Macrophage–NLRP3 Activation Promotes Right Ventricle Failure in Pulmonary Arterial Hypertension

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Abstract

Rationale: Pulmonary arterial hypertension (PAH) often results in death from right ventricular failure (RVF). NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) macrophage activation may promote RVF in PAH.

Objectives: Evaluating the contribution of the NLRP3 inflammasome in RV macrophages to PAH RVF.

Methods: Rats with decompenated RV hypertrophy (monocrotaline [MCT] and Sugen-5416 hypoxia [SuHx]) were compared with compensated hypertrophy rats (pulmonary artery banding). Echocardiography and right heart catheterization were performed. Macrophages, atrial natriuretic peptides, and fibrosis were evaluated by microscopy or flow cytometry. NLRP3 inflammasome activation and cardiotoxicity were confirmed by immunoblot and in vitro strategies. MCT rats were treated with SC-144 (a GP130 antagonist) or MCC950 (an NLRP3 inhibitor). NLRP3 activity was evaluated in patients with PAH RVF.

Measurements and Main Results: Macrophages, fibrosis, and atrial natriuretic peptides were increased in MCT and SuHx RVs but not in left ventricles or pulmonary artery banding rats. Although MCT RV macrophages were inflammatory, lung macrophages were antiinflammatory. CCR2+ macrophages (monocyte-derived) were increased in MCT and SuHx RVs and highly expressed NLRP3. The macroage-NLRP3 pathway was upregulated in patients with PAH with decompenated RVs. Cultured MCT monocytes showed NLRP3 activation, and in coculture experiments resulted in cardiomyocyte mitochondrial damage, which MCC950 prevented. In vivo, MCC950 reduced NLRP3 activation and regressed pulmonary vascular disease and RVF. SC-144 reduced RV macrophages and NLRP3 content, prevented STAT3 (signal transducer and activator of transcription 3) activation, and improved RV function without regressing pulmonary vascular disease.

Conclusions: NLRP3–macrophage activation occurs in the decompenated RV in preclinical PAH models and patients with PAH. Inhibiting GP130 or NLRP3 signaling improves RV function. The concept that PAH RVF results from RV inflammation rather than solely from elevated RV afterload suggests a new therapeutic paradigm.

Keywords: IL-1β; mitochondrial fission; CCR2; SC-144; MCC950

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Pulmonary arterial hypertension (PAH) is a cardiopulmonary syndrome defined by an increased resting mean pulmonary artery pressure (mPAP) greater than 20 mm Hg and pulmonary vascular resistance greater than 5 Wood units (1). The right ventricle (RV) initially adapts to increased pressure by compensatory right ventricular hypertrophy (RVH) and increased RV contractility (2). Eventually, these compensatory mechanisms are insufficient, leading to RV failure (RVF) (3, 4). The current dogma suggests that the transition from compensated to decompensated RVH is primarily a consequence of progression in pulmonary vascular disease and RV afterload.

PAH clinical trials and preclinical studies indicate that immune mediators play a crucial role in the pathogenesis of PAH (5, 6). Inflammatory cytokines such as IL-1β and IL-6 are elevated in the blood of patients with PAH, and their concentrations correlate with disease severity and mortality (7, 8). Macrophages are among the primary effectors of inflammation in pulmonary vascular lesions in PAH and may aggravate disease severity (6, 9). Macrophages display remarkable plasticity in response to the environment (10) and can either be polarized to produce proinflammatory cytokines (M1) or function as anti-inflammatory cells (M2), promoting tissue repair. However, whether their accumulation contributes to impaired RV function remains unknown.

This study evaluates RV inflammation as an independent and therapeutically tractable cause of RVF. More specifically, this study assesses the role of the NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome in PAH. The NLRP3 inflammasome responds to the host damage-associated molecular patterns (DAMPs) by generating proinflammatory cytokines, notably IL-1β (11). Apoptosis-associated Speck-like protein containing a CARD (caspase activation and recruitment domain) (ASC). The assembled NLRP3:ASC complex recruits and activates CASP1 (caspase1), which is essential for the cleavage of IL-1β and GSDMD (gasperdin D), a pore-forming protein that participates in the release of active IL-1β and induction of pyroptosis (12, 13). We hypothesize that RV inflammation, driven by activation of the NLRP3 inflammasome in macrophages, is a significant contributor to RVF in PAH.

We have already reported preliminary results of this study in the form of abstracts (14, 15) and showed that in PAH, there is RV-specific activation of NLRP3 in macrophages associated with RV decompensation, which is rescued through the inhibition of the NLRP3 pathway. This work suggests that RV inflammation is caused by the activation of the macrophage-NLRP3 Inflammasome pathway and identifies new therapeutic targets for PAH, including much-needed therapies for RVF.

Methods

The authors declare that all supporting data are available within the article and the online supplement.

Experimental Animals

Experiments were conducted after the Canadian Council on Animal Care regulations were approved by Queen’s University Animal Care Committee (Protocol #1714, #1971, and #2112) or the University of Minnesota Institutional Animal Care and Use Committee (protocol # 1904–36927A).

Male Sprague-Dawley rats (Charles River) were used to create all models. The pulmonary artery banding (PAB) model was created using surgical banding of the pulmonary trunk with metal clips in rats at age 6–7 weeks (~115 g) (16) (see online supplement). The monocrotaline (MCT) model was created using a single subcutaneous injection of 60 mg/kg MCT in 8- to 9-week-old rats (~250 g) (17). The Sugen-5416 hypoxia (SuHx) model was created by a single subcutaneous injection of Sugen-5416 (20 mg/kg) in rats at the age of 8 to 9 weeks old (~250 g) combined with environmental hypoxia (10% normobaric hypoxia) for 3 weeks followed by 3 weeks of normoxia.

