated time points. (d and h) THP-1 cells were incubated with CORM-2 and/or the transcriptional inhibitor actinomycin D (Act D;  $1 \mu\text{g mL}^{-1}$ ) and cycloheximide (CHX;  $5 \mu\text{g mL}^{-1}$ ) for 12 h. (i) THP-1 cells were pretreated with CORM-2 at the indicated concentrations for 30 min, and then treated with LPS ( $1 \mu\text{g mL}^{-1}$ ) for 12 h. (j and k) THP-1 cells were incubated with CORM-2, LPS, or IL-10 at the indicated concentrations for 12 h. a, b, c, d, j: The levels of pyrin mRNA were analyzed by quantitative real-time PCR. GAPDH served as the standard. e, f, g, h, i, k: The expression of pyrin was determined by western blot analysis.  $\beta$ -actin served as the standard. The relative band density on western blots was normalized to the  $\beta$ -actin loading control and was quantitated. All experiments were performed in triplicate, and representative data are shown. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.005$ .



**Figure 2** (a) THP-1 cells were stimulated with LPS (1 μg mL<sup>-1</sup>) in the absence or presence of CORM-2 (20 μM) at indicated time, and then ATP (5 mM) was added for the last 30 min. Secretion of IL-1β in the supernatants was quantified by ELISA. (b and c) THP-1 cells were pretreated with CORM-2 at indicated concentrations for 30 min, and then treated with LPS (1 μg mL<sup>-1</sup>) for 12 h and ATP (5 mM) was added for the last 30 min. (b) Secreted IL-1β in the supernatants was measured by ELISA. (c) The pro-caspase-1, cleaved caspase-1 (p10), pro-IL-1β, and IL-1β p17 in the supernatants (Sup) and the cell lysates (Lys) were analyzed by western blot analysis. (d and e) The expression of IL-10 mRNA was measured by quantitative real-time PCR in the various concentrations of CORM-2 or RuCl<sub>2</sub> for 12 h (d) and CORM-2 (20 μM) for increasing time intervals (e). (f) IL-10 protein was determined by western blot. (g and h) THP-1 cells were inhaled with different times of CO gas (250 ppm). Expression of IL-10 mRNA (g) and protein (h) was assayed by quantitative real-time PCR and western blot. (i) THP-1 cells were stimulated with LPS (1 μg mL<sup>-1</sup>) in the absence or presence of CORM-2 (20 μM) at indicated time, and then ATP (5 mM) was added for the last 30 min. Secreted IL-10 was detected by ELISA in the supernatants. (j-l) THP-1 cells were transfected with IL-10 siRNA or control siRNA (con), pre-treated with or without 20 μM CORM-2 for 30 min, and then treated with LPS (1 μg mL<sup>-1</sup>) for 12 h and ATP (5 mM) was added for the last 30 min. (j) The effectiveness of pyrin knockdown was confirmed by quantitative real-time PCR. (k) Western blot of the cellular extracts probed for the indicated protein. (l) ELISA for IL-1β in the supernatants. GAPDH and β-actin served as a loading control for quantitative real-time PCR and western blot analysis, respectively. The relative band density on western blots was normalized to the β-actin loading control and was quantitated. Representative data are shown. Data are expressed as mean ± SEM (n = 3). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.005.



including LPS and IL-10 (Figure 1j and k). These results suggest that CO not only induces the basal expression of pyrin but can augment the response to LPS stimulation. These data taken together suggest that CO is sufficient for the induction of pyrin, which is dependent on *de novo* transcription and protein synthesis.

