Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by stenosis and occlusion in distal pulmonary arteries, leading to elevation of pulmonary arterial pressure and right ventricular failure. Pathological changes in the lungs of PAH patients include arterial wall thickness, disorganized proliferation of endothelial cells (plexiform lesions), and perivascular and interstitial inflammatory infiltrates. In the pathogenesis of PAH, the role of inflammatory signaling has been reported. The serum level of interleukin (IL)-6 is elevated in patients with idiopathic PAH (IPAH), and mice that have lung-specific IL-6 overexpression develop pulmonary hypertension (PH) spontaneously. Additionally, we and others demonstrated that IL-6 pathway blockade by anti-IL-6 receptor antibodies or genetic deletion of Il6 attenuates hypoxia-induced PH (HPH) in mice. These studies highlight the importance of immune mechanisms in PH pathobiology.

Connective tissue disease (CTD), including systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), sometimes complicates PAH, and is called CTD-associated PAH (CTD-PAH). Although the prognosis of PH has improved due to the development of novel pulmonary vasodilators, the prognosis of CTD-PAH, especially complicated with interstitial pneumonia, is still poor. Therefore, novel therapeutic agents based on the specific molecular pathogenesis are desired. In the pathogenesis of CTD-PAH, inflammation and immune abnormality play important roles.
example, cytokines including IL-6, IL-1β, and tumor necrotic factor (TNF)-α are elevated in the serum of patients with CTD-PAH, and have a role in proliferation of smooth muscle cells. Fibrosis also plays an important role in the pathogenesis of CTD-PAH.\(^9,18\) The amount of extracellular matrix (ECM) proteins contributes to pulmonary arterial remodeling in PAH. Some studies using the HPH model show that hypoxic stimulation leads to secretion of profibrotic factors. However, the relationship between fibrosis, inflammation, and hypoxia in PAH, particularly in CTD-PAH, is not yet fully understood.

To investigate the role of these immune cells and fibrosis in the pathogenesis of CTD-PAH, appropriate animal models reflecting the disease features are essential. Existing mouse models such as the HPH model and the SU5416/Hypoxia-induced PH (HySu) model\(^9\) do not reflect inflammation and fibrosis enough. To date, several challenges have been made to develop CTD-PAH mouse models. Fos-related antigen 2 (Fra-2) transgenic mice\(^12,13\) and fibroblast-specific high-affinity transforming growth factor receptor knock out mice\(^14\) were reported as mouse models for PH associated with SSc. Another group reported that MRL/lpr mice, which produce various autoimmune antibodies and develop nephritis and vasculitis, also spontaneously developed PH.\(^16\) However, these models require genetically modified mice, and the relationship between inflammation and hypoxia in such autoimmune-associated PH mouse models still remains unclear.

Pristane (2,6,10,14-tetramethylpentadecane) is a kind of mineral oil, which might be a possible environmental factor contributing towards human SLE.\(^7\) It is used for SLE mouse model preparation,\(^18\) and can also cause plasma cell hyperplasia\(^19\) and developing lupus-specific autoantibodies in an IL-6-dependent manner.\(^20\) Although it is known that pulmonary vasculitis and diffuse alveolar hemorrhage occur in the pristane-induced SLE mouse model,\(^22\) the effect of pristane on PH has not been evaluated.

In this study, we present a novel mouse model of PH through combination of pristane administration and exposure to chronic hypoxia. We characterized the infiltrated immune cells and transcriptional alterations in the affected lungs, and also showed that this pristane/hypoxia (PriHx) model reflected inflammation, immune abnormality, and fibrosis, the important pathogenic factors in CTD-PAH.

**Methods**

More details about the Methods are available in the Supplementary Methods section.

