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Calciprotein Particles Induce IL-1β/α–Mediated Inflammation through NLRP3 Inflammasome-Dependent and -Independent Mechanisms

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ABSTRACT

Calciprotein particles (CPPs) are nanoparticles composed of calcium phosphate crystals and fetuin-A and have been implicated in diseases associated with inflammation. In the current study, we investigated the molecular mechanisms underlying CPP-induced inflammation in mice. CPPs predominantly upregulated IL-1β and IL-1α and provided priming and activation signals for the NLRP3 inflammasome in murine macrophages. Pharmacological and genetic inhibition of the NLRP3 inflammasome revealed that CPPs induced the release of IL-1β and IL-1α via NLRP3 inflammasome-dependent and -independent mechanisms, respectively. CPPs also induced necrotic cell death, but gasdermin D was dispensable for CPP-induced IL-1β release and necrotic cell death. Although phagocytosis of CPPs was required for CPP-induced IL-1β/α release and necrotic cell death, lysosomal dysfunction and K+ efflux were mainly involved in CPP-induced NLRP3 inflammasome activation and subsequent IL-1β release but not in CPP-induced IL-1α release and necrotic cell death. In vivo experiments showed that CPP administration evoked acute inflammatory responses characterized by neutrophil accumulation via both IL-1β and IL-1α. In particular, CPP-induced neutrophil inflammation was mediated predominantly through an IL-1α–induced CXCL1/CXCR2 signaling pathway. These results provide new insights into the mechanism underlying CPP-induced inflammation and suggest that targeting both IL-1β and IL-1α is necessary to regulate the CPP-induced inflammatory response and to treat CPP-associated inflammatory disorders. ImmunoHorizons, 2021, 5: 602–614.

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Abbreviations used in this article: CKD, chronic kidney disease; CPP, calciprotein particle; Cy3, cyanine 3; FLICA, fluorescent-labeled inhibitor of caspases; GSDMD, gasdermin D; LDH, lactate dehydrogenase; MSU, monosodium urate; NAC, N-acetyl-L-cysteine; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3; RCD, regulated cell death; ROS, reactive oxygen species; TCP, tricalcium phosphate; WT, wild type.

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INTRODUCTION

An imbalance of metabolism results in the formation of endogenous crystals and particles, which participate in the progression of various diseases (1). Hyperuricemia leads to the formation of monosodium urate (MSU) crystals, which cause gout (2). Hyperlipidemia causes the deposition of cholesterol crystals in atherosclerotic lesions, which promotes the progression of atherosclerosis (3). Furthermore, recent studies have demonstrated that hyperphosphatemia endogenously causes the formation of particles called calciprotein particles (CPPs) (4). CPPs are defined as aggregates of the serum protein fetuin-A laden with tiny calcium phosphate precipitates that are dispersed in blood as a colloid (5). The levels of circulating CPPs increase with serum phosphate levels in patients with chronic kidney disease (CKD) and are positively correlated with the progression of CKD (6). Elevated CPP levels are also associated with increased aortic stiffness and calcification in CKD patients (7, 8). Moreover, a recent study showed that a high CPP level is a surrogate marker for coronary atherosclerosis, especially in lipid-rich plaque, and contributes to an increased risk of plaque vulnerability (9). Another important feature of CPPs is their inflammatory effect. For instance, serum CPP levels are positively correlated with serum C-reactive protein levels in CKD patients (10). Thus, CPPs are implicated in chronic inflammation and the pathogenesis of cardiovascular and kidney diseases.

CPPs can be classified into primary CPPs and secondary CPPs (11). Primary CPPs are composed of amorphous calcium phosphate precipitates and fetuin-A molecules. Primary CPPs spontaneously undergo aggregation and transition of calcium phosphate from the amorphous phase to the crystalline phase to become secondary CPPs. Secondary CPPs but not primary CPPs have the ability to induce calcification in cultured vascular smooth muscle cells. In addition, several studies have suggested that secondary CPPs containing calcium phosphate crystals induce the expression of inflammatory cytokines in macrophages (12). However, the precise mechanisms by which secondary CPPs induce inflammatory responses are not yet fully understood.