On day 14 after MCT, rats received a daily injection of either SC-144...
(10 mg/kg, i.p.; APExBio Technology) or MCC950 (6 mg/kg, i.v.; InvivoGen, CP-456773) (18).

**Human Samples**
Control and decompensated human RV tissue were provided by the Institut de Cardiologie et de Pneumologie de Québec-Laval University (Canada), in accordance with the Comités d’éthique de la recherche (CER20775). The samples were obtained via autopsy or surgery. Patient information is summarized in Tables 1 and E3 in the online supplement.

**Cardiac Assessment**
RVF and response to therapy were assessed using high-resolution Doppler ultrasound (Vevo 2100, Visual Sonics) and closed-chest right heart catheterization (RHC) (Scisense pressure–volume catheter; Transonic) (see online supplement).

**Tissue and Cell Processing**
RV and lung tissue were prepared for immunohistochemistry, flow cytometry, or Western blotting. Peripheral blood mononuclear cells were evaluated via flow cytometry or used to obtain enriched monocytes tested in vitro for the activation of NLRP3 or cocultured with rat neonatal cardiomyocytes (R-CM-561, Lonza). Cardiomyocytes were assessed for number, size, mitochondrial fragmentation, and membrane potential (see online supplement; Table E1).

**Statistical Analysis**
Randomization of experimental animals was implemented as per Provencher and colleagues (19). Results are presented as mean ± interquartile range. Using GraphPad Prism (GraphPad Software), a one-way ANOVA or Student’s t test was used when the sample size was ≥8, whilst Mann-Whitney or Kruskal-Wallis tests were used when the sample size was ≤8. Two-way ANOVA was used to assess intergroup differences. P < 0.05 was considered statistically significant.

**Results**

**An Increase in RV Macrophage Number Is Associated with Reduced RV Function and Increased Fibrosis in MCT PAH and SuHx PAH**
RVH was present in both MCT (4-week group) and PAB models compared with their respective control samples. RVH was evident by increased RV free wall thickness (RVFWT) via echocardiography (P = 0.0006 and P = 0.0003, respectively) (Table 2) and shown on transverse histological sections of the heart (Figures 1A and E1B). Relative to control samples, tricuspid annular plane systolic excursion (TAPSE) was significantly decreased in MCT but not in PAB rats (P = 0.0012) (Table 2). Cardiac output (CO) and pulmonary artery acceleration time (PAAT) were decreased in the MCT 3-week group (P = 0.013 and P = 0.01, respectively), and both parameters were worse in MCT 4-week group compared with control samples (P = 0.0006 and P = 0.0012, respectively). Cardiac ultrasound was not conducted in the SuHx model. The Fulton index (RV/LV [left ventricle] + septum weight ratio) was increased in MCT RVs and PAB RVs compared with respective control samples (P = 0.004 and P = 0.022, respectively) (Table 2).

RHC revealed elevated right ventricular systolic pressure (RVSP) in MCT (3- and 4-week groups), SuHx, and PAB rats compared with control samples (P = 0.009, P = 0.0007, P = 0.0043, and P = 0.0012, respectively) (Tables 2 and E2). In 4-week MCT and SuHx rats, CO and ejection fraction were decreased (CO: MCT P = 0.0006, SuHx P = 0.0159; and ejection fraction: MCT P = 0.0007 and SuHx: P = 0.015) (Table 2). Compared with control samples, RV end-diastolic pressure was also increased in MCT 3-week (P = 0.009). Cardiac index decreased significantly in the 4-week MCT group only (P = 0.025). Pressure–volume loops are shown in Figure E2A. Although the CO significantly decreased in MCT 3-week compared with PBS as measured by cardiac ultrasound, there was no significant change in CO as measured by pressure–volume loops using closed chest RHC.

Atrial natriuretic peptide (ANP) expression was increased in MCT-RV compared with control RV (P = 0.0067) (Figures 1A and E3A). ANP expression in MCT LVs, PAB (RV and LV), and SuHx (RV and LV) was not significantly different from control samples (Figures 1A and E3A).

**Table 1. Patient Clinical Information**

|                  | Male       | Female     | Male       | Female     |
|------------------|------------|------------|------------|------------|
|                  | Control    | Decomp-RVF| Control    | Decomp-RVF|
| Cardiac ultrasound |            |            |            |            |
| NT-proBNP (pg/ml) | 68.57 ± 82.25 (14/15) | 5,583.4 ± 7,202.61 (5/5) | 0.006** | 113.63 ± 47.88 (8/8) | 7,472 ± 5,837.31 (8/9) | 0.004** |
| TAPSE (mm)       | 22.2 ± 2.68 (10/15) | 13.8 ± 5.98 (5/5) | 0.004** | 20 ± 4.40 (8/8) | 15.8 ± 6.30 (8/9) | 0.091 |
| Cardiac output (L/min) | 5.01 ± 1.03 (7/15) | 3.94 ± 0.49 (5/5) | 0.071 | 7.1 ± 1.10 (2/8) | 4.1 ± 0.94 (8/9) | 0.008** |
| Cardiac index (L/min/m²) | 2.86 ± 0.47 (4/15) | 1.99 ± 0.60 (5/5) | 0.878 | 3.28 ± 0.28 (2/8) | 2.38 ± 0.57 (8/9) | 0.078 |
| WBC count        |            |            |            |            |
| Total WBC (×10⁹/L) | 9.61 ± 6.67 | 8.06 ± 1.36 | 0.620 | 6.57 ± 1.25 | 14.21 ± 6.30 | 0.038* |
| Neutrophils (×10⁹/L) | 7.05 ± 6.67 | 5.74 ± 1.52 | 0.840 | 4.12 ± 0.70 | 12.12 ± 5.74 | 0.022* |
| Monocytes (×10⁹/L) | 0.61 ± 0.43 | 0.64 ± 0.39 | 0.690 | 0.57 ± 0.17 | 0.55 ± 0.31 | 0.891 |

**Definition of abbreviations:** Decomp-RVF = decompensated right ventricular failure; NT-proBNP = N-terminal pro-b-type natriuretic peptide; TAPSE = tricuspid annular plane systolic excursion; WBC = white blood cell.