**Experimental Design**

The experimental protocol was approved by the National Cerebral and Cardiovascular Center Animal Experiment Committee (permit number: 17082, 18030, and 19037). All methods were performed in accordance with the relevant guidelines and regulations of the Physiological Society of Japan. Eight-week-old female C57Bl/6 mice (SLC Japan, Shizuoka, Japan) were divided into 4 groups: an age-matched control group administrated 500 µL of normal saline and bred in normal oxygen concentration (Nx group); another group were administrated 500 µL of pristane (Sigma-Aldrich, St Louis, MO, USA) and bred in Nx (PriNx group); and the other 2 groups, which were exposed to hypoxia (10%) for 4 weeks after administration of 500 µL of pristane (PriHx group) or normal saline (Hx group).\(^18\)

To assess the effect of IL-6 blockade, pristane-administered mice were injected with 2 mg of MR16-1 (rat IgG1 monoclonal anti-IL-6 receptor antibody, kindly provided by Chugai Pharmaceutical Co.) or purified rat control IgG (MP Biomedicals) intravenously just before exposure to hypoxia, and subsequently injected with 0.5 mg of MR16-1 or control IgG intraperitoneally once a week.

**Hemodynamic Measurements**

Hemodynamic measurement was conducted as described previously.\(^5,24\) Briefly, mice were anesthetized with isoflurane (induction 3%, maintenance 1.0–1.5% mixed with room air). The pressure signals were relayed to a blood pressure (BP) Amp (ML117; ADInstruments). Data acquisition was performed by using the Powerlab data system (ADInstruments). Heart rate (HR) was derived from the arterial systolic peaks, and mean arterial pressure and right ventricular systolic pressure (RVSP) was calculated online.

**Morphometric Analysis**

After hemodynamic recording, the mice were exsanguinated, and the heart was excised. The atria were removed, and the RV wall was separated from the left ventricle (LV) and septum. The tissues were weighed and bleached. RV hypertrophy was determined by the ratio of RV to LV+S weight (RV/[LV+S]; Fulton’s ratio).\(^5,24\)

**Immunohistochemical Analysis**

For the immunohistochemical analyses of lung tissues, samples were fixed in 4% paraformaldehyde/phosphate buffered salts (PFA/PBS) for 1 h, cryoprotected with PBS containing 5–20% sucrose, frozen in optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan), and cut into 10-μm-thick cryosections as described previously.\(^25\) All lung sections then were washed in PBS, treated with 1% hydrogen peroxide in methanol for 30 min, incubated in blocking solution containing either 1% BSA or 5% skim milk in PBS and 0.1% Triton-X (PBST) for 1 h, and incubated with primary antibody in PBST overnight at 4°C. Images were acquired by using a microscope (BX-800; Keyence, Osaka, Japan).

**Statistical Analysis**

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Data are presented as the mean±SEM. Differences among multiple groups were compared by one-way analysis of variance (ANOVA) followed by a post-hoc comparison tested with Tukey’s multiple comparison test; P<0.05 was considered statistically significant.

**Results**

**Addition of Pristane Exacerbates Hypoxia-Induced PH**

We first evaluated the pathogenic effects of pristane on PH with or without chronic hypoxia (Figure 1A). Peritoneal administration of pristane did not elevate RVSP in normoxic conditions, whereas pristane significantly elevated RVSP in combination with chronic hypoxia (Figure 1B,C). Histological examinations revealed a significant increase in MWT in the PriHx group compared with the Hx group (Figure 1D,E). Pristane-administered mice showed an increase in medial wall thickness (MWT) compared with non-treated mice also in normoxic conditions (Figure 1D,E). In addition, the PriHx group showed a significant increase
confirm the increase of Gr-1+ myeloid-lineage cells in the lungs of the PriHx mice, we performed flow cytometric analysis. The rates of Gr-1 high CD11b+ neutrophils and monocytes were increased in the pristane-administered groups, as expected (Figure 2F, G). Collectively, these results indicate that pristane administration causes alveolar hemorrhages, leading to inflammatory myeloid cell accumulation regardless of oxygen conditions.

Hemosiderin-Laden Macrophages Are Accumulated in the PriHx Lungs

Next, we assessed pathologic features in the lungs of the pristane-administered mice. Although the affected lungs had scattered and heterogenous lesions in the macroscopic observation, immune cell aggregations around arteries and veins were observed in the lungs of the PriHx mice as well as the PriNx mice (Figure 2A). These cells were positive for CD45 (Figure 2B) and CD68 (Figure 2C), indicating that they were macrophages. These macrophages were also positive for Gr-1, which is expressed on the myeloid lineage cells such as neutrophils, inflammatory monocytes, and macrophages (Figure 2D). Iron staining revealed that these macrophages had phagocytosed hemosiderin, implying that pristane administration induced alveolar hemorrhages in hypoxic conditions, as previously reported in normoxic conditions (Figure 2E, Supplementary Figure 1).

