Pathogenesis of diffuse alveolar hemorrhage in murine lupus

Haoyang Zhuang, Ph.D.,1 Shuhong Han, Ph.D.,1 Pui Y. Lee, M.D., Ph.D.,2 Ravil Khaybullin, Ph.D.,3 Stepan Shumyak, B.S.,1 Li Lu, M.D.,4 Amina Chatha, M.D.,1, Anan Afaneh, M.D.,1, Yuan Zhang, M.D., Ph.D.,4 Chao Xie, M.S.,4 Dina Nacionales, M.D.,5 Lyle Moldawer, Ph.D.,5 Xin Qi, Ph.D.,3 Li-Jun Yang, M.D.,4, and Westley H. Reeves, M.D.,1,*

1Division of Rheumatology & Clinical Immunology, Department of Medicine, University of Florida, PO Box 100221, Gainesville, FL 32610-0221
2Division of Immunology, Boston Children’s Hospital, 300 Longwood Ave., Boston, MA 02115
3Department of Medicinal Chemistry, University of Florida, Gainesville, FL 32610
4Department of Pathology, Immunology, & Laboratory Medicine, University of Florida, Gainesville, FL 32610
5Department of Surgery, University of Florida, Gainesville, FL 32610

Abstract

Objective—Diffuse alveolar hemorrhage (DAH) in lupus patients is >50% fatal. The cause is unknown. The pathogenesis of DAH in C57BL/6 mice with pristane-induced lupus, a model of human lupus-associated DAH, was examined.

Methods—Clinical/pathological and immunological manifestations DAH in pristane-lupus were compared with human DAH. Tissue distribution of pristane was examined by mass spectrometry. Cell types responsible for disease were determined by in vivo depletion using clodronate liposomes (CloLip) and anti-neutrophil monoclonal antibodies (GR1). The effect of complement depletion with cobra venom factor (CVF) was examined.

Results—After i.p. injection, pristane migrated to the lung, causing cell death, small vessel vasculitis, and alveolar hemorrhage similar to human DAH. B-cell-deficient mice were resistant to induction of DAH, but susceptibility was restored by infusing IgM. C3-deficient and CD18-deficient mice also were resistant and DAH was prevented in wild-type mice by CVF. Induction of DAH was independent of TLRs, inflammasomes, and inducible nitric oxide (iNOS). Mortality was increased in IL-10-deficient mice and pristane treatment decreased IL-10 receptor expression in monocytes and Stat3 phosphorylation in lung macrophages. In vivo neutrophil depletion was not protective, whereas treatment with CloLip prevented DAH, suggesting that macrophage activation is central to DAH pathogenesis.

Conclusion—The pathogenesis of DAH involves opsonization of dead cells by natural IgM and complement followed by complement receptor-mediated lung inflammation. The disease is

*To whom correspondence should be addressed: Division of Rheumatology & Clinical Immunology, University of Florida, PO Box 100221, Gainesville, FL 32610-0221; Phone: 352-294-8210; Fax: 352-294-8204; whreeves@ufl.edu.

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macrophage-dependent and IL-10 is protective. Complement inhibition and/or macrophage-targeted therapies may reduce mortality in lupus-associated DAH.

Introduction

Although frequently unrecognized, lung disease occurs in half of patients with systemic lupus erythematosus (SLE). Manifestations include pleuritis, pulmonary hypertension, and interstitial lung disease [1]. About 3% of SLE patients develop diffuse alveolar hemorrhage (DAH), but it is a significant problem with >50% mortality [2, 3]. Clinical features include hemoptysis, falling hemoglobin, and a strong association with lupus nephritis [3, 4]. Pathological examination reveals hemosiderin-laden macrophages, bland hemorrhage, and/or pulmonary capillaritis [3]. The pathogenesis remains unclear.

C57BL/6 (B6) mice with pristane-induced lupus develop DAH manifested by alveolar and perivascular inflammation (capillaritis, small vessel vasculitis), hemorrhage, endothelial injury, and infiltration of macrophages, neutrophils, lymphocytes, and eosinophils [5, 6]. Anti-neutrophil cytoplasmic antibodies (ANCA) are absent [6]. Recruitment of macrophages and neutrophils precedes hemorrhage, starting 3-d after pristane injection and peaking at 2-wk [7]. DAH is independent of MyD88, TLR7, FcγR, Fas, and T cells but immunoglobulin-deficient (μMT) mice [7] are resistant. The present studies were carried out to further define the pathogenesis of DAH in this model.

Materials and Methods

Mice and pristane treatment

Mice were bred and maintained under specific pathogen-free conditions. Female, 10–12-week-old C57BL/6 (B6), B6.129S2-Ighm<sup>tm1Cgn</sup>/J (μMT), B6.129P2-Illg<sup>tm1Cgn</sup>/J (IL-10−/−), B6.129X1-Elan<sup>tm1Sdv</sup>/J (Elastase−/−), and B6.129S4-C3<sup>tm1Crr</sup>/J (C3−/−), B6.129S7-<sup>Itgb2</sup><sup>tm1Bay</sup>/J (CD18−/−), B6(Cg)-<sup>Il10</sup><sup>tm1Cgn</sup>/J (IL-10−/−), B6.129S2-<sup>Casp1</sup><sup>tm1Flv</sup>/J (Casp1−/−), and B6.129P2-<sup>Nos2</sup><sup>tm1Lau</sup>/J (Nos2−/−) mice were from Jackson Laboratory (Bar Harbor, ME). C57BL/6J-<sup>Ticam1</sup><sup>Lps2</sup>/J (Trif<sup>Lps2</sup>, TRIF−/−) and B6.129P2(JIL)-<sup>MyD88</sup><sup>tm1Defr</sup>/J (MyD88−/−) mice were bred at the University of Florida. To induce lupus, 0.5 mL of pristane (Sigma-Aldrich, St. Louis, MO) was administered i.p. Controls were left untreated. Peritoneal exudate cells were collected by lavage. In some experiments, bronchoalveolar lavage (BAL) was performed. After euthanizing the mice, a small incision was cut in the trachea and the alveolar spaces were lavaged with 1 ml of PBS. Cells collected by BAL were resuspended in RPMI1640 + 10% fetal bovine serum and incubated at 37°C for 1-hr before treating with IL-10 (1 ng/ml). After 15-min incubation, the cells were fixed, permeabilized, and surface-stained with anti-CD11b antibodies and intracellularly-stained with anti-phospho-Stat3 antibodies as below. These studies were approved by the Institutional Animal Care and Use Committee.