Values are presented as mean ± SEM. Student’s t test: males (control vs. Decomp-RVF) and female (control vs. Decomp-RVF): *P < 0.05 and **P < 0.01. Total number of subjects N = 37: male control = 15, male Decomp-RVF = 5, female control = 8, and female Decomp-RVF = 9. Values in between brackets represent the number of subjects that have the parameter calculated. The female and male control groups were diagnosed with cardiomyopathies other than pulmonary arterial hypertension.
Table 2. Rat Cardiac Function Information

|                  | MCT model | SuHx Model | PAB Model | PBS | MCT, 4 wk | P Value | VeNx | SuHx | P Value | Sham | PAB | P Value |
|------------------|-----------|------------|-----------|-----|-----------|---------|------|------|---------|------|-----|---------|
| **Cardiac ultrasound** |           |            |           |     |           |         |      |      |         |      |     |         |
| RVFWT–dias (mm)  | 0.64 ± 0.024 | 1.36 ± 0.166 | 0.0006*** | —  | —         | —       |      |      |         |      |     |         |
| TAPSE            | 2.88 ± 0.094 | 1.76 ± 0.130 | 0.0012**  | —  | —         | —       |      |      |         |      |     |         |
| CO (ml/min)      | 122.40 ± 5.195 | 56.57 ± 5.879 | 0.0006*** | —  | —         | —       |      |      |         |      |     |         |
| PAAT (ms)        | 31.71 ± 1.409 | 20.01 ± 1.492 | 0.0012**  | —  | —         | —       |      |      |         |      |     |         |
| **RHC**          |            |            |           |     |           |         |      |      |         |      |     |         |
| RVSP (mm Hg)     | 22.69 ± 1.022 | 51.32 ± 2.510 | 0.0007*** | 24.83 ± 4.02 | 48.80 ± 6.45 | 0.0043** | 21.10 ± 1.576 | 61.70 ± 7.058 | 0.0012*** |      |     |         |
| RVEDP            | 0.946 ± 0.304 | 4.851 ± 0.568 | 0.0007*** | 2.01 ± 0.43 | 3.98 ± 0.36 | 0.0159*  | 1.036 ± 0.162 | 2.138 ± 0.17  | 0.007**  |      |     |         |
| EF               | 78.66 ± 1.721 | 51.19 ± 4.914 | 0.0007*** | 70.73 ± 4.91 | 59.22 ± 5.5  | 0.0036** | 75.66 ± 5.98 | 72.15 ± 4.16  | 0.640    |      |     |         |
| CO (ml/min)      | 139.1 ± 4.120 | 54.38 ± 9.33  | 0.0006*** | 113.8 ± 12.15 | 84.23 ± 13.76 | 0.0159*  | 125.0 ± 17.2 | 120.1 ± 19.06 | 0.249    |      |     |         |
| CI               | 0.334 ± 0.088 | 0.207 ± 0.025 | 0.025*    | 0.1980 ± 0.03 | 0.1825 ± 0.02 | 0.45     | 0.300 ± 0.02 | 0.323 ± 0.038 | 0.218    |      |     |         |
| **Anatomical parameter** |           |            |           |     |           |         |      |      |         |      |     |         |
| Fulton index     | 0.213 ± 0.014 | 0.311 ± 0.041 | 0.004**   | 0.266 ± 0.02 | 0.448 ± 0.06 | 0.0043** | 0.227 ± 0.023 | 0.374 ± 0.093 | 0.022**  |      |     |         |

**Definition of abbreviations:** CI = cardiac index; CO = cardiac output; EF = ejection fraction; MCT = monocrotaline; PAAT = pulmonary artery acceleration time; PAB = pulmonary artery banding; RHC = right heart catheterization; RVEDP = right ventricular end-diastolic pressure; RVFWT–dias = right ventricular free wall thickness in diastole; RVSP = right ventricular systolic pressure; SuHx = Sugen-5416 hypoxia; TAPSE = tricuspid annular plane systolic excursion; VeNx = vehicle normoxia.

Values are presented as mean ± SEM. Nonparametric t test (Mann-Whitney) was used to compare MCT, SuHx, and PAB rats with their respective controls: *P < 0.05, **P < 0.01, and ***P < 0.001.