Accumulating evidence indicates that the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3 (NLRP3) inflammasome plays a central role in inflammatory responses induced by crystals and particles (1, 13). The NLRP3 inflammasome is an intracellular molecular complex composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 and is involved in the innate immune response through the recognition of damage/danger-associated molecular patterns or pathogen-associated molecular patterns (14–16). When NLRP3 senses damage/danger–associated molecular patterns or pathogen-associated molecular patterns, it assembles the NLRP3 inflammasome to promote the oligomerization of caspase-1, resulting in its autocleavage and activation. The active caspase-1 cleaves gasdermin D (GSDMD) to induce pyroptosis, which is a highly inflammatory cell death accompanied by the release of intracellular contents (17, 18). Although the cleavage of another member of the IL-1 family, cytokine IL-1α, is not accomplished by caspase-1, IL-1α release is also partially regulated by the NLRP3 inflammasome (19). Furthermore, unlike IL-1β, uncleaved pro–IL-1α can bind IL-1R and activate inflammatory signals (20), suggesting that the IL-1α–mediated inflammatory response is regulated by NLRP3 inflammasome-driven pyroptosis. Thus, the NLRP3 inflammasome can contribute to the release of IL-1β and IL-1α to initiate inflammatory responses.

It is generally accepted that various endogenous/exogenous crystals and particles induce NLRP3 inflammasome activation and subsequent inflammatory responses. MSU crystals, calcium pyrophosphate dihydrate crystals, cholesterol crystals, and nanosilica particles strongly activate the NLRP3 inflammasome and are implicated in the pathogenesis of gout, pseudogout, atherosclerosis, and silicosis, respectively (21–24). Therefore, we hypothesized that secondary CPPs (hereafter referred to for simplicity as CPPs) could activate the NLRP3 inflammasome and induce the release of IL-1β and IL-1α, thereby leading to inflammatory responses. To test this hypothesis, we used mice deficient in IL-1β, IL-1α, and GSDMD and found that secondary CPPs induce the release of IL-1β and IL-1α in an NLRP3 inflammasome-dependent and -independent manner, respectively, in macrophages. We further showed that excess secondary CPPs induce neutrophil accumulation and inflammatory responses via IL-1β and IL-1α in vivo.

MATERIALS AND METHODS

CPP preparation

For CPP preparation, DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FCS was mixed with 30 mM CaCl₂ and 1 M sodium phosphate to make the final concentration of calcium and phosphate 5 mM and 10 mM, respectively. The mixture was incubated at 37°C for 48 h and centrifuged at 16,000 g for 2 h. After the supernatant was discarded, the precipitated labeled CPPs, alendronate–Cy3 (0.05 μM), were added to the mixture for 60 min before centrifugation. Tricalcium phosphate (TCP) crystals were prepared as described previously (25).

Animals

All experiments in this study were carried out in accordance with the Jichi Medical University Guide for Laboratory Animals (permit number 17140-02). C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). NLRP3−/−, caspase-1−/−, and IL-1β−/− mice were kindly provided by Drs. Vishava M. Dixit (Genentech, South San Francisco, CA), Hiroko Tsutsui (Hyogo Medical College, Nishinomiya, Japan), Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan), respectively. GSDMD−/− mice were developed by Genentech (17) and...
provided by Dr. Kate Schroder (University of Queensland, Brisbane, Australia).

**Cell culture and in vitro experiments**

Murine J774 macrophages were maintained with DMEM (FUJIFILM Wako Pure Chemicals, Osaka, Japan) supplemented with 10% FCS. Murine peritoneal macrophages were isolated from mice using the thioglycollate elicitation method as described previously (26). Cells were primed with LPS (100 ng/ml; Sigma-Aldrich) for 6 h and then treated with CPPs for the indicated periods. For inhibitor experiments, cells were pretreated with polymyxin B (Sigma-Aldrich), MCC950 (AdipoGen), N-acetyl-L-cysteine (NAC; FUJIFILM Wako Pure Chemicals, Osaka, Japan), K777 (AdipoGen), cytochalasin D, CA-074Me (FUJIFILM Wako Pure Chemicals, Osaka, Japan), K5, K777 (AdipoGen), N-acetyl-L-cysteine (NAC; FUJIFILM Wako Pure Chemicals), and Mito-TEMPO (Enzo, Farmingdale, NY) for 30 min before CPP treatment. To block K+ efflux, KCl (45 mM) was added to the culture medium 30 min before CPP treatment. NaCl (45 mM) was used as an osmolarity control.

**CRISPR/Cas9-mediated genome editing in J774 macrophages**

The Traf6 gene was mutated by CRISPR/Cas9 in J774 macrophages. The guide RNA targeting Traf6 (5'-GGAGATCCAGGGCTACGATG-3') was designed with CRISPRdirect (http://crispr.dbcls.jp) and subcloned into LentiCRISPRv2, which was a gift from Feng Zhang (Addgene plasmid number 52961). For lentiviral transduction, J774 macrophages were incubated with lentiviral vectors overnight in the presence of 8 μg/ml polybrene (Sigma-Aldrich). The transduced cells were selected by incubating them with 2 μg/ml puromycin (Sigma-Aldrich).