Of Fulton’s index compared with the Hx group (Figure 1F, G). These data indicate that the combination of pristane with chronic hypoxia can produce a significantly more severe phenotype of PH than chronic hypoxia alone.

**Pristane Increases Effector Memory Follicular Helper T Cells in the Lungs**

We next focused on lymphocyte-accumulating lesions in the lungs of the pristane-treated mice. Histological analysis revealed that administration of pristane induced mono-nuclear lymphocyte accumulation, particularly around pulmonary arteries (Figure 3A). However, such lesions were relatively rare and varied from mouse to mouse (Supplementary Figure 2). Therefore, we performed flow cytometric analysis to evaluate all the lymphocytes in the lungs. The percentage of CD4+ helper T lymphocytes increased in the pristane-administered group (Figure 3B, D). Follicular helper T (Tff) cells are a subset of CD4+ helper
Figure 2. Hemosiderin-laden macrophages accumulate in injured areas and around pulmonary arteries and veins after pristane administration. (A) Representative images of the cells around pulmonary arteries by hematoxylin and eosin (H&E) staining (Scale bars: 50 μm). (B) Representative images of the CD45+ cells in the lungs by immunohistochemical staining (Scale bars: 50 μm). (C) Representative images of the CD68+ cells in the lungs by immunohistochemical staining (Scale bars: 50 μm). (D) Representative images of the Gr-1+ cells in the lungs by immunohistochemical staining (Scale bars: 50 μm). (E) Representative images of the hemosiderin-laden macrophages in the lungs by iron staining (Scale bars: 50 μm). The percentages of CD11b+ (F) and Gr-1high CD11b+ cells (G) within the CD45+ CD31− populations in the lungs (n=7). Values shown are the means±SEM. One-way analysis of variance was used to detect differences. *P<0.05, **P<0.01, ***P<0.001.
T lymphocytes, and are required for development of antigen-specific B cells and antibody production. Because pristane is used to induce autoantibody production, we next analyzed PD-1+ cells, including Tfh cells in the lungs. The percentage of PD-1+ cells in CD4+ cells increased in the pristane-administered group (Figure 3C,E). Moreover, such PD-1+ CD4+ cells were mainly CD44+ CD62L− cells, indicating that these cells were effector memory cells (Figure 3F). These results suggest that pristane administration increases activated helper T lymphocytes, including Tfh cells, in the lungs.

Figure 3. Rate of CD44+ CD62L− PD-1+ T lymphocytes increase in the lung after pristane administration. (A) Representative images of lymphocyte accumulation by hematoxylin and eosin (H&E) staining (Scale bars: 50 μm). (B) Representative flow cytometric analysis of CD4+ cells within CD45+ cells from lung tissue in each group. (C) Representative flow cytometric analysis of PD-1+ CD4+ cells within CD4+ cells from lung tissue in each group. (D) Percentages of CD4+ cells within the CD45+ cells from lung tissue in each group, n=7. (E) Percentages of PD-1+ CD4+ cells within the CD4+ cells from lung tissue in each group, n=10–11. (F) Representative flow cytometric analysis which characterize CD44 and CD62L expression of PD-1+ or PD1− CD4+ T lymphocytes in the pristine/hypoxia (PriHx) group. Values shown are the means±SEM. One-way analysis of variance was used to detect differences. **P<0.01, ***P<0.001.
Blood Hemoglobin Concentration Predicts RVSP Elevation in the PriHx Group