PBS n = 6, MCT n = 8, VeNx n = 6, SuHx n = 5, sham n = 5, and PAB n = 5.
Figure 1. An increase in right ventricle (RV) macrophages is associated with worsening RV function and increased RV fibrosis in pulmonary arterial hypertension. (A) The mean fluorescence intensity of atrial natriuretic peptide (ANP; Alexa-fluor 647; red) was measured per cardiomyocyte via confocal microscopy, and a fold of change analysis shows that it is significantly high in 4-week monocrotaline (MCT) RV compared with control samples (n = 5/group; P = 0.003). The fold of change of total collagen deposition [Picrosirius red stain (PS)] was higher in the RV of 4-week MCT and SuHx (Sugen-5416 hypoxia) (n = 5/group; P = 0.0001 and P = 0.0078) compared with control samples. The total collagen deposition was significantly higher in MCT and SuHx compared with pulmonary artery banding (PAB) rats (P = 0.0001 and P = 0.008, respectively). Collagen-III (Alexa-fluor 488; green) expression was higher in 4-week MCT RVs and SuHx RVs compared with control samples.
Using flow cytometry, we were also able to identify and quantify RV macrophage abundance, defined as the percentage of CD68+ within the total of CD45+ live cells. In this experiment, MCT rats treated for 3 and 4 weeks were compared, and it was further confirmed that there was a significant increase in CD68+ cells in MCT RVs compared with control RVs (3 weeks: 45.5%; MCT vs. 35.3% in control samples; and 4 weeks: 74% in MCT vs. 58.4% in control samples). Pulmonary hypertension severity progressed between 3 and 4 weeks after MCT, and the number of RV macrophages significantly increased in parallel (3 weeks vs. 4 weeks MCT: \( P = 0.0095 \)) (Figure 1D). The macrophage number was directly associated with deterioration in RV function in MCT rats (Table 2). We also detected an inverse correlation between CO and macrophage abundance that was more evident at 4 weeks \( (r^2 = 0.483, \ P = 0.0173) \) than 3 weeks \( (r^2 = 0.293, \ P = 0.165) \) after MCT treatment (Figure 1E). In contrast, despite RVH, the number of macrophages was unchanged in PAB vs. sham RVs (Figure 1D).

The Macrophages in MCT and SuHx RV Demonstrate NLRP3 Inflammasome Activation

We used flow cytometry to detect the baseline NLRP3 expression in RV cells. NLRP3 expression was twofold higher in macrophages than in other immune cells \( (CD45^- CD68^-) \); endothelial cells \( (CD45^- CD68^- CD90^- CD31^-); P = 0.0286) \), or fibroblasts \( (CD45^- CD68^- CD90^- CD31^-); P = 0.0286 \) (Figure 2A). No differences were detected in the expression of ARG1 (an anti-inflammatory M2 marker) in MCT RV vs. control RV macrophages (Figure E5), consistent with the polarization of RV macrophages toward an inflammatory M1 phenotype. To confirm the expression of NLRP3 in the RV macrophages as well as its fluctuation after PAH development, we quantified the number of NLRP3+ CD68+ macrophages (relative to total CD68+ macrophages) in MCT and SuHx rats. Macrophages expressing NLRP3 were significantly increased in MCT and SuHx RVs compared with control samples \( (P = 0.011 \text{ and } 0.007, \text{ respectively}) \) (Figure 2B), and no intergroup differences were observed.

Immunoblotting showed significantly higher NLRP3 expression in the MCT RVs \( (P = 0.036) \), but not in PAB RVs, compared with their respective control samples (Figures 2C and E6). Indeed, whereas NLRP3 protein was detectable in 8/9 MCT RVs, only 4/8 PAB RVs, 2 sham RVs, and 2 PBS RVs had detectable NLRP3. Pro-CASP1 and pro–IL-1β were detected in PAB and MCT RVs; however, increased expression of cleaved CASP1 and IL-1β, which indicates activation of the inflammasome, were only observed in MCT RVs \( (P = 0.031 \text{ and } 0.028, \text{ respectively}) \) (Figures 2C and E6). In SuHx rats, the activation of the NLRP3 inflammasome pathway was determined by quantifying cleaved GSDMD, a protein cleaved by CASP1 that participates in the release of IL-1β and pyroptosis (20). Cleaved GSDMD was significantly increased in the SuHx RVs compared with VeNx \( (P = 0.002) \) (Figure E7A). IL-1β was also measured in the serum from SuHx and VeNx rats. Although significant differences were not detected \( (P = 0.1) \) (Figure E7B), there was a trend toward elevated IL-1β concentrations in the serum of SuHx rats.

NLRP3 inflammasome activation was further studied in vitro in blood monocytes of MCT versus control rats on the basis of an assay measuring NLRP3:ASC interaction, a key step in inflammasome activation (Figure 2D). A time course of inflammasome activation, measured 10 to 30 minutes after nigericin activation, showed greater NLRP3:ASC interaction 10 minutes after nigericin in MCT versus control monocytes \( (P = 0.019) \) (Figures 2D and E8). NLRP3 activation in vitro was prevented by MCC950 \( (P = 0.040) \) (Figures 2D and E8). These data indicate that increased NLRP3 activation occurs in MCT monocytes.

SC-144 Improved Cardiac Function, Reduced RV Macrophages, and Suppressed NLRP3 Inflammasome and STAT3 Activation In Vivo