**RT-PCR analysis**

Total RNA was prepared using ISOGEN (NIPPON GENE Co., Toyama, Japan) according to the manufacturer’s instructions. RT-PCR analysis was performed using the Takara TP960 PCR Thermal Cycler Dice Real Time System II (Takara Bio, Shiga, Japan) to detect the mRNA levels of Casp1, Cxcl1, Cxcr2, Il1a, Il6, Ly6g, Nlrp3, Pycard, Tnf, and Actb. The primers are listed in Supplemental Table I.

**Cytokine measurement**

Culture supernatants were centrifuged at 2,000 × g for 15 min to remove CPPs. The levels of IL-1β and IL-1α were determined using a Mouse ELISA kit (R&D Systems, Minneapolis, MN).

**Cell death assay**

Cytotoxicity was determined as lactate dehydrogenase (LDH) activity using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. To determine total cellular LDH activity, the cells were lysed with 2% Triton X-100. L-LDH from rabbit muscle (Roche) was used as a standard.

**Western blotting**

Western blotting was performed as described previously (27) using primary Abs for NLRP3 (clone cryo-2; AdipoGen), caspase-1 (AG-20B-0042-C100; AdipoGen), IL-1β (AF-401NA; R&D Systems), IL-1α (AF-400-SP, R&D Systems), GSDMD (ab209845; Abcam, Cambridge, UK), and β-actin, (A5441; Sigma-Aldrich). Immunoreactive bands were visualized by Western Blot Quant or Ultra Sensitive HRP Substrate (Takara Bio). The expression level of β-actin served as an internal control for protein loading.

**Measurement of caspase-1 activity**

Caspase-1 activity was analyzed using the carboxyfluorescein fluorescent-labeled inhibitors of caspases (FLICA) Caspase-1 Assay kit (98; ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer’s instructions. After the treatment, J774 macrophages were labeled with FLICA for 30 min and examined by flow cytometry (FACSVerse, BD Biosciences, San Jose, CA). The data were analyzed using FlowJo software (version 10; Tree Star, San Carlos, CA).

**Live cell imaging**

Cells were primed with LPS (100 ng/ml) for 6 h and then treated with alendronate–Cy3 (0.05 μM)–conjugated CPPs (30 μl/ml). The cells were stained by Alexa Fluor 488–conjugated dextran (m.w. 10,000; Thermo Fisher Scientific, Waltham, MA) at 50 μg/ml and Hoechst 33342 (Dojindo Molecular Technologies, Kumamoto, Japan) at 1 μg/ml for 1 h and 10 min, respectively, before CPP treatment. The localization of nuclei, lysosome, and CPPs was evaluated with Hoechst 33342 (blue), Alexa Fluor 488–dextran (green), and Cy3–alendronate (red), respectively, by using confocal laser scanning microscopy (FV10i; Olympus Life Science, Tokyo, Japan).

**Incorporation of Cy3-labeled CPPs**

Cells were treated with alendronate–Cy3 (0.05 μM)–conjugated CPPs, washed with PBS, and incubated with Cell Dissociation Solution (Biological Industries, Beit-Haemek, Israel) for 5 min. The detached cells were washed with PBS and examined by flow cytometry (FACSVerse). The data were analyzed using FlowJo software.

**CPP administration in vivo**

For i.p. administration, precipitated CPPs were resuspended in DMEM at 10-fold the original concentration. For i.v. administration, precipitated CPPs were resuspended in DMEM at 50-fold the original concentration and filtered by a 40-μm cell strainer (BD Biosciences).
**Analysis of neutrophil infiltration**

Cells from peritoneal lavage were analyzed using flow cytometry (FACSVerse; BD Biosciences). The cells were labeled with the following Abs: FITC-conjugated anti-CD45R (11-0452; eBioscience, San Diego, CA), PE-conjugated anti–Ly-6G (561104; BD Biosciences), and allophycocyanin-conjugated anti-CD45 (17-0451; eBioscience). The cells were examined by flow cytometry (FACSVerse) and analyzed using FlowJo software. Isotype control Abs were used as negative controls to exclude nonspecific background staining.

**Immunofluorescence staining**

Fresh-frozen sections of the liver, spleen, kidney, heart, and lung were fixed with 10% neutral buffered formalin for 5 min and permeabilized by 0.1% Triton X-100 for 10 min. After blocking with normal goat serum, slides were incubated with anti–Ly-6G Ab (clone 1A8; BioLegend, San Diego, CA). Unbound Abs were washed with PBS, and the slides were incubated with Alexa Fluor 594 goat anti-rat IgG (A-11007; Thermo Fisher Scientific). Nuclei were costained with DAPI, and fluorescence was detected by confocal laser scanning microscopy (FV10i, Olympus Life Science). No signals were detected when an irrelevant IgG (I-4000; Vector Laboratories, Burlingame, CA) was used as a negative control.