Next, we assessed inflammation- and fibrosis-related gene expressions. We observed that the RVSP of the PriHx mice varied widely, and in some PriHx mice, RVSP elevation was similar to that in the Hx mice (Figure 1C). To evaluate gene expression changes characteristically caused by the addition of pristane to chronic hypoxia, we aimed to analyze the mice with RVSP of ≥40mmHg, which was only observed in the PriHx mice. Because catheter manipulation induces undesirable transcriptional changes in the lungs, we searched for a surrogate indicator that can predict mice with elevated RVSP without catheter examinations. As it was reported that blood hemoglobin concentration tends to increase in the patients with SLE-PAH, we measured hemoglobin concentration in the peripheral blood. As the variation of hemoglobin concentration was small in the Nx, the PriNx, and the Hx groups, at ∼13.5 g/dL, 11.5 g/dL and 18.2 g/dL, respectively, the variation of hemoglobin concentration was large in the PriHx group (Figure 4A). In addition, we also found a significant correlation between RVSP and hemoglobin concentration (Figure 4B). There was no difference in the number of white blood cell (WBC) counts among the 4 groups (Figure 4C), neither was there a correlation between RVSP and WBC number in the PriHx group (Figure 4D). Collectively, we used the mice with a hemoglobin concentration of ≥19.0 g/dL, the value corresponding to RVSP of 40 mmHg (Figure 4B), as the

**Figure 4.** Right ventricular systolic pressure (RVSP) positively correlates with blood hemoglobin concentration. (A) Hemoglobin concentration in the blood, n=7–27. (B) The correlation between blood hemoglobin concentration and RVSP of the pristine/hypoxia (PriHx) group, n=14. The dotted line shows a hemoglobin concentration of 19.0 g/dL. (C) The number of white blood cells (WBC) in the blood, n=6–25. (D) The correlation between WBC number in the blood and RVSP of the PriHx group, n=12. Values shown are the means±SEM. One-way analysis of variance was used to detect differences. ***P<0.001.

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Figure 6. Alveolar damage and interstitial thickening are observed in the lungs of the pristine/hypoxia (PriHx) group. (A) Representative images of the whole lung (Scale bars: 5 mm). (B) Representative images of alveolar damage and interstitial thickening in the lungs by hematoxylin and eosin (H&E) staining (Scale bars: 200 μm). (C) Representative images of fibrosis around pulmonary arteries by Masson’s trichrome staining (Scale bars: 50 μm). (D) Quantification of fibrosis area. (E–J) Quantitative real-time polymerase chain reaction (qRT–PCR) analysis of the relative expression level of fibrosis-related mRNA of the total lung (n=3 in each group). (E) Ctgf, (F) Tgfb1, (G) Fn1, (H) Col1a1, (I) Col1a2, (J) Col3a1. Values shown are the means±SEM. One-way analysis of variance was used to detect differences. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
PriHx group in subsequent gene expression analyses.

Inflammatory Cytokines Are Upregulated in the PriHx Mice With High RVSP
As the infiltration of immune cells was remarkable in the pristane-administered mice, we next examined the mRNA levels of inflammatory cytokines and chemokines to evaluate their possible roles in the PriHx model. In addition to strong enhancement of the Il6 mRNA level (Figure 5A), Il1b (Figure 5B) and Tnfα (Figure 5C) also tended to be upregulated in the PriHx group. Although the mRNA level of Ifng was not different among the 4 groups (Figure 5D), one of its receptors, Ifngr1, was upregulated in the hypoxia-exposed mice (Figure 5E). C-C motif chemokine ligand 2 (Cxcl2), also called macrophage inflammatory protein 2-α (MIP-2α), is one of the chemokines secreted from macrophages and is important for immune response and angiogenesis. Although administration of pristane tended to increase the expression level of Cxcl2 even under normoxic conditions, the combination of pristane with hypoxia induced remarkable upregulation of Cxcl2 mRNA (Figure 5F). These results reveal that the addition of pristane enhances the expression of various inflammatory cytokines and chemokines, rather than hypoxia alone.