Immunoglobulin infusion in μMT mice

Similar to interferon production in pristane-treated mice [8], ischemia-perfusion injury in mice is mediated by the early classical complement cascade and natural IgM [9]. Human natural IgM is as effective at inducing ischemia/reperfusion injury as murine IgM [10]. In
light of these observations and in view of the relative ease of obtaining human vs. mouse IgM, the requirement for immunoglobulin in DAH was evaluated by administering purified human IgM (50 or 200 μg/mouse, Sigma-Aldrich), murine IgG (200 μg/mouse, Sigma-Aldrich), or PBS i.v. to μMT mice 1-d before and 7-d after pristane treatment. DAH was assessed at 14-d.

**Cobra venom factor (CVF) treatment**

Mice were treated with CVF (CompTech, Tyler, Texas) 10 μg i.p. 1-d before and 7-d after pristane treatment. C3 depletion was monitored (ELISA) using anti-C3 antibodies from Bioss (Woburn, Massachusetts). DAH was assessed at 14-d.

**Lung pathology**

Formalin-fixed, paraffin-embedded archived human lung biopsy tissue from a 19-year-old woman with lupus nephritis who developed massive hemoptysis and DAH was sectioned (4 μm) and stained with hematoxylin & eosin (H&E). Pristane-treated mice were euthanized at 14-d and lungs were formalin-fixed. DAH was evaluated by gross inspection of excised lungs and confirmed by microscopically. Tissue sections were subjected to antigen retrieval and analyzed by TUNEL (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Chemicon/Millipore, Danvers, MA). Neutrophil elastase was detected by IHC with polyclonal rabbit antibodies (Abcam, Cambridge, MA, 1:50 dilution for 60-min) and quantified morphometrically. The expression area and staining intensity were quantified using MetaMorph Premier Image Analysis Software (Molecular Devices Corporation, Sunnyvale, CA). Staining intensity (thresholded area) was expressed as percentage of total examined lung cell area after subtracting noncellular space from total area.

Staining with oil red-O was performed on 10 μm frozen sections of lung tissue from pristane-treated mice or untreated controls [11]. Tissue was counterstained with Mayer’s hematoxylin and viewed microscopically.

**Pristane-induced in vitro cell death**

Pristane or mineral oil (MO, a hydrocarbon oil that does not cause lupus) were dissolved/emulsified in PBS containing 100 mg/ml BSA. At saturation, the solution contained 37.2 mg/ml of pristane. MO was mixed with PBS-BSA at the same concentration. BW5147 (murine thymoma, ATCC) and RAW264.7 (murine macrophage, ATCC) cells were incubated 24-h at 8 × 10^5/ml in RPMI containing 10% fetal bovine serum and serial 2-fold dilutions of either pristane or MO in PBS-BSA or in medium alone. Cell death (necrosis) was determined by flow cytometry (7-amino actinomycin-D).

**Mass spectrometry**

Lung and bone marrow tissue was collected 1-wk after pristane treatment and frozen in liquid nitrogen. Metabolites were extracted from 15–30 mg of tissue, which was homogenized with mortar and pestle under liquid nitrogen. Pre-cooled 80% methanol (0.7 mL) and chloroform (0.7 mL) was added, and the mixture was kept on ice and vortexed every 5-min for 30-min. Ice-cold double-distilled H_2O (0.5 mL) was added to ensure separation of the aqueous and organic layers. The tube was centrifuged (3200 × g, 10-min at
4°C). The organic layers extracted from pristane-treated and untreated mice were diluted in methanol for determining pristane level using a Thermo Scientific™ LTQ-Orbitrap-XL mass spectrometer (Waltham, MA USA). Mass spectra were analyzed using SCIEX OS Software.

Flow cytometry

Peritoneal exudate and bone marrow cells were surface stained with the following antibodies: anti-CD11b-Pacific Blue (PB), anti-Ly6G-phycocerythrin (PE) (or APC-Cy7), and IL-10 receptor (IL-10R)-PE. In some cases, the cells were fixed, permeabilized with Perm/fix buffer (eBioscience, San Diego, CA), and intracellularly stained with anti-TNFα-allophycocyanin (APC) (BioLegend). In some experiments, the cells were fixed with Cytofix/Perm buffer, permeabilized with PhosFlow Permealization buffer (BD Biosciences, San Jose, CA), and stained with anti-phospho-STAT3-PE antibodies (BD Biosciences). Flow cytometry was performed using a LSRII flow cytometer (Becton Dickinson San Jose, CA). At least 5 × 10⁵ events were collected per sample. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

In vivo depletion of neutrophils and macrophages

Neutrophils were depleted by treating with anti-Ly6G mAb IA8 (BioXcell) (250 μg i.v./mouse 1-d before and 7-d after pristane treatment) [12]. Mice were euthanized at 14-d for pathological analysis of DAH. Depletion of neutrophils was ascertained in the bone marrow, peripheral blood, and peritoneum by flow cytometry (anti-Ly6G-PE, clone RB6-8C5) plus anti-CD11b-PB). Neutrophil depletion in the lung was evaluated by H&E staining and elastase IHC.