GP130 receptor signaling is critical to the proliferation and recruitment of macrophages. It complexes with different members of the IL-6 receptor family, allowing the binding of cytokines and the activation of transcription factors such as STAT3. Therefore, we tested whether SC-144, a GP130 inhibitor, would improve RV function by reducing the influx of macrophages to the RV and consequently preventing NLRP3 inflammation. GP130 antagonist-treated MCT rats (MCT-GP130Antag) showed a significant reduction in RVH compared with vehicle-treated MCT rats (MCT-V). MCT-GP130Antag rats also had increased TAPSE \( (P = 0.004) \) and CO.
Figure 2. Increased number of RV macrophages in PAH-rat models is marked by an increase in NLRP3 inflammasome activity. (A) Baseline flow cytometric assessment of NLRP3 content (n = 4 rats) showing higher relative expression [staining index (SI)] of NLRP3 in rat RV-macrophages (CD45⁺CD68⁻) compared to all other cell types, including: other immune cells (CD45⁺CD68⁻; p = 0.017), endothelial cells (CD45⁻CD31⁺; P = 0.005) and fibroblast (CD45⁻CD90⁺; P = 0.008). (B) Double immunofluorescence detecting CD68 (Alexa Fluor 555; red) and NLRP3 (Alexa Fluor 488; green) in RV tissue of MCT, SuHx and control rats [PBS and Vehicle-normoxia (VeNx)]. The quantification of the relative incidence of CD68⁺NLRP3⁺ cells (normalized by total CD68⁺ cells) revealed an increased number of macrophages expressing NLRP3 in the RV of MCT and
(P = 0.030) and reduced RVFWT (P = 0.0001) (Figure 3A). Ventricular-to-arterial coupling was also improved by SC-144, as assessed by measuring the ratio of RV end-systolic elastance to effective arterial elastance (Ea; Figure 3A). Hypertrophy of RV cardiomyocytes in the MCT- GP130Antag group was also reduced (P < 0.0001) (Figure 3B). This improvement in RV function occurred independently of changes in the severity of pulmonary hypertension, as measured by PAAT, arterial elastance, RVSP (Figure 3A; pressure-volume loops are demonstrated in Figure E2B), or the severity of adverse pulmonary vascular remodeling, as measured by pulmonary arterial medial wall thickness (Figure 3C). A reduction in RV collagen deposition (P = 0.018) (Figure 3D), macrophage accumulation (P = 0.008) (Figure 3E), and phosphorylation of STAT3 in macrophages (P = 0.049) (Figure 3F) was also observed with SC-144 treatment. Furthermore, SC-144 treatment reduced the NLRP3 and pro-ASC1 protein content in the MCT-RV (P = 0.028 and P = 0.028, respectively) (Figure 3G). Cleaved forms of CASP1 or IL-1β were not detected.

In Vivo Treatment with MCC950 Inhibits NLRP3 Inflammasome Activation and Improves RV Function in the MCT Rats While Reducing Monocyte-driven Hypertrophy and Mitochondrial Damage in Normal Cardiomyocytes In Vitro

MCC950 significantly improved RV function in MCT (MCT-MCC950) compared with MCT-V rats (Figure 4A), evidenced by improved TAPSE (P = 0.004) and CO (P = 0.0208). MCC950 also improved ventricular-to-arterial coupling (TAPSE to RVSP ratio, P = 0.0146) (21) and PAAT (P = 0.010) while reducing RVSP (P = 0.042) and pulmonary arterial medial wall thickness (P < 0.0001) (Figures 4A and 4B). The Fulton index improved in all except 3 rats (3/11), creating a trend toward reduction in RVH (P = 0.076) (Figure 4A). RV NLRP3 and cleaved IL-1β content were significantly reduced in MCT-MCC950 versus MCT-V rats (P = 0.035 and P = 0.0082, respectively) (Figure 4C). Although MCC950 did not alter RV collagen deposition (P = 0.161 using a nonparametric test) (Figure 4D), it showed a beneficial trend. MCC950 also did not reduce RV macrophage count (P = 0.843) (Figure 4E).

To assess the specific effects of the in vivo MCC950 treatment on NLRP3 inflammasome inhibition on monocytes (which are precursors of macrophages in the bloodstream) and amelioration of cardiomyocyte function, we treated MCT rats with MCC950 and co-cultured their enriched monocytes with healthy neonatal cardiomyocytes (Figure E9). Compared with control monocytes, MCT monocytes significantly reduced the number of cardiomyocytes in culture (after 48 hours, P = 0.019), induced myocyte hypertrophy (24 and 48 hours, P < 0.0001) (Figure 4F) and caused mitochondrial dysfunction, evidenced by reduced membrane potential (P = 0.005), and increased mitochondrial fragmentation (increased punctate morphology, P < 0.0001 vs. decreased filamentous morphology, P < 0.0001), as measured by machine learning (22) (Figures 4G and E10). Conversely, cardiomyocytes cocultured with monocytes enriched from MCT rats treated in vivo with MCC950 restored cardiomyocyte number (P = 0.0003), reduced myocyte hypertrophy (P < 0.0001), preserved mitochondrial membrane potential (P = 0.045), and reduced mitochondrial fragmentation (P = 0.0007). Interestingly, when monocytes (from PBS, MCT, or MCT-MCC950 rats) were stimulated in vitro with nigericin (a potent NLRP3 inflammasome activator) before the coculture, no cardiomyocytes grew (data not shown because no cells survived). In these experiments, the cardiomyocytes that were cocultured with monocytes were never exposed to MCC950.

RV Macrophage Numbers Are Increased, and the NLRP3 Inflammasome Is Activated in the Decompensated RV from Patients with PAH

Patient information is summarized in Table 1. Fibrosis and CD68+ macrophage numbers were significantly increased in the RV tissue of patients with PAH with decompressed RVs compared with control patients (P = 0.002 and P = 0.029, respectively) (Figures 5A and 5B). Although we could not detect differences in the number of CD68+ NLRP3+ cells by immunofluorescence between patients versus control samples (Figure 5C), immunoblotting showed that the expression of pro- and cleaved IL-1β was higher in PAH RV tissue compared with control RVs (P = 0.012 and P = 0.040, respectively) (Figure 5D). Using immunofluorescence, we did not detect significant changes in the numbers of macrophages expressing p-STAT3 or GP130 (CD68+ GP130+ p-STAT3+, and CD68+ GP130+ p-STAT3−) between PAH and control patients (Figure 5E).

