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*J Immunol* published online 3 October 2014
http://www.jimmunol.org/content/early/2014/10/02/jimmunol.1401051

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/10/02/jimmunol.1401051.DCSupplemental

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Pristane-Induced Granulocyte Recruitment Promotes Phenotypic Conversion of Macrophages and Protects Against Diffuse Pulmonary Hemorrhage in Mac-1 Deficiency

Yiqin Shi,* Naotake Tsuboi,* Kazuhiro Furuhashi,†‡ Qiuna Du,* Asuka Horinouchi,* Kayaho Maeda,* Tomoki Kosugi,* Seiichi Matsuo,* and Shoichi Maruyama*

Diffuse pulmonary hemorrhage (DPH) is an uncommon but critical complication of systemic lupus erythematosus. Peritoneal administration of 2,6,10,14-tetramethylpentadecane (pristane) can recapitulate a lupus-like syndrome in mice, which can develop into DPH within a few weeks, especially in C57BL/6 mice. Mac-1 (CD11b/CD18), a leukocyte adhesion molecule, is known to play a role in inflammation by regulating migration of leukocytes into injured tissue. In this study, we aimed to clarify the role of Mac-1 in pristane-induced DPH, using Mac-1−/− and wild-type (WT) mice on a C57BL/6 background. After pristane injection, Mac-1−/− mice showed reduced prevalence of DPH and attenuated peritonitis compared with WT mice. Analysis of the peritoneal lavage on days 5 and 10 after pristane treatment revealed increased numbers of eosinophils and alternatively activated macrophages, but decreased numbers of neutrophils and classically activated macrophages in Mac-1−/− mice compared with WT. Enhanced production of IL-4 and IL-13, both key mediators of macrophage polarization toward the mannose receptor+(MMR+) phenotype, was observed in the peritoneal cavity of Mac-1−/− mice. Depletion of neutrophils and eosinophils or adoptive transfer of classically activated macrophages resulted in the exacerbation of pristane-mediated DPH in both WT and Mac-1−/− mice. Moreover, peritoneal transfer of F4/80high/MMR+ alternatively activated macrophages successfully reduced the prevalence of DPH in WT mice. Collectively, Mac-1 promoted acute inflammatory responses in the peritoneal cavity and the lungs by downregulating granulocyte migration and subsequent phenotypic conversion of macrophages in a pristane-induced systemic lupus erythematosus model. The Journal of Immunology, 2014, 193: 000–000.

Diffuse pulmonary hemorrhage (DPH) is an infrequent but life-threatening complication in patients with systemic lupus erythematosus (SLE) (1); its prevalence ranges from <2% to 5.4% (2–4) and the mortality rate ranges from 23% to 90% (2, 5). Spontaneous developments can be rapidly unfavorable, with death occurring within the 48 h following the onset of the first symptoms. The histology of DPH in SLE patients is characterized by acute lupus pneumonitis, associated interstitial infiltration by mononuclear and polymonuclear leukocytes, the presence of hyaline membranes, alveolar necrosis, edema, microvascular thrombi, and intima proliferation with deposits of hemosiderin phagocytosing macrophages (6). Several studies reporting histologic evaluations of skin or kidney specimens in human SLE patients showed that the interaction between complement activated leukocytes and endothelial expression of VCAM-1 and ICAM-1 on the vasculature may be important for leukocyte migration into immune complex (IC)-deposited tissue (7, 8). However, the underlying mechanism of SLE-related DPH remains unclear because lung biopsy has not been performed routinely for diagnosis in patients with hemorrhagic complications, and no suitable animal model exists for recapitulating DPH.

The leukocyte integrin Mac-1 (also known as αmβ2, CD11b/CD18), which is expressed on neutrophils (Neu), eosinophils (Eos), monocytes, macrophages, and NK cells, is important for various cell functions including adhesion, chemotaxis, migration, phagocytosis, and cytotoxicity (9). Mac-1 has multiple ligands, including complement fragment C3bi, ICAM-1 on the endothelium, and platelet receptor glycoprotein Ib-α (GPIIb). Animal studies using gene-targeted mice have reported ambivalent roles for Mac-1: promotion of innate immune-mediated inflammation by supporting Neu recruitment, interaction with platelets, triggering of cytotoxic functions, and cooperation with other receptors such as CD14, Dectin, and FcyRs (10–13), whereas protective immunomodulation via inhibition of IFN-α and TLR signaling or T cell proliferation (14, 15). Recent animal studies of SLE have indicated that Mac-1 deficiency promotes target organ damage via regulation of FcyRIIA-mediated Neu recruitment or the maintenance of autoreactive B cell tolerance (13, 16). Moreover, several human genome-wide association studies have reported that Mac-1 may be associated with susceptibility to SLE, and that mutations within the CD11b locus may be a risk factor related to autoantibody production and severe manifestations of SLE (17, 18).
2,6,10,14-Tetramethylpentadecane (pristane) is a hydrocarbon oil that can induce a lupus-like autoimmune syndrome including diffuse proliferative glomerulonephritis combined with glomerular immune complex deposition, arthritis, and autoantibody production of dsDNA/chromatin in BALB/C and SLJ mouse strains several months after a single i.p. injection (19). Pristane can induce cell death by apoptosis in murine peritoneal cells in vivo. Uptake of autoantigens provided by sustained apoptosis in the setting of an inflammatory milieu leads to a break in self-tolerance and ultimately autoimmunity (20). Furthermore, a recent study reported that mice of the C57BL/6 strain frequently developed DPH resembling that seen in SLE within a few weeks of pristane treatment, in which the B cells contribute to DPH via production of proinflammatory cytokines and chemokines to recruit monocytes and Neu to the peritoneal cavity and the lungs or via Ag-presentation-mediated but not Ab-mediated functions (21). However, the mechanism linking pristane-induced peritonitis to pulmonary manifestations, in particular the precise role of granulocyte and monocyte subsets recruited into inflamed sites for the development of DPH, remains largely unknown.