### Carbon monoxide inhibits the release of IL-1 $\beta$ *via* IL-10 induction

CO has been shown to inhibit the expression of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and increase the expression of anti-inflammatory IL-10 by LPS.<sup>20</sup> Although CO suppresses the secretion of IL-1 $\beta$  by LPS, the involvement of caspase-1 remains unclear. In order to investigate this possibility, we first confirmed the inhibitory effect of CO on LPS and ATP-induced IL-1 $\beta$ . THP-1 cells were stimulated with the indicated concentrations of CORM-2 (20, 40  $\mu$ M) in the absence or presence of LPS and ATP, an activator of the NLRP3 inflammasome. Secreted IL-1 $\beta$  levels were measured using ELISA and western blot analysis of cell supernatants. Although LPS treatment alone moderately increased the secretion of IL-1 $\beta$ , LPS, and ATP had a greater effect on IL-1 $\beta$  expression in THP-1 cells than LPS alone. CORM-2 treatment time- and dose-dependently reduced the secretion of IL-1 $\beta$  by LPS and ATP (Figure 2a and b). As shown in Figure 2a and b, CORM-2 treatment alone did not affect the secretion of IL-1 $\beta$ . Since the inflammasome is a multi-protein complex that mediates the activation of caspase-1, which promotes the secretion of IL-1 $\beta$ , we investigated the inhibitory effect of CO on caspase-1 activation in response to LPS and ATP. CORM-2 treatment inhibited caspase-1 activation in THP-1 cells exposed to LPS and ATP (Figure 2c). We then confirmed the effect of CO on the induction of IL-10. Treatment of THP-1 cells in response to CORM-2 dose- and time-dependently increased the expression of IL-10 mRNA (Figure 2d and e) and protein (Figure 2f). We also confirmed that the levels of IL-10 mRNA and protein were increased by exposure to CO gas (250 ppm) (Figure 2g and h). Moreover, we found that CORM-2 treatment time-dependently increased much more the secretion of IL-10 by LPS and ATP (Figure 2i). To determine the involvement of IL-10 in the induction of caspase-1 by CO, we silenced the IL-10 gene using siRNA, and confirmed the effectiveness of IL-10 knockdown by western analysis (Figure 2j). CORM-2 treatment remarkably inhibited caspase-1 activation and IL-1 $\beta$  maturation and secretion, whereas IL-10 siRNA reversed the effects of CORM-2 (Figure 2k and l). These results suggest that CO can regulate the NLRP3 inflammasome response specifically through IL-10-dependent downregulation of the caspase-1 pathway.

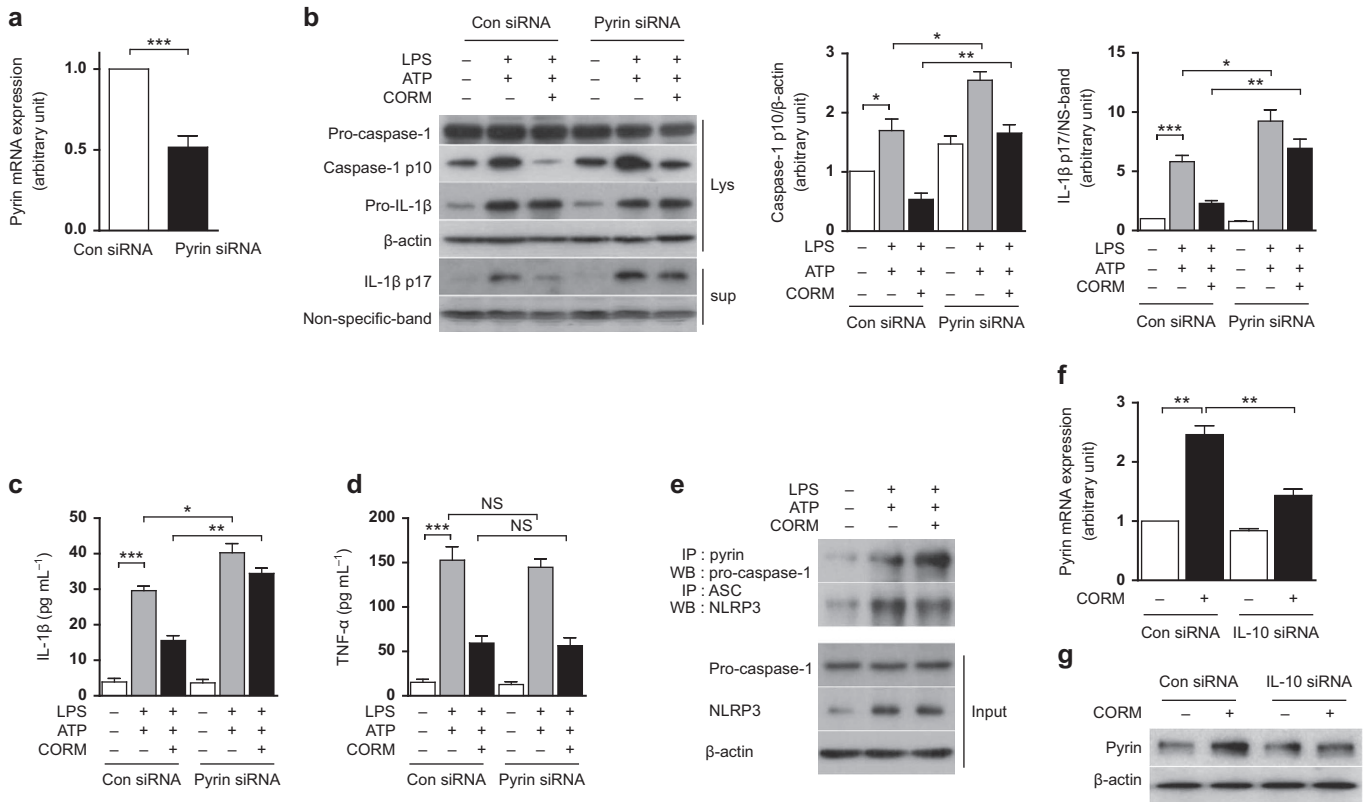
### Carbon monoxide inhibits release of IL-1 $\beta$ *via* IL-10-induced pyrin expression

