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Rationale: Acute lung injury (ALI) is an important cause of morbidity and mortality, with no currently effective pharmacological therapies. Neutrophils have been specifically implicated in the pathogenesis of ALI, and there has been significant research into the mechanisms of early neutrophil recruitment, but those controlling the later phases of neutrophil emigration that characterize disease are poorly understood.

Objectives: To determine the influence of peripheral blood monocytes (PBMs) in established ALI.

Methods: In a murine model of LPS-induced ALI, three separate models of conditional monocyte ablation were used: systemic liposomal clodronate (sLC), inducible depletion using CD11b diphtheria toxin receptor (CD11b DTR) transgenic mice, and antibody-dependent ablation of CCR2^{hi} monocytes.

Measurements and Main Results: PBMs play a critical role in regulating neutrophil emigration in established murine LPS-induced lung injury. Gr1^{hi} and Gr1^{lo} PBM subpopulations contribute to this process. PBM depletion is associated with a significant reduction in measures of lung injury. The specificity of PBM depletion was demonstrated by replenishment studies in which the effects were reversed by systemic PBM infusion but not by systemic or local pulmonary infusion of mature macrophages or lymphocytes.

Conclusions: These results suggest that PBMs, or the mechanisms by which they influence pulmonary neutrophil emigration, could represent therapeutic targets in established ALI.

Keywords: acute lung injury; LPS; monocytes; neutrophils

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Peripheral blood monocytes have been implicated in the pathogenesis of acute lung injury (ALI), but little is known of their contribution to ongoing neutrophil influx in the persistent phase of ALI.

What This Study Adds to the Field

This study provides evidence in a preclinical model of ALI that monocytes play a critical role in the later stages of neutrophil influx and that their temporal targeted depletion is a potential therapeutic strategy.

The innate inflammatory response is geared to the clearance of pathogens, but excessive and persistent granulocyte accumulation is detrimental to the host (1). Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are characterized by neutrophil-mediated lung injury, the most common etiology being severe sepsis (2). Neutrophil-mediated lung injury of the epithelial/endothelial interface and consequent vascular leak are the hallmarks of ALI/ARDS (2–4). There are 200,000 cases per year of ALI in the United States, with a mortality of approximately 40% (2). No pharmacological agents have been shown convincingly to affect mortality. There is thus a pressing need to define critical mediators of neutrophil recruitment and to implement specific, mechanism-based therapeutic interventions.

The neutrophilic response in ALI has been described as occurring in two distinct phases (5): an initial "recruitment phase" mediated by chemokines followed by a "persistent phase" of neutrophil recruitment, possibly mediated in part by stromal-derived factor-1 (SDF-1/CXCL12) (5). Patients often present with established lung inflammation, and interventions must therefore be guided toward this second phase of neutrophil recruitment to reduce lung injury and ventilator dependence. The underlying cellular mechanisms driving this second phase of neutrophil recruitment remain to be fully characterized.

Peripheral blood monocytes (PBMs) are recruited alongside neutrophils in acute inflammation (6–10), and their potential importance in regulating and amplifying the inflammatory response is increasingly recognized (11, 12). Chemokine generation from recruited monocytes is implicated in effecting neutrophil recruitment (12, 13). These descriptions, alongside the recent discovery of patrolling sentinel monocytes within the vasculature (6), highlight PBMs' potential role in sensing and directing the inflammatory response. The importance of PBMs in acute inflammation offers a potential therapeutic window because they mobilize from the bone marrow and circulate in the peripheral vasculature before tissue infiltration in

TABLE 1. SUMMARY OF PERCENTAGE REDUCTION OF ALVEOLAR NEUTROPHILS AFTER MONOCYTE DEPLETION IN LIPOPOLYSACCHARIDE-INDUCED ACUTE LUNG INJURY

	Systemic Clodronate	DT in CD11b-DTR	MC21 mAb
LPS (neutrophils/μl BAL)	3,561 ± 692.3	2,036 ± 413.9	2,488 ± 361.4
LPS plus treatment (neutrophils/μl BAL)	650.6 ± 135.9	408.6 ± 154.3	1,511 ± 177.0
P value	0.0036	0.0040	0.0182
Neutrophil differential of total cell count (LPS alone is 83%)	70%	57%	76%
% Reduction from control	82	80	39
% Total monocyte reduction in peripheral blood	70	95	35
% Gr-1^{hi} reduction	74	95	74
% Gr-1^{lo} reduction	53	90	4

Definition of abbreviations: BAL = bronchoalveolar lavage; CD11b-DTR = CD11b diphtheria toxin receptor mice; DT = diptheria toxin; Gr-1 = granulocyte-1; mAb = monoclonal antibody.