Macrophages were depleted using clodronate liposomes (CloLip, ClodronateLiposomes.com, Amsterdam, The Netherlands) [13], 50 μL i.p. per mouse 1-d before and 7-d after pristane treatment [14]. Mice were euthanized at 14-d for pathological evaluation of DAH. Depletion of peritoneal monocytes and macrophages was ascertained by flow cytometry (anti-Ly6C-FITC plus anti-CD11b-PB). Depletion in lung was evaluated by H&E staining and IHC using peroxidase-conjugated anti-F4/80 (clone BM8, Caltag Medsystems Ltd, UK), anti-CD11b (clone 5C6, AbD Serotec, UK), or anti-CD11c (clone N418, Abcam) antibodies followed by immunoperoxidase-3,3-diaminobenzidine (DAB) staining. Depletion of F4/80⁺ cells was quantified in a blinded manner by a pathologist (LY). Macrophage (F4/80) staining of the perivascular and alveolar regions of lungs from untreated (n = 7) vs. pristane + CloLip-treated (n = 8) mice was graded as strongly positive (2⁺), weakly positive (1⁺), or absent (0⁺).

Statistical analysis

Data are representative of at least two independent experiments and are presented as mean ± SD. For normally distributed data, comparisons were performed by the unpaired 2-tailed Student t test (GraphPad Prism version-5, San Diego, CA). Comparisons for non-normally distributed data were made by Mann-Whitney test. Frequency data were analyzed by Fisher exact test. Correlation was analyzed using Spearman’s rank correlation coefficient. p < 0.05 was considered significant.
Results
DAH requires IgM, C3, and CD18

B6 mice develop severe DAH 2–4 weeks after i.p. pristane injection [5], whereas B cell-deficient (μMT) mice are resistant [7]. We asked whether this is due to the absence of immunoglobulin or another B cell function. About 60–70% of pristane-treated wild type B6 mice developed DAH seen by examining gross lung tissue (Fig. 1A–B). In contrast, μMT mice did not develop DAH, whereas disease could be restored by injecting 200 μg/mouse (but not 50 μg/mouse) of normal human IgM i.v. (Fig. 1A, right) (P < 0.001, Fisher exact test). Infusion of 200 μg/mouse of normal mouse IgG did not restore the ability of pristane to induce disease (Fig. 1A, right). In contrast, μMT mice receiving 100 μL of normal mouse serum i.v. prior to pristane developed DAH (data not shown).

DAH also was absent in C3−/− and CD18−/− mice (Fig. 1B, left). Pretreatment of wild-type mice with CVF 1-d before and 7-d after pristane treatment prevented DAH, whereas 69% of untreated mice developed DAH (Fig. 1B, right) (P < 0.001, Fisher exact-test). A single CVF treatment (1-d before pristane) was partially protective.

H&E-stained lung tissue from wild-type mice showed hemorrhage (Fig. 1C, “H”), pulmonary vasculitis with neutrophilic and mononuclear cell infiltrates surrounding the capillaries and small arteries (“V”), and hemosiderin-laden macrophages within the alveoli (arrows). These changes were absent in C3-deficient mice. Nearly identical changes were seen in H&E stained tissue from a lupus patient with DAH (Fig. 1C). Thus, DAH in pristane-induced lupus closely resembles human lupus-associated DAH pathologically and is mediated by IgM, C3, and CD18, a component of C3b receptors (CR) 3 and 4.

Pristane migrates to lung after i.p. injection

We investigated the mechanism(s) responsible for DAH. Although pristane is injected i.p., we hypothesized that it may gain access to the lungs. To examine that possibility, lung tissue was extracted 7-d after pristane treatment and analyzed by mass spectrometry (Fig. 2A,B). A pristane standard yielded the characteristic fragmentation pattern with mass to charge ratios (m/z) of 268 (M), 267 (M−1), 269 (M+1), and 270 (M+2) (Fig. 2A). The four characteristic peaks were seen in lung tissue of i.p. pristane-treated, but not untreated, wild-type mice (Fig. 2B). Pristane also was detected in the lung from treated, but not untreated, C3−/− mice (Fig. 2B), indicating that C3 is not necessary for migration of pristane from the peritoneum to lung. To confirm the presence of pristane in the lung, cryosections of lung tissue from pristane-treated mice were stained with oil red-O, revealing numerous oil red-O positive droplets within the alveolar walls (Fig. 2C). Oil red stained material was absent in lung from untreated mice, consistent with the mass spectrometry data. Peritoneal pristane injection also causes bone marrow inflammation [15], and pristane was detected in bone marrow of pristane-treated, but not untreated, mice by mass spectrometry (Fig. 2D), suggesting that the oil is widely dispersed following i.p. injection.
**Pristane induces cell death**

Examination of lung tissue by TUNEL revealed dead cells in pristane-treated mice but not untreated controls (Fig. 3A). Many TUNEL+ cells were large, vacuolated cells located within the alveoli, reminiscent of alveolar macrophages, but smaller TUNEL+ cells also were present. Dead cells also accumulate in the bone marrow of pristane-treated mice [15], suggesting that pristane might be cytotoxic. That possibility was examined by incubating mouse BW5147 T cells with pristane or mineral oil (which causes minimal alveolar hemorrhage, not shown). BW5147 cells exposed in vitro to pristane, but not mineral oil, exhibited dose-dependent induction of cell death (7-AAD+) (Fig. 3B). In contrast, RAW264.7 macrophages were resistant to the cytotoxic effects of pristane, with no significant 7-AAD staining (Fig. 3C). Taken together, the data indicate that pristane migrates from the peritoneum to the lungs and other tissues, where it may cause death of certain cell types. Opsonization of these dead cells by IgM and C3 may promote pulmonary inflammation, as also seen in the peritoneum [8].