To determine whether the induction of pyrin was related to suppression of LPS-induced IL-1 $\beta$  production by CO, THP-1 cells were transiently transfected with pyrin small interference RNA (siRNA) or with control (scramble) siRNA. The efficacy of the

knockdown was confirmed by real-time PCR (Figure 3a). In order to assess the role of pyrin in the inhibitory effect of CO on LPS and ATP-triggered inflammasome activation, we analyzed the secretion and production of IL-1 $\beta$ , and caspase-1 activation. Higher secretion levels of IL-1 $\beta$  by LPS and ATP were measured in pyrin siRNA-transfected cells compared to control siRNA-transfected cells. The inhibitory effects of CO on LPS and ATP-induced IL-1 $\beta$  were significantly reduced in pyrin siRNA-transfected cells (Figure 3b and c). Consistent with these observations, the suppression of pyrin by siRNA inhibited the effect of CORM-2 with respect to the reduction of caspase-1 activation by LPS and ATP (Figure 3b). In contrast to the effect of pyrin on CO-dependent regulation of IL-1 $\beta$  expression, pyrin did not antagonize the inhibitory effect of CO on LPS-induced TNF- $\alpha$  production (Figure 3d). This result suggests that the decrease of IL-1 $\beta$  production by CO is caused by the inhibition of NLRP3-dependent caspase-1 activation by CO-dependent pyrin induction. To verify the role of pyrin in inflammasome formation in response to LPS and ATP, the association of pyrin and pro-caspase-1 was demonstrated by an immunoprecipitation assay. CORM-2 increased the binding of pyrin and pro-caspase-1 and decreased the association of NLRP3 and ASC (Figure 3e). Pyrin was previously reported to be induced by LPS or cytokines including IL-10.<sup>17</sup> We hypothesized that suppression by CO of LPS-induced IL-1 $\beta$  production involves the induction of IL-10 which in turn regulates pyrin expression. THP-1 cells were transiently transfected with IL-10 siRNA or control siRNA and then treated with CORM-2. Knockdown of IL-10 reduced CORM-2 dependent expression of pyrin mRNA and protein (Figure 3f and g). These results indicate that IL-10 plays an intermediate role in the effects of CO on pyrin induction, resulting in IL-10-pyrin pathway that contributes to the IL-1 $\beta$  suppression by CO.