**Immunocytochemistry**

Cells were cultured in 4-well glass slide chambers and fixed with 10% neutral buffered formalin for 10 min at room temperature and then permeabilized by PBS containing 0.3% Triton X-100. After blocking with PBS containing 3% BSA, the slides were labeled with anti–NF-κB p65 (sc-372; Santa Cruz Biotechnology, Dallas, TX). Unbound Abs were washed with PBS, and the slides were incubated with Alexa Fluor 594 goat anti-rabbit IgG (A-11012, Thermo Fisher Scientific). Nuclei were costained with DAPI, and fluorescence was detected by confocal laser scanning microscopy (FV10i, Olympus Life Science).

**Statistics**

Data are expressed as mean ± SD. Differences between multiple group means were determined by one-way ANOVA or two-way ANOVA combined with the Tukey post hoc test. Analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). A p value <0.05 was considered to be statistically significant.

**RESULTS**

**CPPs induce IL-1α/β release in macrophages**

To investigate the inflammatory characteristics of CPPs, we treated murine J774 macrophages with CPPs and assessed the expression levels of inflammatory cytokines. CPPs robustly increased mRNA levels of Il1b and Il1a, whereas they moderately and modestly increased mRNA levels of Il6 and Tnf, respectively (Fig. 1A). Although we previously showed that TCP crystals clearly induce the activation of NLRP3 inflammasome and the release of IL-1β in LPS-primed macrophages (25), TCP failed to increase mRNA expression of Il1b, Il1a, Il6, and Tnf. CPPs slightly increased mRNA levels of Nlrp3, but not Asc or Casp1 (Fig. 1B). The possibility of LPS contamination was excluded by the treatment with polymyxin B (Supplemental Fig. 1A, 1B). Because the release of IL-1β is regulated by both transcriptional priming and inflammasome activation signals (15), we tested whether CPP-upregulated Il1b could act as a priming signal. As in LPS-primed cells, the common NLRP3 activator ATP increased the release of IL-1β protein in CPP-primed cells (Fig. 1C), indicating that CPPs predominantly upregulate IL-1β and IL-1α and serve as the priming signal for NLRP3 inflammasome in macrophages. To further clarify the mechanisms of CPP-mediated priming, we examined the TLR/NF-κB axis because TLR4 has been suggested as receptor of CPPs (28). Expectedly, CPPs induced nuclear translocation of NF-κB p65 (Supplemental Fig. 1C). We developed Traf6-deficient J774 macrophages because TRAF6 is an essential downstream mediator of TLR4 (Supplemental Fig. 1D, 1E). Traf6-deficiency significantly inhibited CPP-mediated induction of Il1a, but not Il1b and Nlrp3 (Supplemental Fig. 1F).

Next, we assessed whether CPPs could provide the activation signal for inflammasomes. In unprimed cells, CPPs slightly induced the release of IL-1β but not IL-1α (Fig. 1D). In LPS-primed cells, however, CPPs induced massive IL-1β/α release in a dose-dependent manner. Because inflammasome activation also causes pyroptotic cell death (18), we performed an LDH release assay and found that CPPs clearly increased cell death in LPS-primed cells (Fig. 1E). In accordance with mRNA results, Western blot analysis showed that the protein levels of pro–IL-1β/α and NLRP3 in cell lysates were increased in response to CPPs (Fig. 1F). In addition, CPPs promoted the cleavage and the release of mature IL-1β into culture supernatants in LPS-primed cells, whereas CPPs induced modest IL-1β release in unprimed cells. CPPs also induced IL-1α release in LPS-primed cells. Although most of the released IL-1α were pro–IL-1α, pro–IL-1α is reportedly biologically active (20). CPPs also induced the cleavage of GSDMD, a key executor of pyroptosis, in LPS-primed cells. Moreover, CPPs increased the number of active caspase-1+ cells in LPS-primed J774 macrophages (Fig. 1G). These findings indicate that CPPs also serve as an activation signal for the inflammasome and induce the release of IL-1β/α.
from wild-type (WT), Nlrp3−/−, and Casp1−/− mice. Consistent with the findings obtained with pharmacological inhibitors, CPP-induced IL-1β release was almost completely inhibited in macrophages lacking NLRP3 inflammasome components, whereas CPP-induced IL-1α release was comparable in the macrophages of each genotype (Fig. 2C). Western blot analysis showed that caspase-1 activation and subsequent cleavage of IL-1β were prevented in Nlrp3−/− macrophages (Fig. 2D), indicating that CPP-induced IL-1β release is dependent on NLRP3 inflammasome activation. Although CPP-induced cleavage of IL-1α was blunted in Nlrp3−/− macrophages, pro-IL-1α, which can activate IL-1R, was released into the supernatants by both WT and Nlrp3−/− macrophages. We also found that the CPP-induced cleavage of GSDMD was prevented in Nlrp3−/− macrophages. However, the CPP-induced release of IL-1β, IL-1α, and LDH was not prevented in LPS-primed Gsdmd−/− macrophages (Fig. 2E, 2F).