In the current study, we introduced pristane-induced DPH to Mac-1–deficient mice to investigate the potential role of Mac-1 in critical pulmonary manifestations in patients with SLE. Previous studies of pristane-induced SLE have mainly focused on lymphocyte-mediated autoimmunity, including autoantibody generation, involving type I IFN, IL-6, and IL-12 (19). In this study, we shifted the focus to innate immunity in pristane-induced SLE and explored the critical role of granulocytes for phenotypic conversion of macrophages to immunoregulatory cell types, which is tightly connected to amelioration of pristane-induced DPH.

**Material and Methods**

**Pristane-induced DPH in a murine model**

Mac-1–deficient mice on a C57BL/6 background (Mac-1<sup>−/−</sup>) were obtained from Dr. Tanya N. Mayadas (13), and wild-type (WT) mice were purchased from Chubu Kagaku Shizai (Nagoya, Japan). Mice were maintained in a virus- and Ab-free facility at the animal housing facility at Nagoya University Graduate School of Medicine. At 8–10 wk of age, mice received a single 0.5-ml i.p. injection of pristane (Sigma-Aldrich, St. Louis, MO). All procedures were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine.

**Preparation of lavage fluid and isolation of cells by peritoneal lavage**

Mice were euthanized with diethyl ether, and the peritoneal cavity and the lungs or via Ag-presentation–mediated but not Ab-mediated functions (21). However, the mechanism linking pristane-induced peritonitis to pulmonary manifestations, in particular the precise role of granulocyte and monocyte subsets recruited into inflamed sites for the development of DPH, remains largely unknown.

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Mice were euthanized with diethyl ether, and the peritoneal cavity was lavaged with 8 ml normal saline at the times indicated. Absolute cell numbers were obtained using an Automated Cell Counter (Bio-Rad, Hercules, CA). The collected cells were subjected to flow cytometry to identify leukocyte subsets.

**Lung histology for evaluation of DPH severity**

Lung tissues were harvested and fixed in 4% paraformaldehyde overnight or frozen with OCT compound. The fixed tissues were embedded in paraffin, cut into 5-μm sections, and stained with H&E. The extent of DPH in the transverse section was evaluated by observation of H&E staining. The percentage of lung with hemorrhage was estimated and assigned one of the following scores in the left lobe: 0, no hemorrhage; 1, 0–25%; 2, 25–50%; 3, 50–75%; and 4, 75–100%. Each lobe was randomly cut into five sections. The scores were evaluated independently by two observers in a blinded manner, and the average scores for each animal were used for quantitative analysis.

**Preparation for obtaining single-cell suspensions from lung tissue**

Lungs were obtained from diseased mice at the indicated time points. Single-cell suspensions were obtained using gentleMACS equipment (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Lungs were transferred into gentleMACS C Tubes containing 5 ml HBSS (Life Technologies, Carlsbad, CA) with 120 U/ml collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) and 40 U/ml DNase 1 (Sigma-Aldrich). Tightly closed C Tubes were attached upside down onto the sleeve of the gentleMACS Dissociator, and Program m<sub>_lung_01</sub> was run. Subsequently, samples were incubated for 30 min at 37°C and reattached to the gentleMACS Dissociator again, and Program m<sub>_lung_02</sub> was run. The samples were filtered through 70-μm strainers and washed twice with MACS buffer (0.5% BSA and 2 mM EDTA in PBS). It is notable that each lung was not totally digested to avoid cell damage and degeneration of surface markers on the process of cell preparation. The collected cell suspension was subjected to flow cytometry to identify leukocyte subsets.