### Carbon monoxide inhibits release of IL-1 $\beta$ and induces pyrin induction *in vivo*

In this study, we have observed the effects of CO on pyrin expression *in vitro*. We have performed additional *in vivo* studies to validate the relevance of our findings. C57BL/6 mice were subjected to CO inhalation (250 ppm) for 4 h each day for 5 days. Pyrin expression is detected predominantly in the lung, spleen, and muscle.<sup>34</sup> CO inhalation significantly increased pyrin expression in lung and spleen tissue (Figure 4a and b). To investigate the effect of CO on pyrin expression in an LPS-induced model of inflammation, C57BL/6 mice were pre-exposed to CO inhalation and then given LPS challenge by i.p. injection. While treatment with LPS stimulated pyrin expression in the mice, higher expression was observed when mice were subjected to CO inhalation pre-treatment and then challenged with LPS (Figure 4c, Supplementary Figure 1a for CORM-2 i.p. injection). IL-10, which is known to induce the expression of pyrin, was also increased in the lung and spleen after CO inhalation (Figure 4d, Supplementary Figure 1b for CORM-2 i.p. injection). In contrast, CO inhalation significantly decreased the levels of IL-1 $\beta$  induced by LPS (Figure 4e, Supplementary Figure 1c for CORM-2 i.p. injection). Because lung tissue is one of the most important targets



**Figure 3** (a–d) THP-1 cells were transfected with pyrin siRNA or control siRNA (con), pre-treated with or without 20  $\mu$ M CORM-2 for 30 min, and then treated with LPS ( $1 \mu\text{g mL}^{-1}$ ) for 12 h and ATP (5 mM) was added for the last 30 min. (a) The knockdown of pyrin by siRNA was confirmed by quantitative real-time PCR. (b) The pro-caspase-1, cleaved caspase-1 (p10), pro-IL-1 $\beta$ , and IL-1 $\beta$  p17 in the supernatants (Sup) and the cell lysates (Lys) were analyzed by western blot analysis. Secreted IL-1 $\beta$  (c) and TNF- $\alpha$  (d) in the supernatants were measured by ELISA. (e) THP-1 cells were exposed with LPS ( $1 \mu\text{g mL}^{-1}$ ) + ATP (5 mM) in the absence or presence of CORM-2 (20  $\mu$ M). The interaction of pyrin and pro-caspase-1 was analyzed by immunoprecipitation (IP) of anti-Pyrin followed by western blot (WB) for anti-pro-caspase-1. To evaluate the binding ASC and NLRP3, cell lysates were performed IP with anti-ASC and then followed by WB for anti-NLRP3. Total cell lysates were used as input controls. (f and g) THP-1 cells were transfected with pyrin siRNA or control siRNA (con), cells were exposed with 20  $\mu$ M of CORM-2 for 12 h. The levels of pyrin mRNA (f) and protein (g) were measured. GAPDH and  $\beta$ -actin were used as a loading control for quantitative real-time PCR or western blot analysis, respectively. The relative band density on western blots was normalized to the  $\beta$ -actin loading control and was quantitated. All experiments were performed in triplicate, and representative data are shown. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.005$ .