**Macrophages, but not neutrophils, mediate DAH**

Normal lung contains several subsets of macrophages and dendritic cells, but BM-derived monocytes and neutrophils are recruited during inflammation [16, 17]. We examined the role of monocytes/macrophages vs. neutrophils in DAH by selectively depleting these subsets in wild-type mice. Neutrophil depletion with anti-Ly6G antibodies greatly decreased total peritoneal neutrophils and peritoneal neutrophils containing intracellular TNFα, but had little effect on bone marrow neutrophils (Fig. 4A). In the lung, neutrophils were decreased in H&E- and neutrophil elastase-stained sections (Fig. 4B). Perivascular neutrophilic infiltrates were markedly reduced (Fig. 4B). However, numerous perivascular F4/80+ macrophages remained after anti-Ly6G treatment (Fig. 4C, arrows), despite depletion of elastase+ cells (Fig. 4D).

In contrast, CloLip treatment, which depletes monocytes/macrophages [13], abolished the perivascular infiltrates in pristane-treated mice (Fig. 4E). The frequency of DAH was greatly reduced in CloLip-treated mice ($P < 0.001$, Fisher exact test), but was unaffected by neutrophil depletion (Fig. 4F). Like DAH, the localized Shwartzman reaction in skin is associated with thrombohemorrhagic vasculitis mediated by C3b-CR3 interactions [18]. CR3-mediated neutrophil activation results in vessel damage due to neutrophil elastase production. However, elastase-deficient mice were fully susceptible to pristane-induced DAH (Fig. 4F). They also developed pulmonary vasculitis (not shown). Type I interferon and TNFα production also remained intact in elastase-deficient mice (not shown). Thus, although it superficially resembles the localized Shwartzman reaction, DAH is not mediated by neutrophils and instead appears to be mediated by macrophages.

**CloLip deplete alveolar and perivascular macrophages**

Perivascular inflammatory cell infiltrates in lung from pristane-treated mice contained small, F4/80hi cells (Figs. 4C and 5A, top). Perivascular CD11b+ cells also were present (Fig. 5A, top), but it could not be determined whether CD11b staining was associated with the F4/80hi
macrophages, F4/80− neutrophils, or both. The perivascular cells were uniformly CD11c− (Fig. 5A, top right). The lung alveoli were consolidated by intraalveolar hemorrhage and inflammatory cells (Fig. 5A). Numerous large, F4/80lo alveolar macrophages (AM) were present in the alveolar spaces (Fig. 5A, bottom, yellow arrows). F4/80 staining of AM was less intense than on the smaller perivascular cells, and they also were CD11b+CD11clow (Fig. 5A, bottom, yellow arrows). In addition, small F4/80−CD11b+CD11c− cells (monocytes and/or neutrophils) were present in the lung parenchyma (Fig. 5A, top). Although normal AM are F4/80+CD11b+CD11clow [17], CD11b is induced by inflammation [19], suggesting that the large F4/80lo cells were activated AM. Lung from untreated mice did not contain perivascular F4/80hi cells and had lower numbers of F4/80lo AM (Fig. 5B).

F4/80−CD11b+CD11c− cells were absent in normal lung. CloLip substantially depleted the F4/80hi macrophages (Fig. 5C). Consistent with the marked reduction of alveolar hemorrhage (Fig. 4F), CloLip treatment decreased both vasculitis (perivascular F4/80hi cells, P<0.01, t-test) and the large F4/80lo AM (P<0.015, t-test) (not shown). However, occasional F4/80hi cells were still visualized in the alveolar septa (Fig. 5C). Interestingly, perivascular macrophages and neutrophils both were absent in CloLip-treated mice (Fig. 5C, left panel). The alveolar septa appeared thickened in CloLip-treated mice, despite the absence of alveolar hemorrhage. There was no alveolar hemorrhage in 6 of 8 CloLip-treated mice, whereas two exhibited mild alveolar hemorrhage (p < 0.001, Fisher exact test) (Fig. 4F). The severity of alveolar hemorrhage and vasculitis seemed to be related to the extent of F4/80hi macrophage depletion. Hemorrhage was absent in mice exhibiting complete depletion of the perivascular and alveolar F4/80hi cells, whereas the two mice with mild DAH had only partial depletion of F4/80hi cells, suggesting that CloLip treatment was incomplete. CloLip treatment also depletes Ly6ChiCD11b+ inflammatory macrophages from the peritoneum [14]. Lung from pristane-treated CD18−/− mice showed no evidence of alveolar hemorrhage or F4/80hi macrophages, but in contrast to CloLip-treated wild type mice, alveolar architecture was normal (Fig. 5C). Mild thickening of the alveolar septa in pristane + CloLip-treated mice vs. untreated B6 controls also was apparent on H&E staining and F4/80 immunohistochemistry (Fig. 5D).

**Mechanism of lung inflammation**

Lung interstitial macrophages and epithelial cells are anti-inflammatory and secrete IL-10 [20, 21]. Although AM normally are anti-inflammatory, when activated via TLRs, IL-10 receptor (IL-10R) signal transduction is inhibited and they become pro-inflammatory [21, 22]. Thus, IL-10 is a crucial role regulator of lung inflammation. Expression of CD11b suggested that AM from pristane-treated mice are activated (Fig. 5A). Accordingly, we examined the effect of IL-10 on DAH. IL10−/− mice had significantly increased mortality from DAH (Fig. 6A). Pristane-treated B6 mice had a mortality of ~40% at 1-month. There was little additional mortality afterward. There was no DAH (Fig. 1B) or mortality up to 24-wks after pristane treatment of C3−/− mice (Fig. 6A). In contrast, mortality from DAH was nearly 75% in IL10−/− mice (P < 0.005, Mantel-Cox test) (Fig. 6A).