M2-like Polarization of Lung Macrophages in MCT Rats

To confirm the unique PAH RV macrophage phenotype, we conducted a concomitant investigation of the lung macrophage phenotype in MCT and PAB rats. As expected, lung macrophages were more abundant in MCT rats than in control samples (MCT 3 weeks, P = 0.033) (Figure 6A). In contrast to RV macrophages, we detected a shift toward M2-like macrophages in the lungs of MCT rats, characterized by increased expression of ARG1 (P = 0.002) and CD163 (P = 0.039).
Figure 3. SC-144 improves cardiac function, reduces RV macrophage number, and suppresses NLRP3 inflammasome and STAT3 activation. (A) GP130 Antagonism improves indicators of RV function in MCT rats when compared to MCT rats treated with vehicle (MCT-V): TAPSE, cardiac output, RVFWT, end-systolic elastance (Ees) to effective arterial elastance (Ea) (Ees/Ea) (P = 0.004, 0.030, and 0.0001, respectively; n = 8–10/group). While cardiac function was preserved with the GP130 antagonist, there was no difference in Ees, PAAT, and RVSP when comparing the MCT-GP130 Antagonist treated group with the MCT-V group. (B) Histological assessment of RV cardiomyocyte hypertrophy using H&E staining (cardiomyocyte area µm²; n = 3 rats/group and 94 cardiomyocytes/rat). GP130 antagonist treatment reversed...
and a decreased incidence of NOS2+ inflammatory macrophages (P = 0.014 in MCT 3 weeks) (Figure 6B). When polarization was assessed in blood monocytes, we detected a significant increase in both anti-inflammatory and inflammatory monocyte subsets after MCT treatment: CD43high (which are anti-inflammatory, P = 0.004) and CD43low (which are inflammatory, P = 0.021) (23) (Figure 6C).

**Discussion**

PAH leads to death in approximately half of the patients within 5 years because of RVF (24), and all approved therapies are vasodilators that primarily target the pulmonary vasculature (24, 25). In this study, we considered the possibility that the PAH RV might be a primary target for inflammation-mediated dysfunction and not just a bystander organ responding to an increase in RV afterload. We compared two preclinical rat models of decompressed RVH (MCT and the SuHx models) (26, 27) to the PAB model, in which (at the severity of banding we used) the RV retains a compensated RVH phenotype (16, 28), mimicking pulmonic stenosis. We also compared RV samples of patients with PAH with decompensated RVF to control subjects who had cardiac diseases other than PAH (Table 1).

We show in the MCT and SuHx models that RV infiltration with monocyte-derived macrophages and NLRP3 inflammasome activation creates a state of inflammation that contributes to RVF. This is relevant to patients with PAH because we demonstrate that key aspects of the macrophage–NLRP3 inflammasome pathway are similarly deranged in patients with PAH with decompensated RVF. Our data confirm that macrophages are the most frequent leukocytes in the PAH RV (constituting over 45% of CD45+ cells in MCT rats), and they highly express NLRP3. Notably, over 50% of cells in the RV expressing CCR2, a chemotaxis receptor present in inflammatory cells (27), were macrophages in both PAH models. Thus, not only are the macrophage numbers increased in the RV in PAH, but most macrophages in the pulmonary hypertensive RV are derived from blood monocytes. Moreover, there was no macrophage accumulation in the LV in PAH, suggesting the inflammatory infiltration of the RV is a chamber-specific phenomenon in PAH.

To establish the pathological contribution of macrophage infiltration to RV fibrosis and RVF in PAH, we assessed the therapeutic effect of two unrelated agents that target the NLRP3 pathway in MCT rats SC-144, a GP130 inhibitor (a signal-transducing component of the IL-6 cytokine superfamily, IL-6ST) (29) and MCC950, a specific inhibitor of the NLRP3 inflammasome (18). Both of these therapies substantially improved RV function when administered in vivo, indicating that therapeutic modulation of the NLRP3–macrophage pathway may be a mechanism to augment RV function in PAH (Figure 7).

Important markers of RV dysfunction (ANP and fibrosis (total collagen and collagen–III)) were elevated in the MCT and SuHx models of maladaptive RVH but not in the PAB model (adaptive). In a previous study, we demonstrated that RV fibroblasts isolated from MCT rats are epigenetically and metabolically reprogrammed to a hyperproliferative and fibrogenic phenotype (4, 24). Therefore, we suggest that the RV fibrosis in the MCT model which contributes to a decompensated RV response, may be triggered by inflammation. As predicted, both drugs that inhibited the inflammasome reduced RV fibrosis (Figures 3D and 4D). Although increased RVSP and RVH occurred in all models, only MCT and SuHx rats suffered from RVF.

Unlike PAB rats, MCT and SuHx rats had increased numbers of RV macrophages and evidence of increased NLRP3 inflammasome expression and activation. The macrophage–NLRP3 pathway was not activated in PAB RVs, indicating that RV pressure overload or RVH alone, at the severity we achieved, does not trigger inflammation. A comparison between rats at 3 and 4 weeks after MCT treatment further confirmed progressive RV decomposition of the MCT model at Week 4, evident by worsening of RV function and higher recruitment of macrophages compared with rats killed at Week 3. Comparing the RV and LV, only MCT and SuHx RVs showed higher collagen–III, ANP, and macrophage count. These data confirm that the inflammatory and fibrotic changes in experimental PAH are chamber-specific and not because of any systemic inflammatory effects of any model. We showed that the inhibition of both macrophage recruitment (achieved by SC-144) and NLRP3 inflammasome activation (MCC950) ameliorates fibrosis and improves RV function. Human RV samples also showed augmented fibrosis and increased macrophages in decompensated patients with PAH RVF. The NLRP3 inflammasome pathway was also activated in the RVs of patients with PAH, evidenced by increased expression of both pro- and...
Figure 4. MCC950 inhibits NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome activation, reduces mitochondrial damage in cardiomyocytes, and improves right ventricle (RV) function in pulmonary arterial hypertension.