LPS (10 μg) was instilled intratracheally, followed 6 and 24 h later by monocyte depletion treatments. Data show alveolar neutrophil counts at 48 h after LPS instillation with and without treatment in representative experiments. The average cell differentials were determined in BAL for neutrophils and mononuclear cells. LPS alone had a 83% neutrophilia in BAL. The efficiency of peripheral blood monocyte depletion was evaluated by serial blood analysis, and data show total and subset reductions.

ALI (14–16). Hence, targeting PBMs and the mechanisms by which they could influence neutrophil emigration may provide a valuable interventional strategy. This study aimed to investigate the utility of such an approach in a murine ALI model generated by the direct intratracheal administration of bacterial LPS.

To our knowledge, therapeutic depletion of PBMs timed to occur in the established phase of murine ALI has not previously been reported. Delineating the role of the PBM independently from its tissue descendents in the mononuclear phagocyte system (MPS) has been difficult. The majority of published models investigating depletion in the MPS system *in vivo* affect PBMs and tissue-resident macrophages (17–19). Indeed, studies have highlighted the role played by the resident alveolar macrophage (AM) in eliciting the inflammatory response in ALI through orchestration of early chemokine responses (20, 21). In contrast to models used to study anatomical compartments such as the peritoneum and pleura (18, 19), it is possible to selectively ablate the PBM compartment without affecting resident AMs in murine models of lung inflammation (22). We used three independent techniques to deplete PBMs: systemic liposomal clodronate (sLC), diphtheria toxin (DT) administration in CD11b diphtheria toxin receptor (DTR) mice, and targeted antibody-dependent cell–mediated cytotoxic (ADCC) depletion of monocytes (Table 1). Resident AM populations are unaffected by these three techniques. Compartmental AM depletion and lung macrophage replenishment studies confirmed that the attenuation of neutrophil recruitment was independent of possible effects on the AM population. This study therefore demonstrates the efficacy of temporal, targeted depletion of PBMs in established murine ALI. Some of the results of these studies have been previously reported in the form of an abstract (23).

METHODS

Animals

Adult (25–35 g) specific pathogen-free BALBc/CD1/C57BL/6 mice and CD11b-DTR mice (FVB/N and C57BL/6 genetic background) were used. All studies were done under UK Home Office licenses 60/3545 and 60/3797.

LPS Lung Injury and BALF Retrieval

Unless otherwise indicated, 10 μg LPS from *Escherichia coli* (serotype O111:B4; Sigma-Aldrich, St. Louis, MO) in 50 μl PBS was given intratracheally. BALF was collected and weighed 48 hours later, and cell counts were adjusted per microliter of BALF retrieved. Cells were fixed and stained using a Quik-Diff kit (Reagena, West Sussex, UK), and differential counts were determined.

Neutrophil Depletion

Neutrophil depletion (24) was achieved using monoclonal anti-mouse Ly-6G Ab (IA8 clone, rat IgG2a; BioXCell, West Lebanon, NH). Mice were given two injections of 1 mg Ly-6G Ab or isotype control in 100 μl of sterile saline intraperitoneally 6 and 24 hours after lung injury induction.

Monocyte Depletion

Liposomal clodronate. Liposomal clodronate (LC) was a gift of Roche Diagnostics GmbH (Mannheim, Germany). It was encapsulated in liposomes as previously described (25). LC or liposomal PBS (400 μl of each) were administered intraperitoneally 6 hours after intratracheal LPS, and a further 200 μl was administered 18 hours later. For AM depletion, 100 μl of LC or liposomal PBS were administered intratracheally 48 hours before intratracheal LPS.

DT in CD11b-DTR. DT (10 ng/g) (18, 19) was administered 6 and 24 hours after intratracheal LPS. For adoptive cell add-back experiments, age-, strain-, and sex-matched animals were used as controls, and DT was administered at the same intervals as described above.

MC21 monoclonal antibody. The CCR2-specific antibody MC21 (IgG2b) (20 μg) (26) was administered intraperitoneally 6 and 24 hours after intratracheal LPS. Control animals received isotype (IgG2b, clone 141945, MAB0061; R&D Systems, Minneapolis, MN).

Flow Cytometry Methods

Flow cytometry methods, including assessment of neutrophil transendothelial migration, are detailed in the online supplement.

Lung MPO Activity

Lung MPO activity was determined as previously described (27). Lung vascular permeability was determined as previously described (28) and is described further in the online supplement. Gravimetric determination of lung edema is described in the online supplement.