**Flow cytometric analysis**

Single-cell suspensions from lungs and peritoneal cavities were washed in MACS buffer. After blockade of Fe receptors with rat anti-mouse CD16/CD32 mAb (clone 2.4G2; BD Biosciences, Franklin Lakes, NJ), cells were stained with the corresponding Ab mixtures. The following Abs were used: APC-labeled anti-F4/80 (clone BMS), Alexa Fluor 488-labeled anti-Mannose receptor (anti-MMR clone C068C2), APC-labeled anti-Ly6G (clone 1A8), and PE-labeled anti-sigle-F (clone E50-2440; BD Pharmingen). The following Abs were obtained from BioLegend (San Diego, CA) unless otherwise indicated. Cells were analyzed using a FACSCanto flow cytometry system (BD Immunocytometry Systems). All the data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

**Analysis of phenotypes of peritoneal macrophages**

F/4/80<sup>+/−</sup>M<sub>MR</sub> and F/4/80<sup>+/−</sup>M<sub>MR</sub> macrophages, sorted using a FACSaria cell sorter (BD Biosciences) from peritoneal lavage cells, were harvested from Mac-1<sup>−/−</sup> mice, 10 d following pristane treatment. Total RNAs were purified from each sorted macrophage subset using RNeasy Mini Kits (Qagen, Venlo, the Netherlands). RNA was reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster city, CA). Real-time quantitative PCR (Applied Biosystems) was performed using primers for resistin-like molecule α (Fizz1), arginase 1 (Arg1), chitinase-like 3 (Ym1), IL-4, IL-12, and TNF-α (Applied Biosystems).

**In vivo Neu tracking assay**

WT and Mac-1<sup>−/−</sup> Neu were isolated from bone marrow cells using Percoll (Sigma-Aldrich) gradients (55%, 65%, and 75% Percoll in PBS). The purity of Neu was confirmed to be >80% by flow cytometry. Harvested WT and Mac-1<sup>−/−</sup> Neu were labeled with green fluorescent PKH67 (Sigma-Aldrich) and red fluorescent PKH26 (Sigma-Aldrich), respectively, following the manufacturer’s instructions. Labeled WT and Mac-1<sup>−/−</sup> Neu (4 × 10<sup>5</sup>) were injected i.v. together into WT mice 1 h after pristane treatment. Fifteen hours later, migration and distribution of the infused Neu were observed in peritoneal cavity and lung by flow cytometry.

**In vivo chemotaxis assay**

Both Mac-1<sup>−/−</sup> and WT mice were pretreated by i.v. injection with 4 μg pertussis toxin (PTx; Sigma-Aldrich) 1 h prior to pristane treatment. The peritoneal lavage cell population was evaluated by flow cytometry 6 h following pristane injection.

**In vitro transendothelial Neu migration assay**

Transwell polycarbonate inserts (6.5 mm diameter) with 3-μm-pore-size membrane (Costar, Cambridge, MA) were seeded with RCB1994 cells (2 × 10<sup>5</sup> cells/well; Riken BRC) and mouse endothelial cell lines, and they were cultured overnight (37°C and 5% CO<sub>2</sub>). Confluent inserts were washed twice with DMEM and incubated with LPS (13 ng/ml) for 4 h. Inserts were rinsed twice with PBS and placed in 24-well plates containing RPMI 1640 (10% FBS) with or without the chemottractant MIP-2 (50 ng/ml). After 16 h of pristane treatment, WT and Mac-1<sup>−/−</sup> Neu were harvested from bone marrow cells by Percoll as described before. Purified Neu (2 × 10<sup>5</sup>) in RPMI 1640 (with 10% FBS) were added to the transwell inserts and allowed to migrate to the lower wells for 1.5 h. Migrated Neu were then collected from the lower wells and counted by an Automated Cell Counter (Bio-Rad). Results are expressed as a percentage of cells added.

**Cytokine analysis**

The concentrations of TNF-α, IL-4, IL-6, and IL-13 in peritoneal lavage fluids were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN).
Selective depletion of granulocyte subsets

For Neu depletion, mice were treated with i.v. injection of 0.5 ml pristane in WT and Mac-1−/− mice. Mouse Neu were obtained from peritoneal lavage cells 16 h after i.p. injection of 0.5 ml pristane in WT and Mac-1−/− mice. Mouse Neu were sorted with a FACSAria (BD Biosciences) based on the expression of Ly6G. Apoptotic Neu (APO-PMNs) were obtained by overnight incubation in serum-free DMEM as described previously (22). Aged cells contained >90% Annexin V− by FACS analysis. Mononuclear cells within the pellet resulting from centrifugation (400 × g, 30 min) of bone marrow cells were isolated using Histopaque-1083 (Sigma-Aldrich) and then transferred to culture dishes with macrophage culture medium (MCM; RPMI 1640 medium-FBS containing 20% v/v L929 cell-conditioned medium as a source of M-CSF) (4). After 7 d, 2 × 103 cells/well (in 24-well dishes) of bone marrow–derived macrophages (BMDMs) were treated overnight with 0.5 ng/ml IFN-γ (Cell Signaling Technology, Danvers, MA) and recultured with MCM or APO-PMNs (1 × 106/well) for 3 d. Macrophage polarization into F4/80highMMR+ cells was analyzed by flow cytometry.