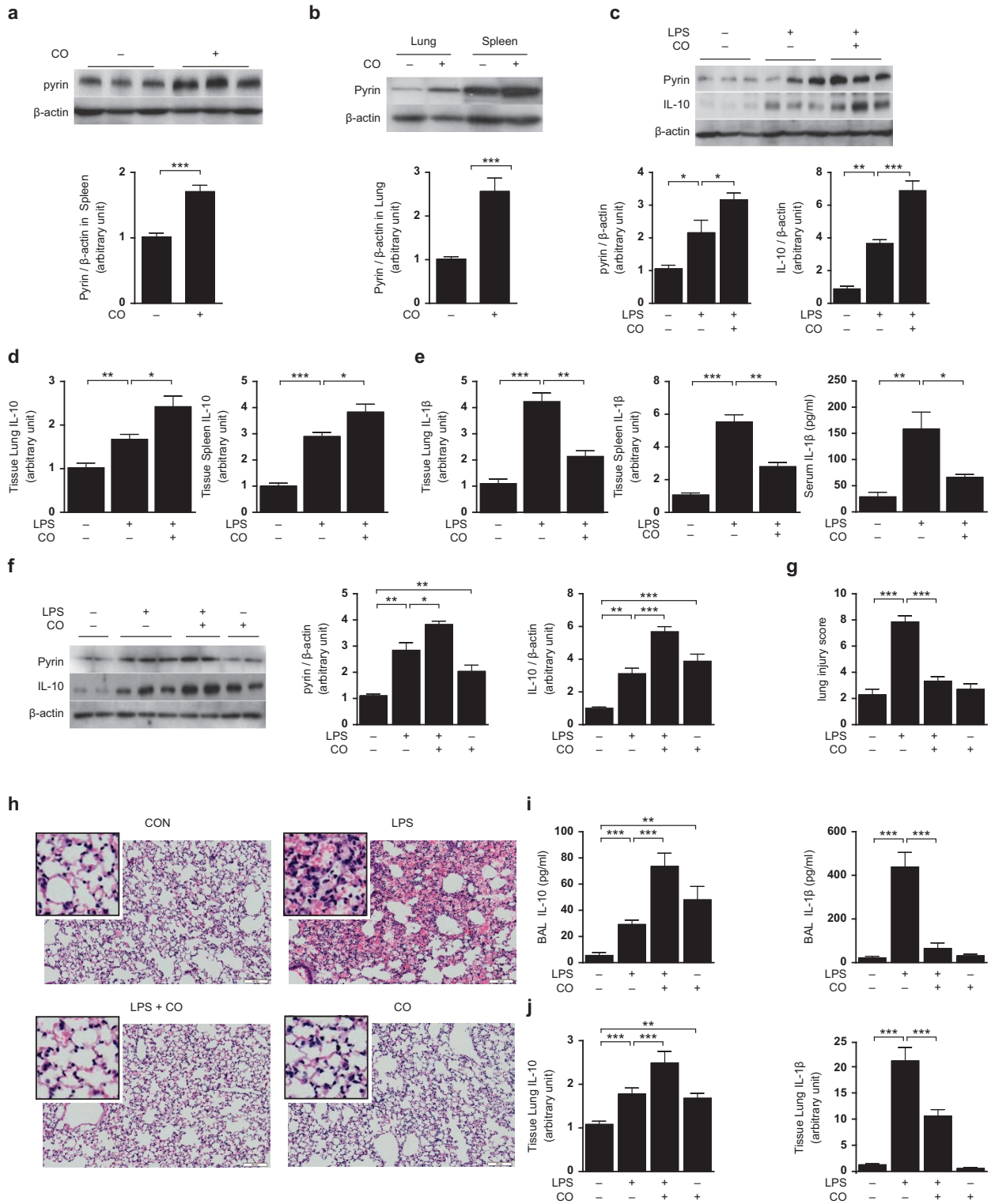
of CO inhalation, we confirmed the effect of CO on pyrin expression using an ALI model. C57BL/6 mice were challenged with intranasal LPS administration after CO inhalation. Consistent with the results observed with the LPS i.p. injection model, pyrin expression could be markedly increased by intranasal LPS. The mice exposed to CO inhalation in the presence of LPS had significantly greater expression of pyrin compared to the LPS-induced ALI group (Figure 4f). Histological examination of lung sections confirmed a protective effect of CO inhalation on LPS-induced ALI. As a consequence of LPS-induced ALI, the lung showed pathologic changes, such as alveolar thickening, interstitial edema, and inflammatory cell infiltration. However, these histopathological changes in lung tissue challenged with LPS were ameliorated by CO inhalation (Figure 4g and h). The levels of IL-1 $\beta$  and IL-10 in the LPS-induced ALI group increased significantly after LPS compared to those in the control group. CO treatment significantly increased the level of IL-10, but at the same time decreased

the production of LPS-induced IL-1 $\beta$  in both BAL fluid (Figure 4i) and lung tissue (Figure 4j). A scheme is provided to illustrate each of these results (Figure 5).