TLR-activated genes are targeted by IL-10 [23] and pristane induces proinflammatory cytokine production via TLR7 [24]. Unexpectedly, MyD88−/− and TRIF−/− mice developed DAH at a frequency similar to wild-type mice (Fig. 6B). Pristane also induced DAH in
TNFα−/− and type I IFN receptor (IFNAR−/−) mice. Consistent with our previous observations that proinflammatory cytokine production in pristane-treated mice is TLR7/MyD88-dependent [15, 24], neutrophils from pristane-treated MyD88−/− mice did not produce TNFα, whereas neutrophils from TRIF−/− and wild-type mice exhibited similar TNFα+ production (Fig. 6C).

As IL-10 inhibits IL-1 production, we tested caspase 1−/− mice, but found no protection from DAH (Fig. 6B). Although nitric oxide production can damage the alveolar wall [25], Nos2−/− mice were susceptible to pristane-induced DAH (Fig. 6B).

To further examine the role of IL-10, we stained bone marrow myeloid cells from untreated and 1-month pristane-treated B6 mice with anti-IL10R antibodies. As expected, CD11b+Ly6G− monocytes expressed IL-10R, whereas neutrophils (CD11b+Ly6G+) were mostly negative (Fig. 6D, left and middle). IL-10R staining of bone marrow monocytes was decreased in pristane- vs. MO-treated mice (Fig. 6D, right).

AM (CD11b+F4/80+CD11c+SiglecF+CD205+) were present in BAL fluid from untreated mice (Fig. 6E, left). As reported [22], they expressed IL-10R. IL-10R also was expressed by AM collected by BAL from MO- and pristane-treated mice, with a trend toward lower expression in pristane-treated mice (data not shown). AM collected by BAL from pristane-vs. MO-treated mice were incubated for 15-min with IL-10 followed by staining with anti-CD11b (expressed by activated AM) and anti-phospho-Stat3 antibodies. As shown in Fig. 6F, Stat3 activation was higher in AM from MO-treated vs. pristane-treated mice, suggesting that pristane treatment decreases the responsiveness of AM to IL-10.

**Discussion**

Lupus-associated DAH has a mortality >50% [2, 3]. Either bland hemorrhage or focal pulmonary capillaritis may be present in DAH due to lupus or vasculitis. Capillaritis occurs in 88% of patients, most commonly in granulomatosis with polyangiitis, microscopic polyangiitis, and SLE [26], and often is associated with fibrin thrombi occluding the intra-alveolar capillaries and fibrinoid necrosis of the small blood vessels (Figs. 5, 6). It may be accompanied by inflammation of larger vessels and IgG/C3 deposition in the alveolar walls. In contrast to leukocytoclastic vasculitis, erythrocytes extravasate into the alveolar spaces (and not the interstitium) in DAH [26] (Figs. 5–6).

C57BL/6 with pristane-induced lupus develop hemorrhage and pulmonary capillaritis morphologically similar to human SLE-associated DAH [5–7] (Fig. 1C). DAH required CD18 (a component of CR3 and CR4) and opsonization of dead cells by natural IgM and the early classical complement pathway (Figs. 1–2). Unexpectedly, macrophages, but not neutrophils, were required for disease (Figs. 4–5). Consistent with the importance of macrophage activation, disease was exacerbated in IL-10−/− mice (Fig. 6A). However, in contrast to the TLR7-dependency of proinflammatory cytokine production [15, 24], DAH was MyD88, TRIF, TNFα, and Nos2 -independent (Fig. 6B). Although both type I IFN [27] and inflammasomes [28] are involved in pristane-induced lupus, IFNAR−/− and caspase-1
deficient mice [also caspase-11 deficient [29]], were not protected. Thus, DAH was independent of interferon and the canonical and non-canonical inflammasome pathways.

**Opsonization of dead cells may initiate lung injury**

Defective clearance of dead cells by resident lung phagocytes contributes to inflammation in acute lung injury, asthma, cystic fibrosis, and chronic obstructive pulmonary disease [30]. Human SLE and pristane-induced lupus both are associated with impaired apoptotic cell clearance and mouse models suggest this promotes lupus [31]. Monocyte-derived macrophages from SLE patients are poorly phagocytic [32]. Both patients and lupus mice exhibit increased numbers of uncleared apoptotic cells in tissues [15, 33]. Accumulation of dead cells in the lungs of pristane-treated mice (Fig. 3) may be partly due to the cytotoxicity of pristane. Consistent with an earlier report [34], pristane caused dose-dependent T cell death (Fig. 3B). But RAW264.7 macrophages were resistant (Fig. 3C). Widespread dissemination of pristane after peritoneal injection (Fig. 2) may promote apoptosis and defective removal of dead cells in the lung (S Han, unpublished observations) as suggested by the TUNEL + cells in lungs of pristane-treated mice (Fig. 3A) and consistent with the presence of dead cells in the lungs of SLE patients with DAH (data not shown). Interestingly, TUNEL staining suggested that large cells consistent with the appearance of AM were undergoing cell death (Fig. 3A). That possibility is supported by evidence that the recovery of AMs by bronchoalveolar lavage was decreased in pristane-treated vs. MO-treated mice (not shown). Thus, there may be differences in the susceptibility of various macrophage subsets to the induction of cell death by pristane. Although resident AM generally are more resistant to apoptosis than macrophages recruited to the lung [35], engagement of the scavenger receptor MARCO sensitizes them to undergo apoptosis following uptake of silica [36]. As MARCO is involved in the uptake of apoptotic cells in pristane-treated mice (S Han, et al. submitted), the uptake of dead cells may sensitize AM to apoptosis. Further studies will be necessary to determine the cause of the differential susceptibility of AM vs. RAW264.7 cells to pristane-induced apoptosis.