Preparation and adoptive cell transfer of classically activated macrophages and F4/80highMMR+ alternatively activated macrophages

For preparation and adoptive cell transfer of classically activated macrophages (M1 macrophages) polarization, BMDMs (5 × 105/ml) were cultured with MCM for 7 d, and then stimulated with LPS (4 μg/ml) and IFN-γ (20 ng/ml; Cell Signaling Technology) in RPMI-10% FBS for 2 d. For F4/80highMMR+ alternatively activated macrophages (M2 macrophages) polarization, BMDMs (5 × 105/ml) were cultured with MCM for 3 d and then incubated with IL-4 (20 ng/ml; Cell Signaling Technology) and IL-13 (20 ng/ml; Cell Signaling Technology) for 4 d. The purity of F4/80highCD166+ M1 macrophage and F4/80highMMR+ M2 macrophages was confirmed to be >90% by flow cytometry as macrophages. Mice were injected i.v. with 2 × 105 M1 macrophages or treated i.p. with 4 × 105 F4/80highMMR+ M2 macrophages 2 d after pristane challenge; they were sacrificed on day 10 in different groups.

Results

Mac-1 promotes pristane-induced DPH in C57BL/6 mice

Intraperitoneal injection of pristane frequently results in pulmonary hemorrhage within a few weeks in C57BL/6 mice (21), which can induce early mortality. We compared Mac-1−/− and WT mice and found that, within 8 wk of pristane treatment, mortality was significantly lower in Mac-1−/− mice than in WT mice. Because most of the mortality occurred between 2 and 4 wk (Fig. 1A), we further assessed lung pathology in DPH on days 5, 10, and 14 following pristane injection. According to classification of lung gross pathology (Fig. 1D), in Mac-1−/− mice did on days 10 or 14 (Fig. 1B). To confirm these results further, we established the disease activity index for DPH (DPH score) according to the area of erythrocytes filled in the H&E-stained lung tissue (Fig. 1C, 1D). On days 10 and 14, the DPH score was significantly higher in the WT mice than in the Mac-1−/− mice. We also observed leukocyte recruitment in lung tissue with both partial and complete DPH (Fig. 1D), which corresponded to results observed in lungs of human SLE patients with DPH (23). To determine which dominant leukocyte subsets initiate DPH after pristane treatment, we assessed pulmonary Ly6G+ Neu, F4/80+ siglec-F−MMR− Eos, F4/80+ siglec-F+MMR+ M2 macrophages and F4/80+ siglec-F−MMR− M1 macrophages using flow cytometry (24, 25) (Fig. 1E). In the very early stage, 16 h after pristane injection, Mac-1−/− mice demonstrated 2-fold more Neu (Ly6G+) accumulation in the lung than WT mice did. But until days 5 and 10, Mac-1−/− mice demonstrated markedly lower numbers of Neu and M1 macrophage population compared with WT animals. These results suggest that Mac-1 protects initial Neu accumulation to the lung, but subsequently promotes pristane-induced DPH through pulmonary infiltration of Neu and M1 macrophages.

Mac-1 modulates peritoneal leukocyte recruitment and cytokine production after pristane treatment

The innate immune response to i.p. pristane exposure may affect the development of DPH (26); therefore, we profiled peritoneal leukocyte subsets from the initial inflammatory state to the onset of pristane-induced DPH. Peritoneal leukocytes were classified into three subsets—Eos, macrophages, and Neu—as evidenced by Siglec-F, F4/80, and Ly6G expression, respectively (Fig. 2A). In addition to the pulmonary macrophage phenotypes, peritoneal F4/80+ macrophages were further classified into two subtypes by cell surface expression of MMR. Real-time RT-PCR of sorted Mac-1−/− peritoneal macrophages showed that mRNA transcription of Fizz1, Arg1, Ym1, and IL-4, which are commonly considered to be regulatory M2 macrophage markers (27), was significantly higher in F4/80highMMR+ (Gate R2) cells than in F4/80highMMR− (Gate R3) cells 10 d after pristane injection. In addition, expression of IL-12 and TNF-α was markedly higher in F4/80highMMR− cells, indicating that these were classically activated M1 macrophages (Fig. 2B). Despite the low incidence of DPH, Mac-1−/− mice had 3-fold more Neu (Ly6G+) and 2-fold more Eos (F4/80+ siglec-F−) in the peritoneal exudate than WT mice did, as early as 16 h after pristane injection (Fig. 2C). Mac-1−/− mice showed significantly higher levels of Eos until day 10, whereas Neu gradually decreased from then on and were significantly impaired from day 5 compared with WT mice. The early accumulated Neu could phagocytose a portion of the pristane, as evidenced by oil red O staining (Supplementary Fig. 1). Peritoneal macrophages gradually accumulated in the cavity from day 5 following the pristane injection. Infiltration of F4/80+ MMR+ M1 macrophages markedly increased along with Neu elevation in WT mice and reached a level 3-fold higher than that in Mac-1−/− mice on day 5. In contrast, levels of F4/80highMMR+ M2 macrophages, which play an important role in resolving inflammation (28), were significantly higher in Mac-1−/− mice than in WT and continued to increase until day 10 (Fig. 2A, 2C). The difference in peritoneal leukocyte infiltration between WT and Mac-1−/− mice was further corroborated by the peritoneal cytokine profiles. In concordance with the accumulation of M1 macrophages, WT mice exhibited significantly higher levels of proinflammatory cytokine IL-6 and TNF-α on days 5 and 10 (Fig. 2D). However, peritoneal IL-4 and IL-13, both of which are Th2 cytokines critical for M2 macrophage polarization, were significantly higher on day 5 in Mac-1−/− mice than in WT mice. Notably, serum levels of IL-6, TNF-α, IL-4, IFN-γ, and IL-13 were below detectable levels in both Mac-1−/− and WT mice (data not shown). Collectively, these results show that Mac-1 deficiency can modulate inflammation by promotion of initial granulocyte accumulation and subsequent polarization of M2 macrophages in the pristane-exposed peritoneal cavity.