ELISA

ELISA kits for measurement of IL-10, SDF-1, and KC in BAL fluids (DuoSet; R&D Systems) were used according to the manufacturer's protocols.

In Vivo Optical Imaging

In vivo optical imaging was performed on the Kodak MS FX Pro (Kodak Carestream, Rochester, NY) and the Visen FMT 2500 (Perkin-Elmer, Waltham, MA) as previously described (29). For vascular leak, 1.5 nmol of SAIVI albumin 680 (Invitrogen, Paisley, UK) was injected via the

lateral tail vein, and animals were imaged 30 minutes later. For lung protease activity, 1 nmol of a cathepsin-activatable probe (Prosense 750; Perkin-Elmer) or PBS control was instilled intratracheally 12 hours after intratracheal LPS. Images were acquired 24 hours after Prosense administration.

Cell Isolations

Bone marrow–derived macrophages (BMDMs) were harvested and generated from 8- to 12-week-old female mice as described previously (27). Viable cells (1×10^6) were instilled intratracheally 24 hours after intratracheal LPS. For intravenous reinfusions, 5×10^6 viable cells were administered by intravenous tail vein injection at 6 and 24 hours after intratracheal LPS. Mononuclear cell (MNC) isolation is detailed in the online supplement and is based on a published method (30). Cells (5×10^6) were infused intravenously 6 and 24 hours after intratracheal LPS. Lymphocyte controls were generated by negative magnetic selection using CD11b microbeads (Miltenyi Biotec, Surrey, UK). For optical imaging, cells were labeled with DiR (10 μM in PBS) (Invitrogen, Paisley, UK) (31).

Histology and F4/80 Immunohistochemistry

Histology and F4/80 immunohistochemistry were performed as previously described in subsets of animals without prior BAL (32). Histology scoring was conducted by a blinded observer (33).

Statistics

Student's *t* test and Mann-Whitney U test were used for comparisons between two groups and one-way ANOVA with Bonferroni's *post hoc* test for greater than two groups. For sequential samples, repeated measures two-way ANOVA with Bonferroni's *post hoc* test was used. All analyses were conducted using GraphPad prism (V5.0, La Jolla, CA). In all data presented, $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$.

RESULTS

Characterization of LPS-induced Lung Injury

A LPS-mediated model of lung injury was used (*see* Figure E1 in the online supplement) with intratracheal delivery of LPS. Maximum lung injury was observed at 48 hours, and the peak neutrophil count was observed at 24 hours. To demonstrate the continued influx of neutrophils between 12 and 48 hours after insult in this model, we used the neutrophil-specific depleting monoclonal antibody (mAb) Ly-6G (IA8 clone) (24). Systemic administration of the mAb 12 hours after LPS instillation yielded a 60% reduction in mean alveolar neutrophil count in bronchoalveolar lavage fluid (BALF) retrieved at 2 days (Figure E1). Forty-eight hours was chosen as the time point for BALF retrieval and tissue analysis in all further murine experimentation because it afforded time to apply therapeutic interventions after LPS administration and corresponded to maximal vascular leak and extravasated total protein in BALF.