Our data suggest that DAH is mediated by opsonin-dependent uptake of dead cells, as natural IgM, C3, and CD18 are required (Fig. 1). Induction of the interferon signature by pristane also requires IgM and C3 due to opsonization of dead cells and phagocytosis via CR3 and/or CR4 [8]. Since CD11b−/− mice are protected from pristane-induced DAH [37], CR3 is likely to play an important role in the pathogenesis of lung inflammation. Our data suggest that the importance of CD11b and CD18 in lung inflammation lies in the role of CR3 as a phagocytic receptor rather than as a mediator of cell adhesion and migration. An additional role for CR4 (CD11c/CD18) cannot be excluded.

**DAH is macrophage-dependent**

Alveolar hemorrhage is thought to emanate from neutrophilic inflammation of capillaries (capillaritis) [38] and neutrophils also are critical to the pathogenesis of acute lung injury induced by endotoxin, shock, and ischemia-reperfusion injury [39]. The lungs of pristane-treated mice exhibited neutrophilic infiltration (Fig. 4B), but F4/80+ macrophages and other myeloid cells also were present (Fig. 5). Although the pulmonary vasculitis superficially resembled a localized Shwartzman reaction (neutrophilic infiltration, erythrocyte
extravasation, and dependence on C3 and neutrophil CR3 [18]. Neutrophil depletion had little effect on DAH (Fig. 4F). It also was unaffected by the absence of neutrophil elastase (Fig. 4F), a key mediator of the Schwartzman reaction [18]. DAH was greatly attenuated by CloLip treatment (Fig. 4F, Fig. 5C), suggesting that cells of the monocyte-macroage lineage, rather than neutrophils, are central to the pathogenesis of lung hemorrhage. Although acute lung injury can be associated with alveolar hemorrhage, neutrophils, IL-1, and TNFα are key inflammatory mediators [39], suggesting that pristane-induced DAH differs from acute lung injury.

At least two F4/80+ cell subsets were present in lungs of pristane-treated mice: small perivascular F4/80hiCD11b−CD11c− cells and large F4/80hiCD11b+CD11c0 cells (Fig. 5A). Both were depleted by CloLip (Fig. 5C). AM are long-lived, self-renewing resident macrophages [40]. Although normally CD11b−, CD11b expression is induced on AM by inflammation [19]. Thus, the large F4/80hiCD11b+CD11c0 cells are likely to be AM activated by the inflammatory response to pristane. The perivascular F4/80hiCD11b+CD11c− cells are probably bone marrow-derived inflammatory macrophages recruited to the inflamed lung. As both were depleted by CloLip and absent in CD18−/− mice, we cannot determine which subset causes lung injury.

**IL-10 modulates the severity of DAH**

IL-10 down-regulates proinflammatory cytokine production by macrophages at multiple (transcriptional and post-transcriptional) levels [41]. Its constitutive production by lung interstitial macrophages and epithelial cells helps maintain the anti-inflammatory phenotype of AM and BAL fluid from untreated mice contains IL-10 thought to be derived mainly from lung epithelial cells [20, 42]. DAH was more severe in IL10−/− mice than controls, suggesting that IL-10 protects against pristane-induced lung injury (Fig. 6A) by interacting with IL-10R on AM (Fig. 6E) or bone marrow-derived cells.

During inflammation, bone marrow-derived macrophages are recruited to the alveoli and differentiate into proinflammatory (M1-like) macrophages [25]. IL-10R expression in untreated bone marrow was restricted to CD11b+Ly6G− cells of the monocyte lineage (Fig. 6D). After pristane injection, these cells are recruited to the inflamed peritoneum via CCL2-CCR2 [43]. In the absence of type I interferon, they develop into Ly6Clo macrophages, whereas maturation is inhibited by interferon. Decreased IL-10R expression on bone marrow monocytes from pristane- vs. untreated mice (Fig. 6D), suggests that monocytes recruited to the inflamed lung in pristane-treated mice may be poorly responsive to the anti-inflammatory effects of locally produced IL-10, consistent with the decreased Stat3 phosphorylation in BAL cells from pristane vs. MO treated mice (Fig. 6F). It will be of interest to see if the higher IL-10R expression by bone marrow monocytes from MO-treated mice promotes differentiation into anti-inflammatory (M2-like) macrophages, whereas low IL-10R expression in pristane-treated mice results in M1-like polarization.

**Inflammatory pathways in DAH**

The inflammatory pathway(s) activated when IgM/C3-opsonized dead cells engage CR3/Cr4 on macrophages remain to be determined. IL-10 down-regulates TLR-stimulated
transcription of proinflammatory cytokines by inducing ubiquitination and degradation of MyD88-dependent signaling molecules, such as TRAF6 and IRAK-4 [44]. In view of the importance of TLR7 and MyD88 in pristane-induced proinflammatory cytokine production, it was surprising that DAH develops in TLR7−/− [7] and MyD88−/− (Fig. 6B) mice. DAH also developed in pristane-treated TRIF−/− mice (Fig. 6B). Thus, the pathogenesis of DAH may not involve TLR signaling, although it will be necessary to examine the susceptibility of MyD88/TRIF double knock-out mice to exclude the possibility that the TRIF and MyD88 can compensate for one another.

Pristane causes sterile inflammation, which is caused by innate immune recognition of damage associated molecular patterns released from dead cells [45]. Sterile inflammation is mediated by both neutrophils and monocytes. Neutrophil-mediated inflammation requires MyD88, IL-1α, and the IL-1 receptor (IL-1R), but not TRIF or TLRs whereas the monocytic response is MyD88/IL-1R independent [46, 47]. The susceptibility of MyD88−/− and caspase-1/caspase-11 deficient mice (Fig. 6B) further supports the idea that macrophages rather than neutrophils mediate DAH. Although Nos2 has been implicated in bleomycin-induced lung injury [48], it was not involved in pristane-induced DAH (Fig. 6B). Further studies are needed to define the inflammatory pathway(s) downstream of CR3/CR4 that lead to DAH.