Mac-1 inhibits chemokine-dependent Neu recruitment in pristane model

As early as 16 h after pristane treatment, the accumulation of Mac-1−/− Neu to the peritoneal cavity and lung was significantly higher
than in WT groups. To ensure the result further, we i.v. injected the same number of WT and Mac-1−/− bone marrow Neu that were labeled with the different fluorescent dye of PKH67 and PKH26, to one pristane treated mice. Fifteen hours later, flow cytometric analysis revealed higher accumulation of PKH26+Mac-1−/− Neu but lower PKH67+ WT Neu in the lung and peritoneal cavity (Fig. 3A). This result further proved that Mac-1−/−deficient mice displayed increased granulocyte accumulation in the pristane-exposed peritoneal cavity and lung (Figs. 1E, 2C). A similar inhibitory function of Mac-1 in Neu recruitment has been described in the reverse passive Arthus reaction, in which Mac-1−/− Neu showed slow rolling velocity and increased chemoattractant-induced adhesion of Neu (13). To determine whether pristane-mediated extravasation of Neu and Eos from peripheral circulation into the peritoneal cavity was an active process that involved chemoattractant receptor signaling (29), we pretreated Mac-1−/− and WT mice with an i.v. injection of PTx, an inhibitor of Gαi2-coupled receptors that can prevent most chemotactic responses. At 6 h following the pristane treatment, we observed marked inhibition of the granulocyte accumulations of Neu and Eos in PTx-pretreated groups of Mac-1−/− and WT mice (Fig. 3B). This result strongly indicates that pristane-induced leukocyte recruitment is a chemokine-dependent process. Doerschuk et al. (30) first reported a CD18-independent mechanism of Neu emigration in the lung and systemic microcirculation in response to certain stimuli. As MIP-2 is the major Neu chemoattract mediator strongly induced by pristane (31), we assessed the in vitro transendothelial Neu migration assay in response to MIP-2. The result indicated that MIP-mediated Neu migration from Mac-1−/− mice significantly increased than that from WT mice (Fig. 3C). These data collectively suggest that Mac-1 plays an inhibitory role in chemokine-dependent granulocyte recruitment in response to pristane.

Initial Neu recruitment ameliorated pristane-induced DPH

Despite the low prevalence of DPH, Mac-1−/−deficient mice demonstrated more peritoneal Neu during the initial phase of pristane-induced inflammation (Fig. 2C). To explore the potential contribution of Neu in pristane-induced DPH, we used anti-Ly6G mAbs to deplete Neu in WT mice at the time of pristane injection and every other day thereafter. All neutropenic WT mice developed DPH on day 10 (Fig. 4A) and showed significantly higher DPH scores than isotype IgG-treated control animals did (Fig. 4B). Furthermore, Neu depletion resulted in a significant increase in M1 macrophages and a marked decrease in M2 macrophages in the lungs on day 5 after pristane treatment (Fig. 4C). Levels of peritoneal M2 macrophages were significantly lower in the neutropenic group than in the control group on day 10, but no significant difference was observed in the number of Eos or M1 macrophages. Proinflammatory cytokines were also evaluated in peritoneal fluids. Five days after pristane injection, the level of IL-6 was markedly lower in neutropenic WT mice than in controls, but contrarily became significantly higher on day 10. The level of TNF-α was slightly higher in the anti-Ly6G-treated group than in isotype group (Fig. 4E). Similar results were obtained in neutropenic Mac-1−/− mice (Supplementary Fig. 2).
When Neu depletion was delayed until day 2 after pristane injection, we did not observe any differences in DPH prevalence or M2 macrophage populations (data not shown), indicating that the protective role of Neu in DPH was limited to the early phase of the disease, and that initial accumulation of Neu was also critical for M2 macrophage polarization.