Peripheral Administration of LC Depletes PBMs and Attenuates ALI

sLC has been shown to deplete 90% of PBMs within 24 hours in mice (25, 34) and tissue macrophages in the spleen and liver. In preliminary studies, biodistribution of fluorescently labeled sLC showed accumulation in the liver, spleen, and bone marrow with minimal accumulation in the lungs (Figure E2). Consistent with the biodistribution studies, F4/80-positive hepatic (112.7 ± 8.4
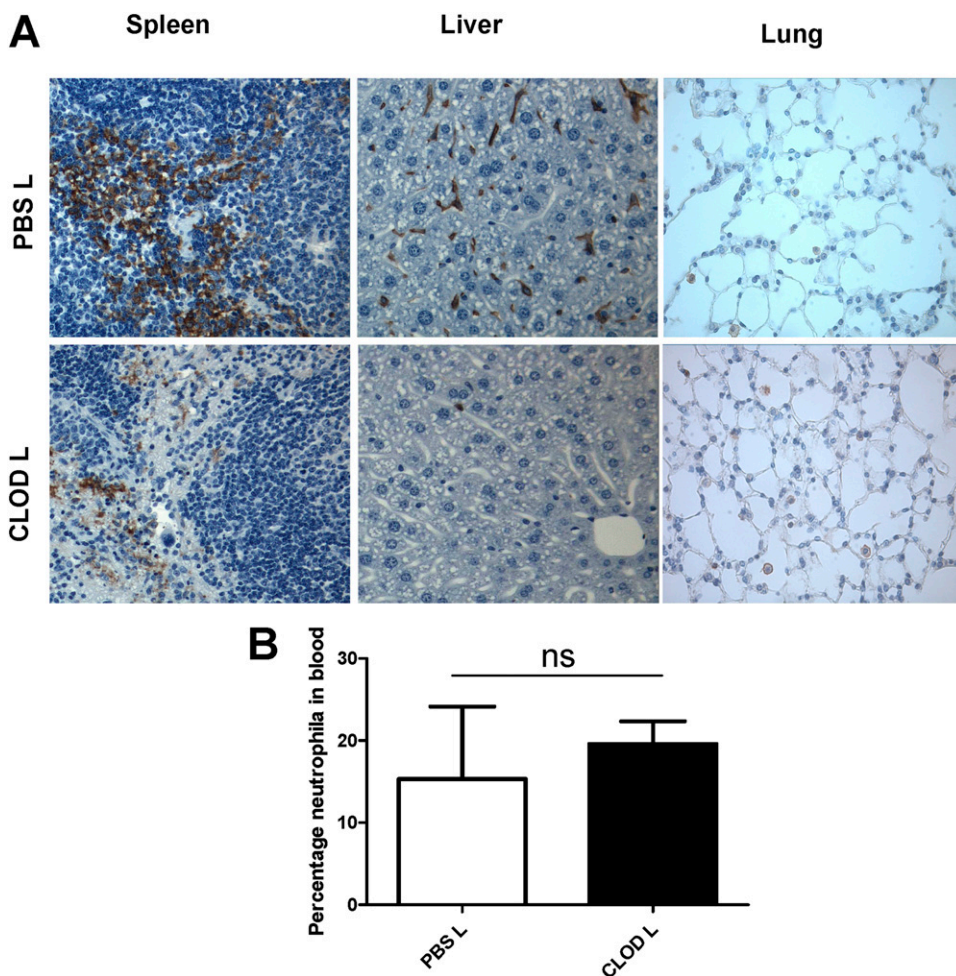
Figure 1. Characterization of the systemic administration of systemic liposomal clodronate (sLC). (*A*) sLC induces widespread resident F4/80 macrophage depletion in spleen and liver (original magnification: ×200) with no effect on resident alveolar macrophages (original magnification: ×40) compared with PBS liposomes (PBS L) (F4/80 staining indicated by brown color). (*B*) sLC does not affect blood neutrophil numbers in naive mice compared with PBS L (i.e., mice that have not been given LPS) (n = 7 per group). Data are presented as mean and 95% confidence interval. Not significant (*P* = 0.323) by Student's *t* test.

vs. 4.66 ± 0.88 [mean ± SEM] cells per high-power field; P = 0.0003 by Student's *t* test) and splenic (35.69 ± 1.9 vs. 5.55 ± 0.223 percentage of area of F4/80 staining per high power field determined on Image J; $P < 0.0001$ by Student's *t* test) macrophages were ablated by sLC (Figure 1A) compared with animals receiving PBS liposomes. sLC did not reduce peripheral blood neutrophils (Figure 1B) or AM numbers retrieved from BALF (Figure E2). sLC did not lead to significant elevations in inflammatory cytokines in BALF (data not shown). The temporal depletion of PBMs using sLC is well characterized, with a nadir at 6 to 8 hours after LC administration with recrudescence of Gr-1^{hi} PBMs 18 hours later (34, 35). Hence, two sequential doses of intraperitoneal LC (6 and 24 h after LPS insult) were used to ensure sustained PBM depletion (Figure 2B). F4/80 staining of lungs in LPS-treated mice receiving PBS liposomes showed prominence of F4/80-positive infiltrating monocytes. In contrast, in sLC-treated animals, resident AMs were evident with reduced influx of F4/80-positive cells (Figure 2B). Histological assessment of lungs at 48 hours (Figure 2B) showed reduced neutrophil ingress and lung injury. Blinded scoring of histology showed a significant reduction in injury score (12.50 ± 0.76 vs. 6.0 ± 0.33; $P < 0.0001$ by Student's *t* test). PBM depletion after LPS administration significantly attenuated neutrophil numbers in BALF at 48 hours (Figure 2C). Additionally, total protein in BAL fluid (Figure 2D), lung edema (Figure 2E), and pulmonary vascular permeability were significantly reduced (Figure E2).