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FIGURE 2. CPPs induce NLRP3-dependent IL-1β release and -independent IL-1α release.

(A and B) After being primed with LPS (100 ng/ml) for 6 h, J774 macrophages were treated with MCC950 (1, 3, 10 μM) or VX765 (1, 3, 10 μM) for 30 min and then treated with CPPs (30 μl/ml) for 18 h. The IL-1β, IL-1α, and LDH levels in the supernatants were assessed. (C) Primary macrophages from WT, Nlrp3−/−, and Casp1−/− mice were primed with LPS for 6 h and then treated with CPPs for 18 h. The levels of IL-1β and IL-1α in the supernatants were assessed (n = 3–4 mice). (D) WT and Nlrp3−/− macrophages were primed with LPS for 6 h and then treated with CPPs for 18 h. The cell lysates and culture supernatants were analyzed by Western blot. Data are expressed as a dot plot and means ± SD. ***p < 0.001.

Consistently, Western blot analysis showed that mature IL-1β was released into the supernatant of Gsdmd−/− macrophages (Fig. 2G). These findings suggest that CPPs induce NLRP3 inflammasome-dependent IL-1β release and -independent IL-1α release and that GSDMD is dispensable for CPP-induced IL-1β release and necrotic cell death in macrophages.

**CPPs are incorporated into macrophages by phagocytosis**

Because CPPs are extracellular particles, we investigated whether the incorporation of CPPs could affect NLRP3 inflammasome activation. To assess the incorporation and localization of CPPs, we treated J774 macrophages with CPPs labeled with Cy3-conjugated alendronate. Flow cytometric analysis showed that the incorporation of CPPs was increased in a dose-dependent manner (Fig. 3A). The incorporation was mediated by phagocytosis because it was significantly inhibited in the presence of cytochalasin D (an actin polymerization inhibitor) (Fig. 3B). To assess the localization of incorporated CPPs, cells labeled with Alexa Fluor 488–dextran (a lysosome marker; green) were treated with Cy3-labeled CPPs (red). Live cell
imaging clearly showed that CPPs were incorporated into macrophages and colocalized with lysosome within 60 min (Fig. 3C). Furthermore, CPP-induced release of IL-1β/a and LDH was inhibited by treatment with cytochalasin D (Fig. 3D, 3E). These findings suggest that phagocytosis of CPPs is required for CPP-induced IL-1β/a release and necrotic cell death.

**Mechanisms of CPP-induced NLRP3 inflammasome activation**

The proposed common upstream pathways of NLRP3 inflammasome activation include lysosomal dysfunction-mediated cathepsin B release, K⁺ efflux, and mitochondrial reactive oxygen species (ROS) generation (13). Of these, lysosomal dysfunction is mainly involved in NLRP3 inflammasome activation in response to particles and crystals. Treatment with CA-074Me (a cathepsin B inhibitor) dose dependently suppressed CPP-induced IL-1β release in LPS-primed J774 macrophages (Fig. 4A). In contrast, CA-074Me slightly suppressed CPP-induced IL-1α release. In addition, CA-074Me had no effect on CPP-induced LDH release (Fig. 4B). To determine whether CPPs could induce lysosomal dysfunction, we labeled the cells with Alexa Fluor 488–dextran and analyzed them by live cell imaging. We clearly found that CPPs induced lysosomal rupture (Fig. 4C, Supplemental Video 1). Lysosomal cell death has been suggested to be a form of regulated cell death (RCD) and is thought to be inhibited by cathepsin inhibitors (29). However, K777 (a pan-cathepsin inhibitor) failed to inhibit CPP-induced LDH release (Fig. 4D). We also examined the role of K⁺ efflux and mitochondrial ROS and found that inhibition of K⁺ efflux by high extracellular KCl prevented CPP-induced release of