## DISCUSSION

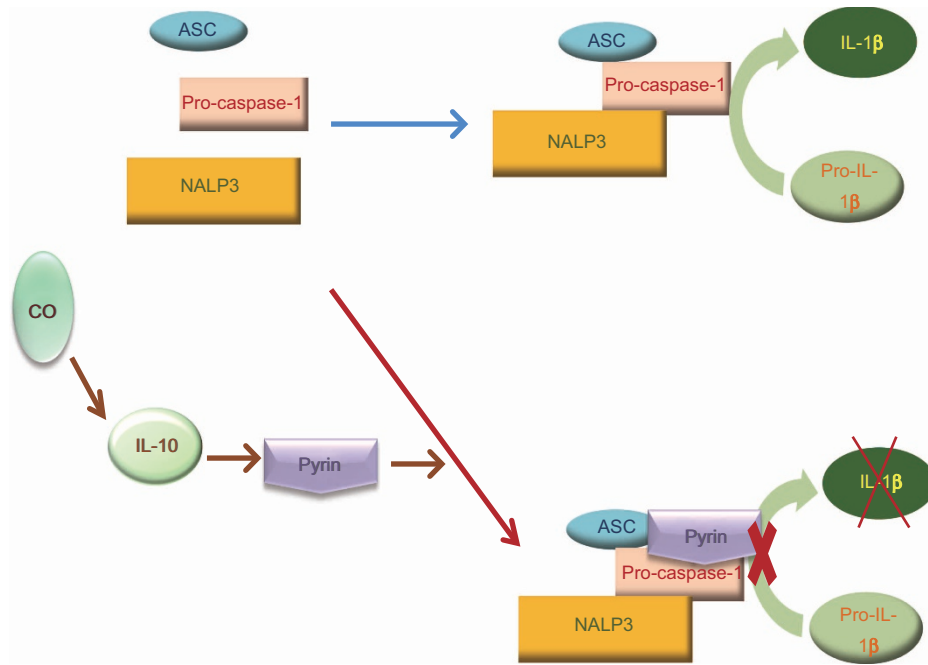
CO, a reaction product of HO-1 enzymatic activity, can exert protective effects in multiple models of organ injury, including vascular injury,<sup>35</sup> transplantation,<sup>36,37</sup> and I/R injury.<sup>38</sup> In several lung injury models, CO inhalation at a low concentration conferred cyto- and tissue protection through modulation of inflammation and apoptosis.<sup>39</sup> Although several studies have now reported tissue protective effects of CO in models of lung inflammation,<sup>20</sup> the underlying mechanisms remain incompletely understood.

The anti-inflammatory effect of CO demonstrated in LPS-stimulated macrophages, required activation of p38 MAPK pathway,<sup>20</sup> and inhibition of NADPH oxidase-dependent signaling.<sup>40</sup> Additional signal transduction pathway molecules



**Figure 4** (a–e) C57BL/6 mice were exposed to air or CO gas (250 ppm) for 4 h each day for 5 days and then injected with LPS (10 mg kg<sup>-1</sup>, i.p.) for 24 h. The levels of pyrin and IL-10 were detected in the lung (a and c) and spleen (b) tissue. Protein expression was measured using western blot analysis. β-actin served as the standard. IL-10 (d) and IL-1β (e) in the lung, spleen tissue, and serum measured by ELISA. (f–j) C57BL/6 mice were exposed to air or CO gas (250 ppm) for 4 h each day for 5 days and then subjected to intranasal administration of LPS (2.5 mg kg<sup>-1</sup>). (f) The levels of pyrin and IL-10 in the lung after 24 h. (g and h). Lung sections were stained with H&E (h) and lung injury was scored (g). (i) IL-10 and IL-1β levels in BAL fluid were quantified by ELISA. (j) IL-10 and IL-1β levels were detected in lung tissue. The relative band density on western blots was normalized to the β-actin loading control and was quantitated. All experiments were performed in triplicate (n = 5/group), and representative data are shown. Quantitative data are expressed mean ± SEM. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.005.