**Clinical implications**

DAH is an unusual manifestation of SLE with a high mortality and unclear etiology [3, 4]. Although a variety of therapeutic interventions are used, their efficacy is unclear [3, 4]. The pathological changes in pristane-induced DAH, especially the presence of capillaritis, closely resemble those in patients with DAH (Fig. 1C). Like DAH in SLE patients, murine DAH is not associated with antineutrophil cytoplasmic antibodies (ANCA) and hemosiderin-laden macrophages are present [6] (Fig. 1C). Moreover, dead cells accumulate in lung tissue from human DAH (H Zhuang, unpublished data) as well as pristane-induced DAH (Fig. 3A). In contrast to chemical pneumonitis and ANCA+ vasculitis syndromes, such as microscopic polyangiitis and granulomatosis with polyangiitis, SLE-associated DAH is thought to be immune complex-mediated with granular deposits of immunoglobulin, C3, and DNA in the alveolar capillary walls [49]. The requirement for immunoglobulin and C3 in pristane-induced DAH (Fig. 1A–B) is consistent with the role of immune complexes in this animal model, further underscoring the similarity between DAH in SLE patients and mice. Interestingly, hemoptysis and lung fibrosis have been seen in humans with inhalation, aspiration, or injection of mineral and other oils [50, 51]. Although humans generally are not exposed to pristane, the strong phenotypic and pathological similarities [15, 52] suggest that pristane exposure may activate inflammatory pathways similar to those in SLE patients. Respiratory infections, seen in 40–60% of SLE-DAH patients [3, 4, 53], represent one mechanism that could drive the accumulation of apoptotic cells in the lungs, promoting DAH. It will be of interest to investigate the role of these infections in the pathogenesis of human DAH.

The response of pristane-induced DAH to CVF (Fig. 1B) raises the possibility that complement depletion/inhibition may be a viable strategy for treating DAH in human SLE.
Although CVF is too immunogenic for human use, “humanized” CVF is undergoing clinical testing [54] and compstatin, a 13-residue cyclic peptide that inhibits activation of the classical and alternative complement pathways, is in clinical trials for macular degeneration [55]. Complement inhibition also blocks pulmonary fibrosis [56], a long-term complication of DAH [57]. As pulmonary fibrosis also is seen in lupus patients without prior overt episodes of DAH [1], it will be of interest to examine whether clinically silent DAH with alveolar micro-hemorrhage can cause pulmonary fibrosis. Finally, in view of the severity of disease in IL-10 deficient mice (Fig. 6A), it may be of interest to evaluate recombinant IL-10 as a potential therapy.

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References


Figure 1. DAH requires IgM, C3, and CD18

A, Wild type (WT) and B6 μMT mice were treated with pristane and lungs were examined 14-d later. Some μMT mice received human IgM (50 or 200 μg i.v.) or murine IgG (200 μg i.v.) before pristane treatment (n = 4–6 per group). *Left*, gross lung pathology; *right*, prevalence of DAH. B, WT (n = 7), B6 C3−/− (n = 12), and B6 CD18−/− (n = 4) mice were treated with pristane and lungs were examined for DAH 14-d later. Some WT mice were treated with cobra venom factor (CVF) to deplete complement 1-d before (X1, n = 6) or 1-d before and 7-d after (X2, n = 10) pristane. *Left*, gross lung pathology; *right*, prevalence of...
DAH. C, H&E staining of lung tissue from an SLE patient with DAH (upper left) and a WT mouse with pristane-induced DAH (upper right and lower left). Lungs show bland hemorrhage (H), neutrophil-predominant small vessel vasculitis (V), and hemosiderin-laden macrophages (МΦ, arrows). These pathological changes were absent in lungs from pristane-treated C3−/− mice (lower right). Murine lung was examined 14-d after pristane treatment. *** p < 0.001 vs. WT, Fisher exact test; **** p < 0.001 vs. μMT, Fisher exact test
Figure 2. Pristane migrates from peritoneum to lung

A, mass spectrum showing the four peaks characteristic of pristane: M (m/z value 268), M−1 (m/z 267), M+1 (m/z 269), and M+2 (m/z 270). B, representative mass spectra (m/z values between 265–271) from the organic phase of extracted lung tissue from mice treated 2-wk earlier with pristane or left untreated. WT, wild type mice; C3−/−, C3-deficient mice. C, unfixed tissue (frozen sections) from untreated mice and mice treated 2-wk previously with pristane was stained with oil red-O. D, representative mass spectra (m/z values between 265–271) from the organic phase of extracted lung tissue from mice treated 2-wk earlier with pristane or left untreated.
265–271) from the organic phase of extracted bone marrow from wild type (WT) mice treated 2-wk earlier with pristane or left untreated.
Figure 3. Pristane induces cell death
A, Fixed lung tissue from untreated mice and mice treated 2-wk earlier with pristane was subjected to TUNEL staining. TUNEL+ cells are brown. B, BW5147 cells were incubated 24-h at 37°C in medium containing the indicated concentrations of either pristane or mineral oil (MO). Afterward, cells were analyzed by flow cytometry after staining with 7-amino actinomycin D (7-AAD). Percentage of 7-AAD+ cells and mean fluorescence intensity (MFI) were determined. C, Comparison of 7-AAD staining of pristane-treated BW5147 and RAW264.7 cells. Cells were incubated in vitro with pristane as in B followed by
determination of 7-AAD\textsuperscript{+} cells (%) and MFI. *** p<0.001, ** p<0.01, * p<0.05, multiple t-test
Figure 4. Role of neutrophils and monocytes/macrophages in DAH

A. Peritoneal exudate cells (PEC) and bone marrow (BM) neutrophils from wild type mice treated 14-d earlier with pristane, with or without anti-Ly6G depletion (mAb IA8), were analyzed by flow cytometry. Left, surface staining with anti-Ly6G (mAb RB6-8C5)-APC-Cy7 and anti-CD11b-Pacific blue showing depletion of neutrophils (box) from the peritoneum but not bone marrow. Right, staining with anti-Ly6G (mAb RB6-8C5)-PE (surface stain) and anti-TNFα-APC (intracellular stain) showing loss of intracellular TNFα⁺ peritoneal, but not bone marrow, Ly6G⁺ cells.