**FIGURE 3. CPPs are incorporated into macrophages by phagocytosis.**

(A) J774 macrophages were primed with LPS (100 ng/ml) for 6 h and then treated with Cy3-labeled CPPs (3, 10, 30 μM) for 18 h. The number of Cy3⁺ cells and mean fluorescence intensity (MFI) of Cy3 were analyzed by flow cytometry. (B) After being primed with LPS for 6 h, cells were treated with cytochalasin D (10 μM) for 30 min, and then treated with Cy3-labeled CPPs (30 μM) for 18 h. The number of Cy3⁺ cells and MFI of Cy3 were analyzed by flow cytometry. (C) Cells were primed with LPS for 6 h and then treated with Cy3-labeled CPPs. The localization of nuclei, lysosomes, and CPPs were detected at 20 min intervals with Hoechst 33342 (blue), Alexa Fluor 488–dextran (green) and Cy3-alendronate (red), respectively. (D and E) After being primed with LPS for 6 h, cells were treated with cytochalasin D (1 μM) for 30 min and then treated with CPPs for 18 h. The levels of IL-1β, IL-1α, and LDH in the supernatants were assessed. Data are expressed as a dot plot and means ± SD. ***p < 0.001.
IL-1β but not IL-1α or LDH (Fig. 4E, 4F). Furthermore, NAC (a general antioxidant) and Mito-TEMPO (a mitochondria-targeted antioxidant) failed to prevent CPP-induced IL-1β/α release (Fig. 4G). These findings suggest that lysosomal dysfunction and K⁺ efflux are mainly involved in CPP-induced NLRP3 inflammasome activation and subsequent IL-1β release but not in CPP-induced IL-1α release or necrotic cell death.

**Excess CPPs induce acute inflammatory responses in vivo**

Because CPPs induced NLRP3 inflammasome-dependent IL-1β release and -independent IL-1α release in macrophages in vitro, we investigated whether CPPs could evoke an inflammatory response in vivo. To clarify the organ where CPPs accumulate, we i.v. administered Cy3-labeled CPPs into WT mice. As reported previously (28), we observed the deposition of CPPs in the liver (Fig. 5A). In addition, we also detected the deposition in the spleen but not in the kidney, heart, or lung (Fig. 5A, Supplemental Fig. 2A). Because immune cells in the spleen respond to circulating foreign bodies, we focused on the spleen to evaluate whether CPPs induce inflammatory responses. Although the administration of low-dose CPPs (prepared from 1 ml of medium) caused no apparent changes, high-dose CPPs (prepared from 10 ml of medium) clearly upregulated mRNA levels of Il1b, Il1a, and Tnf (Fig. 5B). Increased IL-1β levels were confirmed by Western blot (Supplemental Fig. 2B). Furthermore, the expression levels of Cxcl1 (a
neutrophil chemokine) and Ly6g (a neutrophil marker) were significantly increased in the spleen of CPP-administered mice (Fig. 5C). Infiltration of neutrophils into the spleen was confirmed by immunofluorescence staining with anti–Ly-6G Ab (Fig. 5D). We also ascertained that the FCS used for CPP preparation did not affect these inflammatory effects (Supplemental Fig. 2C). These findings suggest that excess CPPs evokes acute inflammatory responses characterized by neutrophil infiltration.

CPP-induced inflammatory responses are mediated by both IL-1β and IL-1α

To investigate the role of IL-1β and IL-1α in CPP-induced inflammatory responses, we i.v. administered CPPs in WT, Il1b−/−, and Il1a−/− mice. IL-1β deficiency partially but significantly inhibited CPP-induced upregulation of Il1a, Ly6g, and Cxcr2, but not Cxcl1, Cxcr1, Il6, or Tnf (Fig. 6A). In contrast, IL-1α deficiency prevented the expression of not only Ly6g, but also Cxcl1, indicating that IL-1α contributes to the infiltration of neutrophils by CXCL1 production (Fig. 6B). In accordance with the decreased expression of Cxcl1, the expression of Cxcr2 (a CXCL1 receptor) was decreased in CPP-administered Il1a−/− mice, suggesting that the CXCL1/CXCR2 axis is involved in IL-1α–mediated neutrophil accumulation. The expression of Tnf and Cxcr1 was not downregulated by IL-1α deficiency.