**Figure 5** Schema representing the downregulation of IL-1 $\beta$  production by CO *via* suppressing the inflammasome through induction of pyrin. CO increases pyrin expression in an IL-10-dependent manner. The increased pyrin downregulates IL-1 $\beta$  production *via* suppressing the NLRP3 inflammasome and associated caspase-1 cleavage.

have been implicated in CO dependent anti-inflammatory effects, including mitochondrial reactive oxygen species-dependent modulation of the PPAR $\gamma$ /Egr-1 pathway,<sup>41</sup> caveolin-1, which inhibits TLR4 signaling<sup>42</sup> and immunoresponsive gene 1 (IRG1).<sup>43</sup>

During the activation of the inflammasome, pyrin associates with ASC through its pyrin domain and also interacts with caspase-1, and thereby regulates caspase-1 activation and IL-1 $\beta$  production.<sup>12</sup> Pyrin, which is predominantly expressed in the lung and spleen,<sup>16</sup> can inhibit IL-1 $\beta$  expression by inhibiting NLRP3 inflammasome formation. Pyrin expression is increased by various cytokines and LPS. Under various conditions, pyrin may exert either a pro-inflammatory or anti-inflammatory function.<sup>33,44,45</sup> In our model, we found that pyrin induced by CO plays an important role as an anti-inflammatory mediator. We demonstrated that CO-increased the expression of pyrin which in turn inhibited the maturation of IL-1 $\beta$  *via* the suppression of caspase-1 activity. The importance of pyrin in CO-dependent inflammasome regulation was validated using siRNA-dependent knockdown of pyrin (Figure 3). However, genetic deficiency of pyrin did not affect the reduction of TNF- $\alpha$  by CO treatment (Figure 3). Our results suggest that CO can down regulate the NLRP3 inflammasome through the upregulation of pyrin.<sup>12</sup> In contrast, the downregulation of TNF- $\alpha$  expression by CO potentially involves other mechanisms, which we have previously described.<sup>46</sup> For example, CO increased tristetrarolin expression, resulting in the downregulation of TNF- $\alpha$  expression through a post-transcriptional mechanism involving the AU-rich region (ARE) in the 3'-UTR of the corresponding mRNA.<sup>42</sup> Furthermore, CO has been

reported to ameliorate hepatic I/R injury through the downregulation of pro-inflammatory cytokines, and the upregulation of IL-10.<sup>29,30</sup> In other inflammatory disease models such as inflammatory bowel disease, CO exposure or pharmacologic HO-1 induction increased IL-10 protein secretion and suppressed IL-1 $\beta$ , TNF- $\alpha$ , and IL-4 production.<sup>29,47</sup> The induction of IL-10 can stimulate the increase of pyrin expression, which inhibits inflammasome activation in alveolar macrophages and lung endothelial cells.<sup>17</sup> Therefore, we demonstrated, using siRNA against IL-10, that CO induced IL-10 expression inhibits inflammasome activation *via* an increase of pyrin. In non-hematopoietic tissues, pyrin mRNA was detected predominantly in the spleen, lung, and muscle tissue.<sup>16</sup> This indicates that expression of pyrin is regulated in a tissue-specific manner with preferential expression in hematopoietic cells. Expression of pyrin in the lung and muscle may result to some extent from infiltrating macrophages or neutrophils. We addressed the effect of CO on pyrin expression in macrophage cell lines as well as lung tissue and infiltrated cells (Figures 1 and 4). A direct role of pyrin in suppressing NLRP3 inflammasome activation in lung cells was previously demonstrated by Xu *et al.*<sup>17</sup> These studies suggest that the formation of a pyrin-NLRP3 inflammasome complex suppressed the NLRP3-ASC association. Furthermore, deficiency of pyrin expression using siRNA increased IL-1 $\beta$  secretion through NLRP3 inflammasome activation. Thus, pyrin expression exerts an important function in the suppression of IL-1 $\beta$  secretion. Even though LPS can induce pyrin expression through autocrine and paracrine pathways,<sup>17</sup> treatment with CO highly augmented LPS-induced pyrin expression in our study. These findings provide

a novel, and previously unidentified mechanism for the anti-inflammatory effects of CO in lung. In our *in vivo* experiments, we showed that CO attenuated lung injury by LPS through the increase of pyrin levels. In our study, we provide a novel mechanism for the therapeutic effects of CO in ALI. Specifically, CO-induced pyrin expression plays an important role in attenuating lung inflammation responses through the downregulation of IL-1 $\beta$  production *via* suppressing the NLRP3 inflammasome.

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