B. Neutrophil depletion with anti-Ly6G
antibodies. Wild type (WT) mice were treated with anti-Ly6G mAb 1-d before and 7-d after pristane treatment or left untreated (Control) (n = 6 per group). H&E staining showed perivascular inflammatory cell infiltrates in controls, but not anti-Ly6G-treated mice. Efficacy of neutrophil depletion in lung was verified by staining with anti-neutrophil elastase antibodies (right). C, anti-F4/80 staining of macrophages in WT mice after depleting neutrophils with anti-Ly6G antibodies. F4/80+ cells are indicated by arrows. D, morphometric quantification of neutrophil elastase staining in lung from control vs. anti-Ly6G-treated mice. E, H&E staining (left) and gross pathology (right) of lungs (14-d after pristane) from WT mice either treated with clodronate liposomes (CloLip, n = 8) 1-d before and 7-d after pristane or left untreated (No Rx, n = 6). Perivascular inflammatory cell infiltrates and DAH were absent after CloLip treatment. F, frequency of DAH in wild type (WT) and elastase-deficient (Ela−/−) mice 14-d after pristane treatment and in WT mice treated with anti-Ly6G antibodies (Ly6G) or with CloLip 1-d before and 7-d after pristane treatment. Pathology was assessed at 14-d. **, P < 0.01 (t-test); *** P < 0.001 vs. WT (Fisher exact test)
Figure 5. Macrophage subsets in DAH

A, immunohistochemistry of lungs from wild type (WT) mice treated with pristane or left untreated and stained at 14-d with horse radish peroxidase (HRP)-conjugated anti-F4/80, CD11b, or CD11c antibodies. **Top row**, perivascular F4/80\textsuperscript{hi} cells (black arrows); **Bottom row**, large F4/80\textsuperscript{hi}CD11b\textsuperscript{hi}CD11c\textsuperscript{lo} AM (yellow arrows); B, staining of lung from untreated mice. **C, Left and middle.** F4/80 staining (14-d) of lung from mice receiving clodronate liposomes (CloLip) i.p. 1-day before and 7-day after pristane treatment. The perivascular region (left) and alveolar spaces (middle) do not contain F4/80\textsuperscript{+} cells. Note absence of
perivascular and alveolar F4/80+ cells and increased cellularity of the lung parenchyma. **Right,** F4/80 staining of lung from CD18−/− mice treated 14-d earlier with pristane showing normal alveolar architecture and absence of perivascular and alveolar F4/80+ cells. **D,** hematoxylin and eosin (H&E) staining (left) and F4/80 immunohistochemistry (right) of normal lung from an untreated B6 mouse (top) compared with lung from a B6 mouse treated with clodronate liposomes (CloLip) + pristane (bottom) showing mild thickening of the alveolar septa (black arrows) despite the absence of alveolar hemorrhage.
Figure 6. Pathogenesis of lung inflammation

A, Wild type (WT), IL-10−/−, and C3−/− mice were injected with pristane and percent survival was compared among groups 0–24 weeks after treatment (Mantel-Cox test). B, WT, TRIF−/−, MyD88−/−, TNFα−/−, type I interferon receptor deficient (IFNAR−/−), caspase 1/11 deficient (Casp1), and inducible nitric oxide deficient mice (Nos2−/−) (n = 5–6 per group) were treated with pristane and the prevalence of DAH was determined 14-d later. C, flow cytometry of intracellular TNFα in Ly6G+ neutrophils from WT, TRIF−/−, and MyD88−/− mice. D, IL-10 receptor (IL-10R) expression on bone marrow cells from
untreated B6 mice (flow cytometry). Myeloid cells were identified by forward/side scatter characteristics and IL-10R expression was determined on CD11b^+Ly6G^+ neutrophils (Neut) and CD11b^+Ly6G^- monocytes (Mono). **Middle**, IL-10R^+ cells as a percentage of total CD11b^+Ly6G^+ and CD11b^+Ly6G^- cells; **Right**, IL-10R expression on CD11b^+Ly6G^- bone marrow cells (mean fluorescence intensity, flow cytometry) from mineral oil (MO) vs. pristane- treated mice. **E**, Flow cytometry showing forward scatter and surface marker (F4/80, CD11c, Siglec-F, CD205, and IL-10 receptor) expression on CD11b^+ alveolar macrophages from untreated B6 mice collected by bronchoalveolar lavage (BAL). **F**, Stat3 activation (phosphorylation, pStat3) in IL-10 treated CD11b^+ alveolar macrophages from mineral oil (MO, n = 8) vs. pristane (n = 9) treated mice (collected by BAL). **Left**, flow cytometry showing intracellular activated Stat3 (pStat3) staining in CD11b^+ alveolar macrophages. **Right**, percentage of CD11b^+ cells (from MO-treated vs. pristane-treated mice) staining for pStat3 after a 15-min exposure *in vitro* to IL-10. *, P < 0.05; ** P < 0.02; ****, P < 0.0001, t-test.