Finally, we investigated the role of IL-1β and IL-1α in CPP-induced neutrophil infiltration using a particle-induced peritonitis model by the i.p. administration of CPPs. Massive infiltration of neutrophils into the peritoneal cavity was detected 6 h after CPP administration in WT mice (Fig. 7A, 7B). However, neutrophil infiltration was significantly blunted in Il1b−/− and Il1a−/− mice. Notably, the infiltration in Il1a−/− mice was significantly less than that in Il1b−/− mice. There was no

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FIGURE 6. CPP-induced inflammatory responses are mediated by IL-1β and IL-1α. (A) WT and II1b<sup>−/−</sup> mice were i.v. administered CPPs (prepared from 10 ml of media). After 4 h, spleen samples were harvested, and the expression levels of II1b, II1a, Ly6g, Cxcl1, Cxcr2, Cxcr1, Il6, and Tnf were analyzed by real-time RT-PCR (n = 8–11 for each group). (B) WT and II1a<sup>−/−</sup> mice were i.v. injected with CPPs. After 4 h, spleen samples were harvested, and expression levels of II1a, II1b, Ly6g, Cxcl1, Cxcr2, Cxcr1, Il6, and Tnf were analyzed by RT-PCR (n = 6–9 for each group). Data are expressed as a dot plot and means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

significant difference in the total number of cells between the groups (Fig. 7C). These findings suggest that CXCR2 contributes to the process of CPP-induced neutrophil inflammation that is mediated, at least in part, via IL-1β and IL-1α.

DISCUSSION

In the current study, we found that CPP-induced inflammatory responses are mainly mediated through IL-1β and IL-1α, released by NLRP3 inflammasome-dependent and -independent mechanisms, respectively (Supplemental Fig. 2D). We also demonstrated that, although phagocytosis of CPPs is required for CPP-induced IL-1β/α release, lysosomal dysfunction and K<sup>+</sup> efflux are required for IL-1β release but not for IL-1α release. In vivo experiments showed that excess CPPs evoked acute inflammatory responses characterized by neutrophil infiltration via both IL-1β and IL-1α. In particular, CPP-induced neutrophil accumulation is mediated predominantly through an IL-1α-induced CXCL1/CXCR2 signaling pathway. These results provide new insights into the mechanism underlying CPP-induced inflammation and suggest that targeting both IL-1β and IL-1α is necessary to regulate inflammatory responses induced by CPPs.

Multiple studies have suggested that crystals and particles activate NLRP3 inflammasome via lysosomal dysfunction. In the current study, we clearly demonstrated that CPPs are phagocytosed by macrophages and cause lysosomal rupture, which in turn leads to leakage of cathepsin B, resulting in NLRP3 inflammasome activation and subsequent IL-1β release.

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We further showed that CPP-induced NLRP3 inflammasome activation is mediated via K\(^+\) efflux. In this regard, Munoz-Planillo et al. (30) showed that phagocytosis of particulate matter, such as alum, silica, and calcium pyrophosphate dihydrate, triggers K\(^+\) efflux and activates the NLRP3 inflammasome. Therefore, we assume that lysosomal dysfunction and subsequent K\(^+\) efflux is a central mechanism of CPP-induced NLRP3 inflammasome activation. In contrast, recent studies have reported that Ca\(^{2+}\) signal contributes to CPP-induced inflammasome activation (28, 31). Jäger et al. (31) have suggested that CPP failed to induce lysosomal leakage. In addition, CPP-induced inflammasome activation in monocytes is dependent on Ca\(^{2+}\). The phagocytic activity may explain divergence between macrophages and monocytes. In the current study, CPPs were incorporated by phagocytosis that were inhibited by cytochalasin D. In contrast, a previous study showed that monocytes have been shown to incorporate CPPs via macropinocytosis (28). Massive incorporation of CPPs by phagocytosis might result in lysosomal leakage and subsequent NLRP3 inflammasome activation in macrophages. In accordance, we detected inflammasome activation in LPS-primed macrophages that exhibited higher phagocytic activity than unprimed macrophages.

Another issue is the role of GSDMD in IL-1\(\beta\) release. IL-1 family proteins are known to lack secretory signal sequence (32), and the molecular mechanisms of IL-1\(\beta\) release were unclear until the recent identification of GSDMD. Previous investigations have suggested that cleaved mature IL-1\(\beta\) is released outside of cells via GSDMD-forming pores (33). In the current study, although CPPs induce the cleavage of GSDMD in an NLRP3-dependent manner, GSDMD deficiency failed to inhibit CPP-induced IL-1\(\beta\) release. This result indicates that GSDMD-forming pores are dispensable for CPP-induced IL-1\(\beta\) release. Consistent with this result, Rashidi et al. (34) recently showed that MSU-induced IL-1\(\beta\) release is mediated by the NLRP3 inflammasome but is independent of GSDMD. Thus, we assume that the pores formed by GSDMD are not required for IL-1\(\beta\) release induced by crystal or particle.