FIGURE 2. Mac-1 deficiency ameliorated the peritoneal inflammatory response to pristane. (A) Definition of peritoneal leukocyte subsets by flow cytometry. Gating strategy for F4/80<sup>+</sup>siglec-F<sup>+</sup> Eos (R1), F4/80<sup>+</sup>MMR<sup>+</sup> macrophages (R2), F4/80<sup>+</sup>MMR<sup>+</sup> macrophages (R3), and Ly6G<sup>+</sup> Neu (R4) in the peritoneal cavity and representative images at 0 h, 16 h, 5 d, and 10 d after pristane treatment are shown. (B) RT-PCR of peritoneal macrophages to identify their phenotypes in Mac-1<sup>−/−</sup> mice. Ten days after pristane treatment, total RNAs were isolated from peritoneal macrophages sorted from peritoneal fluid cells according to F4/80 and MMR expression. Data are expressed relative to the expression of the respective 18s rRNAs, used as a reference gene (n = 6 per group). (C) Kinetics of peritoneal leukocyte subsets at indicated time (n = 4–10 per group). (D) Peritoneal cytokine profiles after pristane-treatment. IL-6, TNF-α, IL-4, and IL-13 concentrations were determined by ELISA on days 5 and 10 (n = 10–12 per group). All data are mean ± SEM. *p < 0.05, **p < 0.01, WT versus Mac-1<sup>−/−</sup> mice.
Apoptotic Neu induce an F4/80+MMR+ phenotype in macrophages in vitro

To investigate the mechanism by which initial Neu accumulation promotes macrophage polarization to the M2 macrophage phenotype, we cocultured IFN-γ–primed BMDMs with APO-PMNs in vitro (22). Flow cytometric analysis clearly showed that APO-PMN induced MMR expression on BMDMs (Fig. 5). This finding was consistent with the results of the in vivo Neu depletion experiment, which showed that the M2 macrophage population in the lungs and the peritoneal cavity significantly decreased in the neutropenic group. Notably, the capacity for macrophage conversion into F4/80+MMR+ cells by Mac-1−/− or WT APO-PMN was comparable in Mac-1−/− and WT BMDMs, which indicates that Mac-1 is not involved in the process.

Depletion of Eos exacerbates pristane-induced DPH

Eos are considered a primary source of IL-4 during the initiation phase of the type 2 immune response (32). Moreover, IL-4 has recently been shown to be a key cytokine in polarization of macrophages into M2 macrophages, promoting resolution of inflammation (33). Profiles of peritoneal infiltration of leukocytes showed higher Eos accumulation in Mac-1−/− mice than in WT mice until 10 d after pristane injection. Eos depletion by anti–IL-5 mAb (34) in Mac-1−/− mice resulted in increased DPH prevalence.
on day 10, both in gross pathology and histologic scores, compared with isotype IgG-pretreated control mice (Fig. 6A, 6B). Eosinopenic Mac-1−/− mice also exhibited an increase in M1 macrophages accumulation and a significant decrease in M2 macrophages in the lungs compared with the control group 5 d after pristane treatment (Fig. 6C). The peritoneal M2 macrophage population also decreased significantly 10 d after pristane injection in the absence of Eos in Mac-1−/− mice (Fig. 6D). In concordance with these results, the peritoneal IL-4 concentration was almost undetectable with no remarkable changes in IL-13 levels in the eosinopenic group 5 and 10 d after pristane injection (Fig. 6E), which indicated that Eos were critical for IL-4 secretion and subsequently modulated M2 macrophage polarization. Eos depletion in WT mice demonstrated the same tendency as Mac-1−/− group (Supplemental Fig. 3). These results clearly demonstrate that in Mac-1−/− mice Eos play a critical role in promoting the resolution of inflammation.

**Transfer of M1 macrophage exacerbates pristane-induced DPH in WT mice**

After pristane treatment, the accumulation of M1 macrophage to the lung and peritoneal cavity became markedly higher in WT mice than in Mac-1−/− mice from day 5 to day 10 (Figs. 1E, 2C). Because of intimate association of M1 macrophage and inflammatory tissue damage (35), we stimulated WT and Mac-1−/− BMDMs by LPS/IFN-γ (36) and transferred the F4/80⁺CD86⁺ M1 macrophages separately to WT mice via i.v. injection 2 d after pristane treatment. Transferred M1 macrophage accumulated in the lung of pristane-exposed mice 15 h after injection (data not shown). Mice were sacrificed on day 10, and transfusing of M1 macrophages elevated the prevalence of DPH (Fig. 7A). The DPH score in the WT M1 macrophage–transfused group was significantly increased compared with the control group, and the efficiency of WT and Mac-1−/− M1 macrophage showed no difference (Fig. 7B). This result suggests that increased M1 macrophages accumulation in the lung can exacerbate pristane-induced DPH, but this inflammatory function of M1 macrophages is Mac-1 independent.