LC Delivered Locally to the Lung Depletes AMs but Does Not Attenuate ALI

In contrast to sLC, compartmental pulmonary LC delivery depletes AMs (32) (Figure E3) without having systemic effects. In keeping
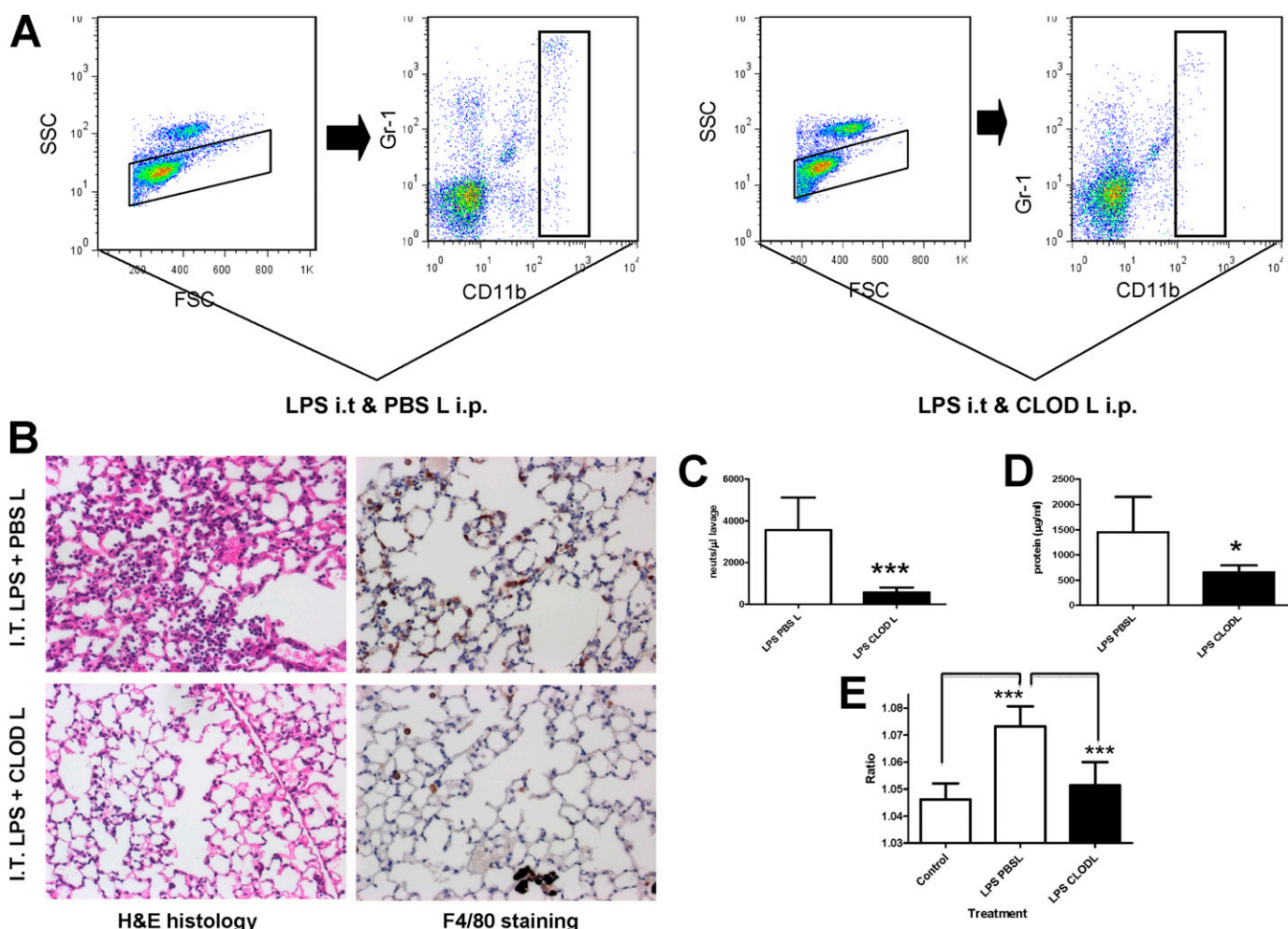