We showed that genetic or pharmacological inhibition of NLRP3 inflammasome or cathepsins failed to inhibit the CPP-induced release of IL-1\(\alpha\) (both pro and mature forms) and necrotic cell death. Interestingly, NLRP3 deficiency prevented CPP-induced cleavage of pro–IL-1\(\alpha\) into its mature form. Because pro–IL-1\(\alpha\) can bind IL-1R and activate inflammatory signals (35), IL-1\(\alpha\)-driven inflammation is independent of NLRP3 inflammasome. Supporting this result, CPP-induced IL-1\(\beta\) release...
1α release was almost independent of K+ efflux and lysosomal dysfunction. Similar to IL-1β, because IL-1α also lacks a secretory signal sequence (35), necrotic cell death with loss of membrane integrity is the likely mechanism of CPP-induced IL-1α release. In this regard, we recently reported that IL-1α release from pyroptotic cells occurs in the absence of caspase-1, whereas IL-1β release requires its cleavage by caspase-1 (27). With respect to cell death, CPPs induced necrotic cell death independent of the NLRP3 inflammasome and GSDMD, although CPPs clearly induced the cleavage of GSDMD. Furthermore, CPP-induced necrotic cell death was not blocked by inhibition of cathepsins, K+ efflux, or ROS. To explore other forms of RCD involved in CPP-induced necrotic cell death, we tested the effects of inhibitors of several forms of RCD, including apoptosis (the pan-caspase inhibitor Z-VAD), necroptosis (necrostatin-1 or GSK872 [a RIPK3 inhibitor] together with Z-VAD), and ferroptosis (ferrostatin-1). Unfortunately, however, none of these inhibitors prevented CPP-induced necrotic cell death (data not shown). Therefore, we speculate that CPP-induced necrotic cell death might be an accidental form of cell death or some other form of RCD.

To explore the role of CPP-induced IL-1β/α release and inflammatory responses in vivo, we used two murine inflammation models, i.v. and i.p. administration of CPPs. We clearly observed that CPPs induce acute inflammatory responses that are characterized by the accumulation of neutrophils in both models. Interestingly, CPP-induced inflammatory responses are inhibited by both IL-1β and IL-1α deficiency, but IL-1α contributes more to inflammatory responses than IL-1β. Notably, expression of the neutrophil chemokine CXCL1 and its receptor CXCR2 is downregulated in CPP-administered Il1a−/− mice, suggesting that IL-1α deficiency prevents neutrophil accumulation by limiting the production of CXCL1. Supporting this, several studies have reported that IL-1α induces the production of CXCL1 (36, 37). Although the precise mechanism remains unclear, it is likely that IL-1α–induced CXCL1/CXCR2 signals play an important role in CPP-induced accumulation of neutrophils.

This study has several limitations. First, CPPs used in this study were prepared from medium containing FCS. Although this is an established protocol for preparing CPPs in vitro (38), the effect of CPPs prepared from the patients with CKD remains to be examined. Second, IL-1β is predominantly expressed in innate immune cells. Unlike IL-1β, however, although IL-1α is inducible in innate immune cells, including macrophages, pro–IL-1α is constitutively expressed in epithelial and mesenchymal cells (35). In particular, because pro–IL-1α is biologically active and functions as an alarmin, CPP-induced IL-1β/α release should be examined in other types of cells. Third, the inflammatory effects of CPPs were evaluated in murine models of acute inflammation. Although the administration of excess CPPs induces IL-1β/α–dependent inflammatory responses, the role of CPP-induced IL-1β/α release in chronic disease remains undetermined. Further investigations are needed to clarify the role of CPP-induced IL-1β/α release and inflammatory responses.

In conclusion, our results demonstrate that CPPs induce the release of IL-1β and IL-1α through NLRP3 inflammasome-dependent and -independent mechanisms, respectively, in macrophages. Excess CPPs also induce inflammatory responses characterized by neutrophil accumulation in vivo, which is mediated by IL-1β/α. In particular, IL-1α–induced CXCL1/CXCR2 signaling predominantly contributes to neutrophil inflammation in vivo. Recent clinical trials, including the Canakinumab Anti-inflammatory Thrombosis Outcome Study, have suggested that targeting IL-1β and the NLRP3 inflammasome has a great potential for preventing and treating cardiovascular diseases (39). However, our results suggest that targeting IL-1β and the NLRP3 inflammasome is insufficient and that targeting both IL-1β and IL-1α is necessary for preventing and treating CPP-associated inflammatory disorders. Furthermore, our findings provide new insights into the role and mechanisms of CPP-induced inflammation.

DISCLOSURES

The authors have no financial conflicts of interest.

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