(A) The cardiac function in MCC950-treated group, assessed by cardiac ultrasound, was improved in the MCT-MCC950 group compared with monocrotaline (MCT) rats treated with vehicle (MCT-V) (n=8 PBS, 8 MCT-V, and 11 MCT-MCC950), as demonstrated by increased tricuspid annular plane systolic excursion (P=0.016), cardiac output (P=0.042), and cardiac index (P=0.025). A reduction of the Fulton index and RV...
cleaved IL-1β. However, we failed to detect an increase in NLRP3 protein, which may be a function of sample size and potential variability in the antigen retrieval of this protein in biobanked, human, histological specimens.

The priming and activation of the NLRP3 inflammasome in macrophages may result from multiple signals such as DAMPs, K⁺ efflux, Ca²⁺ influx, and mitochondrial dysfunction (13, 30, 31). Disturbances in these signals are well described in PAH (32), supporting the idea that the RV tissue is a fertile environment for the activation of this inflammatory pathway. Indeed, our results demonstrated that the coculture of healthy cardiomyocytes with monocytes isolated from MCT rats (which have an activated NLRP3 inflammasome) developed cardiotoxicity evidenced by impaired growth, lower mitochondrial membrane potential, and increased mitochondrial fragmentation. Once activated, NLRP3 recruits ASC and pro-CASP1, resulting in CASP1 activation and the cleavage of IL-1β to its mature form (19). Therefore, our data suggest that RV inflammation in PAH is maintained by a vicious cycle of cardiomyocyte mitochondrial damage, with the production of DAMPs and the release of cleaved IL-1β by macrophages, which is known to mediate cardiomyocyte apoptosis (33).

In patients with PAH, IL-1β is elevated in the serum (34) and is associated with an increased risk of death (8). Consistent with this observation, we show for the first time that IL-1β is upregulated in the RV of both MCT rats and patients with decompensated RVF. Higher serum concentrations of IL-1β were also reported in patients with PAH carrying somatic mutations in ten-eleven TET2 (translocation methylcytosine dioxygenase) compared with noncarrier patients with PAH (5, 35). TET2 is a critical regulator of DNA methylation predominantly expressed in monocyctic (monocytes and macrophages) and granulocytic cell lineages. Indeed, a hematopoietic-specific knockout of TET2 was sufficient to cause an inflammatory mouse model of PAH, which was reversed by canakinumab, an IL-1β antibody (6). In the current study, we used MCC950, which binds to NLRP3 and inhibits ASC oligomerization (36). MCC950 remarkably reduced NLRP3 expression and cleaved IL-1β content in the MCT RV while improving cardiac function without changing the number of RV macrophages. This was expected because MCC950 blocks the interaction between NLRP3 and ASC and prevents the cleavage of IL-1β. Indeed, the cardioprotective benefits of blocking the NLRP3 inflammasome, specifically in monocytes, were seen when monocytes from MCT rats treated in vivo with MCC950 were cocultured with healthy cardiomyocytes. In contrast to MCT monocytes, which induced hypertrophy, growth restriction, and mitochondria dysfunction, the effects of MCT MCC950 monocytes on cardiomyocytes were comparable to PBS monocytes. These data are consistent with monocyte-derived RV macrophages being the effector cell in which NLRP3 activation, relevant to cardiotoxicity in RVF, occurs. Consistent with this hypothesis, previous work shows NLRP3 knockout mice exposed to hypoxia have less RV hypertrophy than control mice despite having the same severity of PH (37). Taken together, these data indicate that MCC950 may have therapeutic potential in the treatment of inflammatory RVF (33, 38, 39).

We investigated the polarization of macrophages in MCT rats by measuring the expression of M1-like (NOS2) and M2-like (CD163 and ARG1) macrophage markers. Although lung macrophages acquired an M2-like phenotype (high expression of CD163 and ARG1 and low NOS2 expression), the RV macrophages had no change in ARG1, strongly suggesting a unique pattern in the activation of RV macrophages favoring an inflammatory, M1 phenotype in PAH RV. We also demonstrated that p-STAT3⁺ macrophages are more abundant in the MCT RVs compared with control samples. IL-6 signaling and STAT3 activation are essential in the proliferation and differentiation of monocytes and macrophages (40). IL-6 is upregulated in the serum of patients with PAH (41), and this is directly associated with RVF. Moreover, overexpression of IL-6 in murine models leads to the development of...
Figure 5. Increased incidence of macrophages and NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome activation in the right ventricle (RV) from patients with pulmonary arterial hypertension (PAH) with decompensated (decomp) RVs. 

(A) Collagen deposition assessment of PAH RV tissue using picrosirius red staining (n = 8 PAH and 4 control samples) demonstrated that PAH RV tissue was significantly richer in collagen compared with control RV tissue (P = 0.002). Scale bars, 100 μm.

(B) Identification and quantification of human RV macrophages (CD68+ cells per 0.18 mm²) showing a significantly higher incidence of macrophages in the PAH group compared with control samples (P = 0.029; n = 8 PAH and 4 control samples). Scale bars, 50 μm.

(C) Identification and quantification of human RV macrophages expressing NLRP3 (CD68+, red; NLRP3+, green). A nonsignificant increase in the number of RV macrophages expressing NLRP3 was detected (P = 0.713). Scale bars, 20 μm. 

(D) Assessment of NLRP3, CASP1, and IL-1β content of human PAH RV using Western blot. Pro- and cleaved IL-1β are significantly increased in PAH RV compared with control samples (P = 0.012 and P = 0.040; n = 3/group). The content of target proteins was normalized using the Ponceau stain (total protein).