Figure 2. Peripheral blood monocyte depletion with systemic liposomal clodronate (sLC) attenuates acute lung injury (ALI). (*A*) sLC administered 6 and 24 hours after intratracheal LPS. sLC induces the depletion of granulocyte-1 (Gr-1)^{hi} and Gr-1^{lo} peripheral blood monocytes (PBMs) in the context of ongoing LPS-induced pulmonary inflammation. *Left panel* of each represents forward (cell size) and side (granularity) scatter plots (FSC/SSC plots) and the gating strategy for mononuclear cells. *Right panels* show CD11b versus Gr-1 staining of mononuclear cells in peripheral blood of mice showing depletion of CD11b^{hi} Gr-1^{hi} and Gr-1^{lo} monocytes in mice receiving sLC. Double-positive cells reduce from 4.93 to 2.44%. Representative flow plot of n = 5. (*B*) sLC administered 6 and 24 hours after intratracheal LPS results in reduced histologic features of ALI and reduced infiltration of F4/80 PBMs (*brown staining*) into the lung. Original magnification: ×40. Representative of n = 3. (*C*) sLC markedly significantly reduced LPS-induced bronchoalveolar lavage fluid (BALF) neutrophilia at 48 hours. Data are presented as mean and 95% confidence interval (CI). Significant by Student's *t* test ($P = 0.0005$; n = 10 per group). (*D*) Accompanying the reduced neutrophil influx, there was reduced total protein in BALF in clodronate liposome (CLOD L)-treated mice compared with PBS L–treated mice, reflecting a reduction in epithelial-endothelial injury (n = 5 mice per group). Data are presented as mean and 95% CI. $P = 0.028$ by Student's *t* test. (*E*) sLC signficantly reduced lung edema at 48 hours measured by wet/dry ratios of whole murine lungs after drying for 3 days in a 60°C oven. Data are presented as mean and 95% CI. Significant by one-way ANOVA and Bonferroni's post test. Overall P value < 0.0001 (n = 5 mice per group).

with previous studies in rodents (36, 37), prior compartmental depletion of resident AMs did not reduce experimental pulmonary inflammation (Figure E3).

Characterization of the CD11b-DTR Mouse for PBM Depletion in ALI Models

To refine PBM depletion, we characterized ALI in the CD11b-DTR mouse, which expresses human DTR from CD11b promoter sequences, directing transgene expression to monocytes/ macrophages after administration of very low concentrations of DT (18). A range of DT doses were tested *in vivo*, with 10 ng/g showing reduction of PBM by 90%. Despite the presence of CD11b on neutrophils, DT administration to CD11b-DTR mice did not affect the ability of granulocytes to up-regulate CD11b in response to LPS (Figure 3A) or neutrophil counts (Figures 3B–3D). BALF cells characterized morphologically as AMs retrieved from CD11b-DTR mice were phenotypically CD11b^{lo} and CD11c^{hi}, explaining their resistance to DT (Figure 3E). Similar to the effects of sLC treatment, DT administration to CD11b-DTR did not affect the numbers of AMs in BALF (Figure 3F). However, in contrast to sLC,
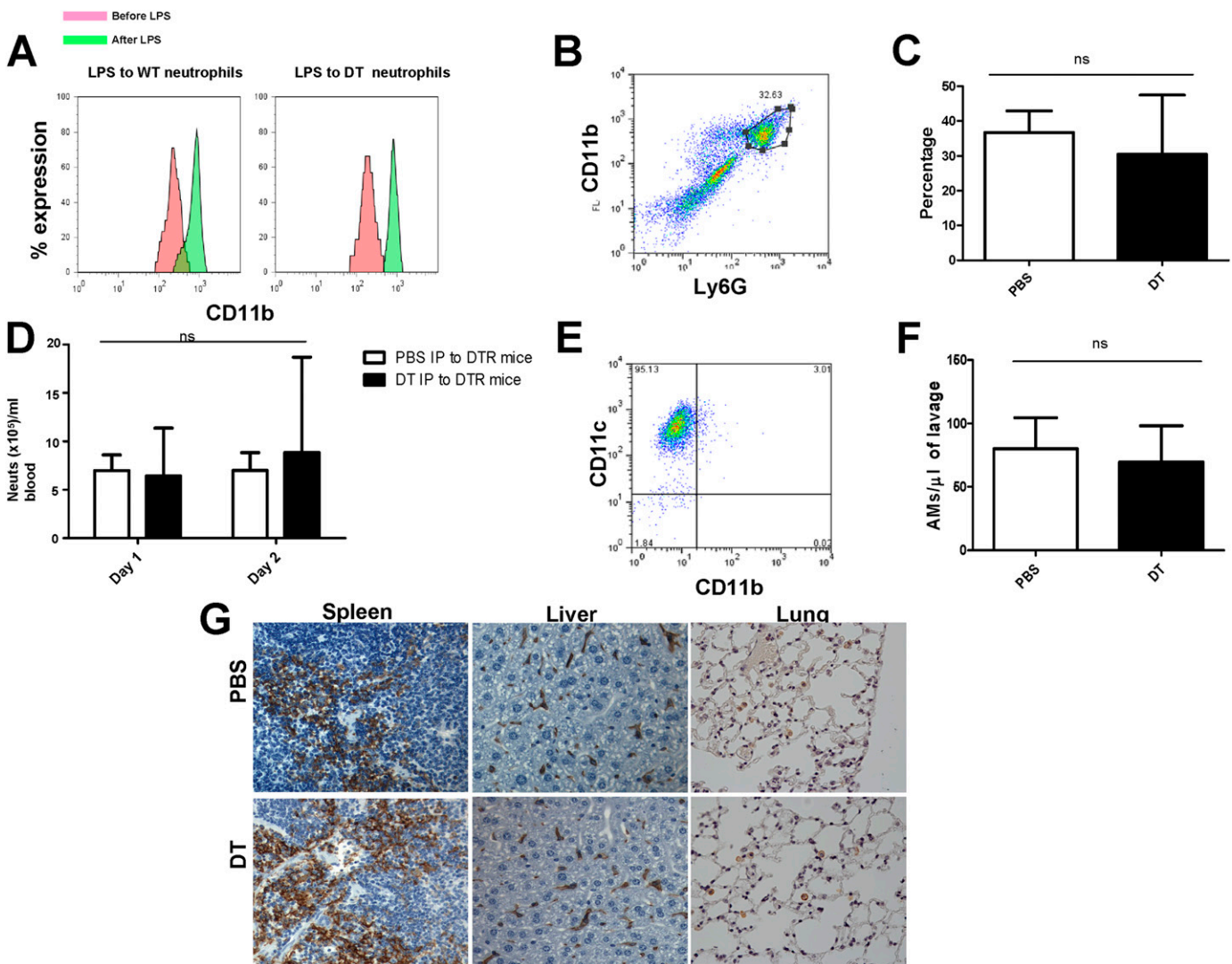