**Transfer of F4/80highMMR⁺ M2 macrophages protects WT mice from pristane-induced DPH**

The phenotypic balance of M1 and M2 macrophages in the pristane-exposed peritoneal cavity appears tightly connect to development of DPH. Therefore, we hypothesized that the resolution of peritoneal inflammation by M2 macrophages in Mac-1 deficiency would affect the prevalence of pristane-induced DPH. We expanded both WT and Mac-1−/− F4/80highMMR⁺ M2 macrophages from BMDMs (Supplemental Fig. 4) by combined stimulation with IL-4/IL-13 (28, 37), and we introduced the cells to WT mice separately to confirm whether M2 macrophages can play...
a protective role against pristane-induced DPH. Intraperitoneal injection of \(4 \times 10^6\) M2 macrophages into WT mice 2 d after 0.5 ml pristane treatment successfully decreased the prevalence of DPH and the disease scores compared with untreated mice (Fig. 8A, 8B). In the peritoneal cavity treated with WT or Mac-1\(^{-/-}\) M2 macrophages, infiltration of leukocyte (data not shown) and proinflammatory cytokines, including IL-6 and TNF-\(\alpha\) (Fig. 8C), were markedly decreased on day 10, indicating that inflammation was suppressed, and this immune-regulatory function of M2 macrophages had no difference between WT or Mac-1\(^{-/-}\).

Thus, impairment of pristane-induced peritoneal inflammation by M2 macrophages is linked to the development of DPH but is independent on Mac-1.

**Discussion**

DPH is a rare, life-threatening complication in patients with SLE. Several case reports, primarily those based on early autopsy or lung biopsy during the recovery state of the disease, have shown that IC-mediated systemic vasculitis is one of the most common causes of SLE-related DPH (38). However, some SLE cases have presented with DPH without pulmonary IC-deposition or serologic disease activity (2). In addition, in the present model, DPH without pulmonary IC-deposition appears before the generation of autoantibodies (39), which becomes evident a few months following pristane injection (40). Collectively, these data indicate that other inflammatory factors, such as infection, cancer, and drug, trigger the pulmonary injury (41).

In the current study, we clearly demonstrated the role of the leukocyte integrin Mac-1 in promoting pristane-induced DPH, as Mac-1\(^{-/-}\) mice showed a significantly reduced prevalence of DPH than WT animals did, as evidenced by decreased mortality and mild pathology. Neutrophilic alveolar capillaritis and the subsequent macrophage infiltration are also observed in the human pathology of SLE-mediated DPH (23, 42). Pulmonary leukocyte profiles of pristane-treated WT animals showed significantly higher Neu and M1 and M2 macrophages in the lung were analyzed on day 5 after pristane treatment, as described in Fig. 1E (\(n = 10\) per group). (B) Peritoneal neutrophils, eosinophils, and M1 and M2 macrophages were determined by ELISA on days 5 and 10 (\(n = 10\) per group). Data are shown as mean ± SEM. *\(p < 0.05\), **\(p < 0.01\) control versus eosinopenic group.
models of rheumatoid arthritis, reverse Arthus reaction, and lupus nephritis (13, 44, 45), but resulted in decreased Neu accumulation and attenuated inflammation in models of acute anti-GBM, thrombotic glomerulonephritis, and bullous pemphigoid (13, 46). In the current study, we observed significantly enhanced initial recruitment of Neu and Eos into the peritoneal cavity in Mac-1 mutant animals; however, the kinetics of subsequent accumulation differed in these granulocytes, as Neu generally decreased whereas Eos levels remained high until day 2. Neu and Eos also express another $\beta_2$ integrin LFA-1 ($\alpha_\beta_2$, CD11a/CD18), which has been demonstrated to play important roles in cell recruitment under inflammatory conditions (47). However, treatment with neutralizing Ab for LFA-1 (48) did not attenuate granulocyte recruitment following pristane challenge in either WT or Mac-1–

deficient mice (data not shown). In the cell tracking experiment, it is further confirmed that Mac-1$^{-/-}$ Neu predominantly accumulated to the lung and peritoneal cavity compared with WT following pristane treatment. Moreover, PTx pretreatment decreased peritoneal granulocytes infiltration in both mouse strains. Results from in vitro transendothelial Neu migration assay in response to MIP-2 clearly supported our in vivo findings. Thus, we conclude that granulocyte integrins displayed protective roles for chemokine-dependent cell recruitment in the present model.