Lung Injury and Fibrosis Is Enhanced in the PriHx Mice
Additionally, we performed histological and gene expression analysis to evaluate fibrosis in each group. Lung damage was observed macroscopically in the PriNx and the PriHx groups (Figure 6A), although the degree of damage varied among the mice, even in the same group (data not shown). After hematoxylin and eosin (H&E) staining, alveolar collapse, enlargement, and interstitial thickening were observed in the pristane-administered group (Figure 6B). Furthermore, we also assessed fibrosis around pulmonary arteries by using Masson’s trichrome (MT) staining, which revealed more deposition of collagen fiber in the pristane-administered group than other groups (Figure 6C, D). Neither plexiform nor intimal lesions were observed. To confirm this observation, we analyzed fibrosis-related gene expressions. The expression of Ctgf (Figure 6E) and Tgfb1 (Figure 6F), which are key humoral factors for fibrosis, were elevated in the Hx group, and pristane administration markedly enhanced the expression of these genes. Consistently, the expression of ECM molecules, such as Fln1 (Figure 6G), Col1a1 (Figure 6H), Col1a2 (Figure 6I), and Colla3 (Figure 6J) were upregulated in the PriHx group. These results indicate that administration of pristane concomitant with hypoxia induced alveolar injury, particularly interstitial lung lesions, and enhanced fibrosis.

IL-6 Blockade by MR16-1 Ameliorated PH of the PriHx Mice
Finally, we assess the importance of IL-6 signaling in the pathogenesis of the PriHx mice. We previously reported the effect of anti-IL-6 receptor monoclonal antibody, MR16-1, in HPH mice,5 and a similar treatment strategy was applied to the PriHx model. Four weeks after pristane administration, mice were divided into 2 groups, 1 group administered anti-IL-6 receptor monoclonal antibody, MR16-1, and the other administered control IgG concomitant with hypoxic exposure (Figure 7A). Mice administered MR16-1 had significantly reduced RVSP (Figure 7B) and right ventricular hypertrophy (Figure 7C), suggesting that IL-6 is a critical factor in the pathogenesis of PH in PriHx mice.

Discussion
In the present study, we developed a PriHx model, a novel PH mouse model that combines administration of pristane and chronic hypoxic exposure. Hemodynamic and histological analyses revealed that this combination increased RVSP, progressed pulmonary vascular remodeling, and exacerbated right ventricular hypertrophy, meaning that the addition of pristane to hypoxia can produce a more severe PH phenotype than pristane or hypoxia alone. Accumulation of immune cells, including macrophages and CD4+ helper T lymphocytes, was confirmed in the lungs of the PriHx mice. Gene expression analysis revealed that inflammatory cytokines, including Il6 and Cxcl2, were upregulated in the PriHx mice. We also showed that the addition of pristane to hypoxia exacerbated lung fibrosis, indicating that the PriHx model has features of both group 1 and 3 PAH. These results collectively suggest that the PriHx model reflects the characteristics of CTD-PAH better than the conventional HPH model.

We used pristane to induce severe PH in mice, reflecting
the feature of CTD-PAH. Pristane was originally isolated from shark liver oil, which may be taken as a supplement, and is also reported to be contained in diesel exhaust. \textsuperscript{29} There have been several reports that diesel exhaust gas and particulate matter 2.5 (PM2.5) are involved in PH. \textsuperscript{30,31} Furthermore, pristane has been widely used in experiments as an adjuvant to promote autoantibody production. \textsuperscript{32} Considering these together, the PriHx model may be a clue to elucidating the mechanisms by which environmental pollutants, especially those with adjuvant effects, are involved in the development and exacerbation of PH. Interestingly, pristane administration alone caused vascular remodeling, but did not increase RVSP (Figure 1). The addition of hypoxia stimulation as a second hit to vascular remodeling against pristane-induced inflammation may be the reason why the PriHx model exhibited more severe PH than hypoxia stimulation alone. \textsuperscript{35}