Figure 3. Characterization of the systemic administration of diphtheria toxin (DT) in the CD11b-DTR mouse. (*A*) Neutrophils were gated in whole blood based on their characteristic FSC/SSC profile. CD11b expression was then assessed on the gated neutrophils. Representative histogram plot of CD11b expression on whole neutrophils in murine blood. Whole blood was incubated with 100 ng/ml of *Escherichia coli* LPS for 30 minutes and then stained and analyzed for CD11b expression. CD11b is up-regulated on blood neutrophils from CD11b-DTR mice that had two doses of DT to the same degree as CD11b-DTR mice that had PBS control (n = 3 mice per group). (*B*) Bone marrow neutrophils were identified by flow cytometry in whole marrow perfusates by CD11b and Ly6G antibody staining. (*C*) Percentage of neutrophils in marrow perfusates from CD11b-DTR mice given DT or PBS control shown as percentage of total cells in marrow (n = 3 mice per group). Data are presented as mean and 95% confidence interval (CI). Not significant (*P* = 0.2) by Mann-Whitney test. (*D*) Peripheral blood neutrophils are not depleted after intraperitoneal DT in CD11b-DTR mice. Whole blood was analyzed by flow cytometry for neutrophil count on Day 1 and Day 2 of after DT treatment in naive mice. Data are presented as mean and 95% CI. Not significant (*P* = 0.4245) by repeated measures two-way ANOVA (n = 3 mice per group). (*E*) Alveolar macrophages (AMs) from CD11b-DTR mice express low/absent levels of CD11b and are CD11c^{hi}. (*F*) DT administered to CD11b-DTR mice does not affect the number of AMs in bronchoalveolar lavage fluid. Data are presented as mean and 95% CI. Not significant (*P* = 0.5614) by Mann-Whitney test (n = 3 mice per group). (*G*) Representative histology in CD11b-DTR mice receiving DT. F4/80 (*brown staining*) macrophages remain in spleen (original magnification: ×200), liver (×200), and lung (×40).

DT administration to CD11b-DTR mice did not ablate hepatic cells (95.4 ± 16.36 vs. 92.20 ± 11.89 cells per high-power field; $P = 0.82$ by Student's t test) and had a much reduced depletion effect on splenic F4/80 staining (35.69 ± 1.9 vs. 17.47 ± 0.96 percentage of area of F4/80 staining per high-power field determined on Image J; $P = 0.0011$ by Student's t test) (Figure 3G).