(E) Immunofluorescence staining was used to detect p-STAT3 (signal transducer and activator of transcription 3) (signal green), GP130 (yellow), and macrophages (CD68, red). The macrophages subsets: GP130+p-STAT3+CD68+, GP130+p-STAT3−CD68+, and GP130−p-STAT3−CD68− were quantified, but no subset differences were detected when comparing control and PAH RV tissue. Scale bars, 40 μm. Decomp = decompensated. *P < 0.05, **P < 0.01, and ***P < 0.001.
Figure 6. Distinctive M2 polarization of lung macrophages is consistent with regional heterogeneity in macrophage polarization between the right ventricle (RV) and the lung in pulmonary arterial hypertension. (A) Flow cytometric assessment of live lung macrophages (CD45+CD68+) of monocrotaline (MCT)-treated and pulmonary arterial banding rats (n = 4/group). The incidence of macrophages was at its highest degree in the lungs of MCT-treated rats at Week 3 after receiving MCT versus PBS control samples (a fold of change, \( P = 0.033 \)), MCT at 4 weeks (\( P = 0.068 \)), and pulmonary arterial banding rats (\( P = 0.092 \)). The comparison between 3 and 4 weeks of MCT showed no significant differences. Two-way ANOVA test was used to compare treatment (control vs. treated groups) and type of treatment (3 weeks vs. 4 weeks MCT). (B) The polarization of lung macrophages was determined on the basis of the degree of expression [staining index (SI)] of the M2-like macrophage markers.
pulmonary vascular lesions and PAH (42). On the other hand, STAT3 is also linked to the activation of M2-like macrophages through IL-6 signaling (43) and the regulation of ARG1-expressing alveolar macrophages in hypoxia-induced PH in mice (44). Thus, whereas in lung macrophages, the activation of STAT3 may lead to antiinflammatory polarization, its activation in the RV may be linked to the recruitment and differentiation of monocytes into inflammatory macrophages. Of note, both subsets of monocytes, inflammatory (CD43low) and antiinflammatory (CD43high), were increased in the blood of MCT rats, supporting the potential for heterogeneous recruitment of cells with different inflammatory profiles to the RV versus the lung.

Our findings using the SC-144 in MCT rats also reinforce the importance of IL-6 signaling in recruiting monocyte-derived macrophages to the RV. SC-144 reduced RV macrophages in MCT rats and diminished the NLRP3 and pro-CASP1 content of the RV. These data suggest that reducing the incidence of RV macrophages, which are the main site of NLRP3 inflammasome complex activation, can prevent the accumulation of these inflammasome components in the RV tissue. SC-144 also reduced fibrosis and improved RV function independent of the regression of pulmonary vascular remodeling (46). It is noteworthy that the drug was given 1 week after MCT injection to Wister rats, a week earlier than given in our study, which might increase its opportunity to improve PAH by preventing pulmonary vascular obstruction, indicating that the benefits of blocking IL-6 signaling are RV-specific. Similar findings were recently reported by Prisco and colleagues (45). Although a delay in pulmonary vascular disease was shown by Tamura and colleagues when rats were treated with IL-6R/sIL-6R antagonist (TB-2–081), only a slight improvement in mPAP was reported despite a significant reduction in total pulmonary resistance, the ratio of mPAP/CO (46). This suggests that the decrease in total pulmonary resistance was mainly because of improved CO rather than positive pulmonary vascular remodeling (46). It is noteworthy that the drug was given 1 week after MCT injection to Wister rats, a week earlier than given in our study, which might increase its opportunity to improve PAH by preventing
(rather than regressing) adverse pulmonary vascular remodeling. Toschner and colleagues reported that ticlozumab (an IL-6 specific antibody) did not significantly alter PAH severity in human PAH (47). On the other hand, a study showed that cardiotoxin-1, a member of the IL-6 superfamily of cytokines that activates the GP130 receptor (i.e., an agonist of this pathway), is protective against pulmonary vascular remodeling in hypoxic PH (48), making it unlikely that a GP130 antagonist would cause beneficial pulmonary vascular remodeling. These data align with our findings that SC-144 selectively improved RV function without improving pulmonary vascular remodeling.

In another study, SC-144 was used at a different dose, and although the authors reported that the drug regressed pulmonary vascular disease, the representative images of their histological findings showed larger-sized pulmonary arteries than the ones usually used to measure pulmonary medial wall thickness, making data comparison between our studies difficult (49).

Our work suggests a critical role of RV inflammation in controlling adverse RV remodeling and dysfunction in PAH. Still, this study has important limitations. First, we acknowledge the lack of assessment of sex differences in the role of NLRP3 in PAH. Second, the relatively small sample size for the human study and the lack of lung tissue prevented us from contrasting macrophage polarity or NLRP3 inflammasome activation in the patient’s lungs versus the heart. Third, although we show mitochondrial damage in cardiomyocytes can occur in the presence of MCT monocytes, we did not establish which DAMPs activate the NLRP3 inflammasome in PAH RVs. Fourth, we only used three markers to determine the polarization of RV and lung macrophages in rats, a weakness that affects this field of research because there is a paucity of well-characterized markers to define macrophage polarization in rats (unlike mice, for which there are abundant markers). Finally, the numbers of p-STAT3+ macrophages in human PAH RV tissue did not differ from control samples. Although this difference may be related to tissue handling or the terminal stage of disease in which these patients were studied, disparities between species in the cell signaling could be at play, and this merits future investigation.

Conclusions

The activation of the NLRP3 inflammasome pathway within the RV-macrophages is present in two robust preclinical models of PAH and patients with decompensated RVH. In vivo, blockade of the IL-6 cytokine pathway via SC-144 or the NLRP3 inflammasome signaling via MCC950 prevented RV macrophage accumulation and NLRP3 inflammasome activation while improving RV function. These findings indicate that NLRP3 activation contributes to the pathogenesis of RVF. Moreover, our research suggests that RVF is chamber-specific and can occur because of macrophage-mediated inflammation rather than simply being reflective of a failure to adapt to increased RV afterload. Additional research will be required to determine the translational value of GP130 antagonists and MCC950 as potential PAH therapies in humans.

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