The role of Tfh cells in systemic autoimmunity has been described. \textsuperscript{36} Tfh cells are characterized by the expression of surface markers such as chemokine receptor-5 (CXCR5) and PD-1. \textsuperscript{37} It was reported that the population of Tfh increased in the pristane-induced SLE model mice. \textsuperscript{37} Additionally, in the lungs of patients with IPAH, the accumulation of PD-1\textsuperscript{+} Tfh cells was observed in tertiary lymphoid tissue around remodeled vessels. \textsuperscript{38} Our study revealed the increase of PD-1\textsuperscript{+} CD4\textsuperscript{+} T lymphocytes in the lungs of the PriHx mice, and most of these cells were CD44\textsuperscript{+} CD62L\textsuperscript{−} CD4\textsuperscript{+} T lymphocytes, known as effector memory T cells (T\textsubscript{EM}), are present in secondary lymphoid tissues and have features of rapid proliferation after the same antigen stimulation. \textsuperscript{39,40} Moreover, the expression of CD44 on CD45RO\textsuperscript{−} positive T lymphocytes was detected in plexiform lesions of lungs from patients with IPAH. \textsuperscript{41} The increase of CD44\textsuperscript{+} CD62L\textsuperscript{−} CD4\textsuperscript{+} T lymphocytes in the PriHx mice may indicate that there is chronic inflammatory stimuli, and that T\textsubscript{EM} cells continue to proliferate. As a result, proliferation and maturation of B cells that recognize specific antigens, followed by production of autoantibodies, might be involved in the pathogenesis of the PriHx mice.

There have been many reports that inflammatory cytokines play important roles in the pathogenesis of PAH. In the lungs of a HPH mouse model, the mRNA level of Il6 peaked on day 2 and returned to baseline level by day 7. \textsuperscript{5} After day 7, the level was kept at baseline level until day 28, the day of assessment. \textsuperscript{5} Consistent with this observation, we showed that the mRNA level of Il6 in the lungs after a 28-day exposure to hypoxia was equivalent to that of the Nx group (Figure 5A). Pristane administration under normoxic conditions did not change the expression level of Il6. In contrast, interestingly, the combination of pristane with chronic hypoxia significantly enhanced Il6 expression in the lungs even at the time of assessment (Figure 5A). These results imply that pristane administration concomitant with chronic hypoxia maintains high levels of Il6 expression in the lungs. \textsuperscript{5} It has been reported that serum levels of IL-6 are elevated in patients with PAH including CTD-PAH, \textsuperscript{42} and overexpression of Il6 in the lungs induces PH in a mouse model. \textsuperscript{4} Severe PH phenotype observed in our model may be attributed to sustained elevation of Il6 level in the lungs. In fact, IL-6 blockade by MR16-1, an anti-IL-6 receptor monoclonal antibody, significantly ameliorated the elevation of RVSP as well as right ventricular hypertrophy (Figure 7). These results suggest that the IL-6 signaling pathway may be a promising therapeutic target in the treatment CTD-PAH. Further clinical studies are warranted to demonstrate the efficacy of IL-6 blockade in patients with CTD-PAH.

The PriHx mice also exhibited pulmonary fibrosis, which is another characteristic of pathologic manifestation of CTD-PAH (Figure 6). Although the mRNA levels of Ctgf, Fln1, Colla1, and Colla3 were upregulated by hypoxic stimulation alone, pristane administration significantly enhanced their expression. As fibronectin, encoded by the Fnn gene, has been identified in ectopic lymph nodes in IPAH patients, \textsuperscript{43} the elevated Fnn mRNA expression may be, in part, attributed to ectopic lymph node formation by an immune response caused by pristane administration. Our study also revealed that the expression of Cxcl2 (MIP-2a) significantly increased in the lungs of the PriHx mice (Figure 5A). Cxcl2 is important not only for immune response and angiogenesis, but also for pulmonary fibrosis. \textsuperscript{44} It has been reported that the expression level of Cxcl2 was increased in the lungs of pulmonary fibrosis model mice treated with bleomycin, and inhibition of Cxcl2 by neutralizing antibodies attenuated fibrosis. \textsuperscript{45} Additionally, the expression of Cxcl2 was elevated in the patients with scleroderma complicated by PAH. \textsuperscript{44} Taken together, we infer that the addition of pristane to hypoxia promotes pulmonary fibrosis, some of which is due to elevated Cxcl2 expression, resulting in worsening of PH phenotype.

In conclusion, we have developed a PriHx model, a novel murine model of PH reflecting the pathologic features of CTD-PAH, through combination of pristane administration and hypoxia. This model is expected to help improve understanding of the pathophysiology of CTD-PAH and develop effective therapeutic agents.

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