Neu are always the first line of defense against invading tissue injury. Effective resolution of inflammation requires cessation of Neu recruitment and timely removal of immigrant Neu from the site of inflammation (49, 50). Furthermore, Neu undergoing apoptosis release lipid mediators PGE$_2$ and PGD$_2$, and nucleotides that attract macrophages. Phagocytosis of apoptotic Neu can stimulate macrophage to express a suppressive phenotype and release mediators such as TGF-$\beta$ and IL-10, which would then act in an autocrine or paracrine fashion to inhibit the production of proinflammatory cytokines and further suppress the inflammatory response (22, 51). Mice in which Neu were depleted by anti-Ly6G mAb at the onset of disease showed enhanced virus replication and strongly increased mortality in pulmonary infection with influenza (52). In the current study, Neu depletion during the initial phase after pristane injection in WT mice markedly elevated DPH prevalence, accompanied by increased M1 macrophage accumulation in the lungs and enhanced proinflammatory cytokine IL-6 expression in the peritoneal cavity. Similar tendencies were observed when Neu were depleted in Mac-1$^{-/-}$ mice. These results can be explained by Neu function in pristane clearance, which was evidenced by phagocytic Neu containing oil particles during the early phase of peritoneal inflammation after pristane exposure. Moreover, we observed decreased M2 macrophage accumulation in both the peritoneal cavity and the lungs in neutropenic groups, which suggested that Neu promoted polarization of M2 macrophages. This result was confirmed by the results of the in vitro coculture experiment, which showed that apoptotic Neu could polarize BMDMs to the M2 macrophage phenotype. Furthermore, the severity of DPH did not differ significantly with delayed Neu depletion (data not shown), indicating that the protective effects of Neu were critical during the early phase of pristane exposure. Collectively, these findings revealed a crucial role of Neu in resolution of pristane-mediated inflammation.

Eos have commonly been considered a type of immune cells associated with allergic inflammation and parasitic infestation, but recent studies have shown that they also have important roles in humoral immunity as the main source of prosurvival factors for long-lived plasma cells in the bone marrow, and they can subsequently influence autoantibody production in autoimmune diseases (53, 54). Eos have a definitive effect on the actions of other leukocytes. Wu et al. (33) reported that Eos in adipose tissue are essential for M2 macrophage polarization via IL-4 production, which contributed to maintaining glucose homeostasis. Another study has reported that Eos function in acute peritonitis by downregulating PMN accumulation via the lipid mediator protectin-1 (PD1) (55). Our data showed that significantly increased Mac-1$^{-/-}$ Eos were associated with higher levels of IL-4 and IL-13, which subsequently promoted M2 macrophage polarization after pristane injection. To confirm this result, we depleted Eos using anti–IL-5 mAb in Mac-1$^{-/-}$ mice and found that pristane-induced DPH was exacerbated. Eosinopenia nearly eradicated cells’ ability to secrete IL-4 and led to the reduction of M2 macrophages in the peritoneal cavity and the lungs; therefore, Eos played a pivotal role in amelioration of pristane-induced DPH.
The M1 macrophage phenotype is characterized by the expression of high levels of proinflammatory cytokines and high production of reactive nitrogen and oxygen intermediates. M1 macrophages mediate tissue damage and initiate inflammatory responses in several diseases (56). In the current study, we observed abundant accumulation of M1 macrophages to the lung and peritoneal cavity in WT mice after pristane treatment. We supposed the classical activated M1 macrophages are responsible for increased prevalence of DPH in WT mice. Wang et al. (57) transfused ex vivo programmed M1 macrophages to Adriamycin-induced nephropathy and observed exacerbated function. We injected bone marrow-derived M1 macrophage i.v. into WT mice and found that the prevalence of DPH is elevated together with DPH score. It has been reported that the activity of M1 macrophage can be inhibited via IkB kinaseβ (58), which may be a therapeutic opportunity for DPH.

Clinical evidence that remote organ infection or malignancy can initiate DPH has been reported (41), and we speculated that peritoneal inflammation is tightly linked to DPH in the current model and that the increased peritoneal M2 macrophages observed in Mac-1−/− mice negatively regulate the pristane-mediated immune response both in the lungs and the peritoneal cavity. The significant efficacy of i.p. transfer of ex vivo–expanded F4/80high M2 macrophage–dominant polarization in the peritoneal cavity through its immunoregulatory functions. Essential roles in protection against DPH. Moreover, M2 macrophages play important roles in protection against DPH. We suggested abundant accumulation of M1 macrophages to the lung and peritoneal inflammation is tightly linked to DPH in the current model and that the increased peritoneal M2 macrophages observed in Mac-1−/− mice negatively regulate the pristane-mediated immune response both in the lungs and the peritoneal cavity.

Acknowledgments
We thank N. Asano, Y. Sawa, and N. Suzuki for technical assistance and Dr. Tanya N. Mayadas (Brigham and Women’s Hospital and Harvard Medical School) for providing Mac-1−/− deficient mice.

Disclosures
The authors have no financial interests of conflict.

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