DT Administration in CD11b-DTR Mice Attenuates ALI

Having established that DT administration ablated blood monocytes (Figure 4A) (but not hepatic cells, AMs, or blood or bone marrow neutrophils) *in vivo*, we used the CD11b-DTR mouse to investigate the role of the PBM in ALI. PBM ablation using two consecutive doses of DT (10 ng/g) at 6 and 24 hours after LPS administration significantly attenuated neutrophil
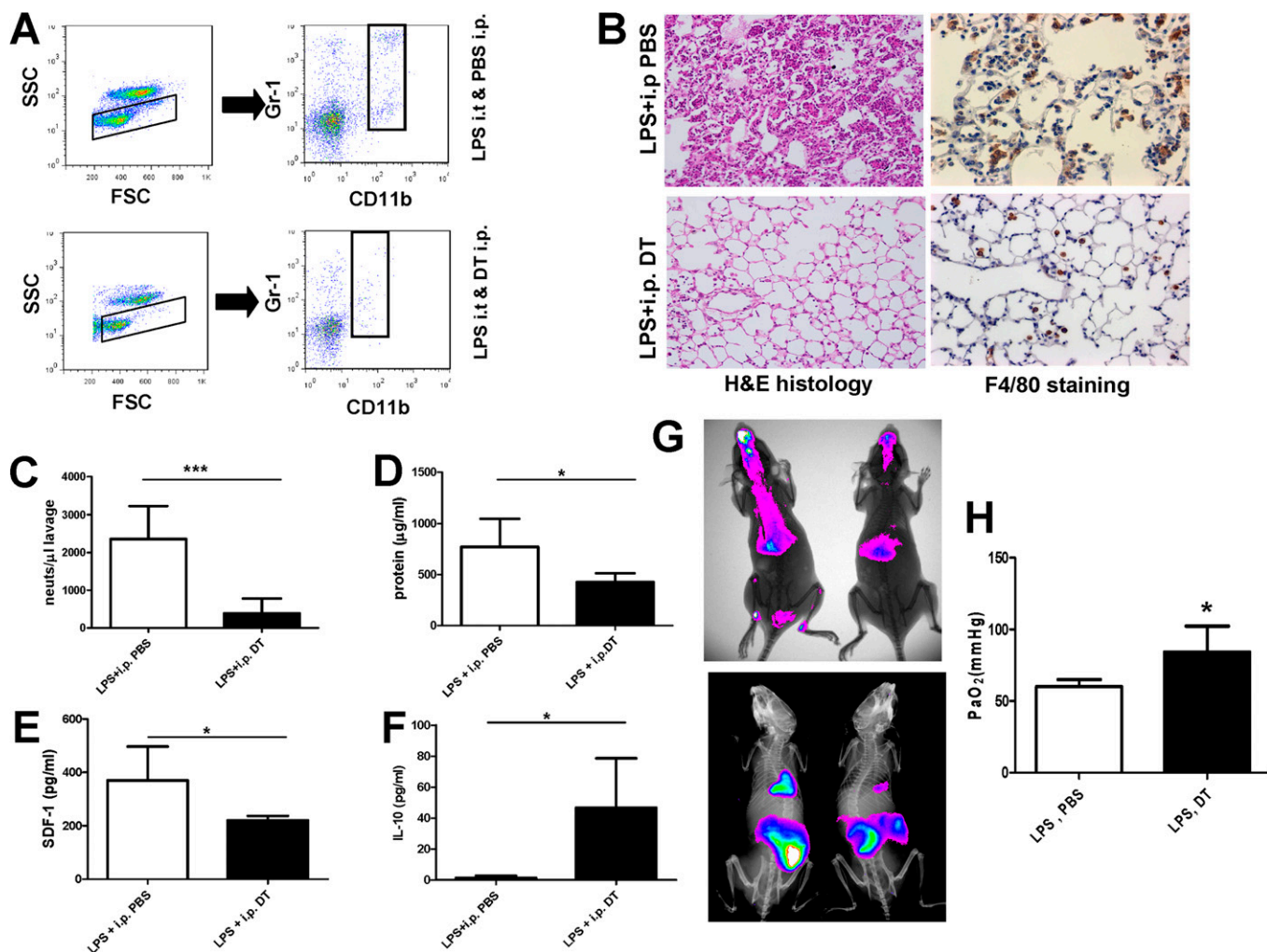

Figure 4. Peripheral blood monocyte (PBM) depletion in the CD11b-DTR mouse reduces ALI as evidenced by decreased neutrophil numbers, vascular leak, inflammatory cytokines, and improved oxygenation. (*A*) Diphtheria toxin (DT) in the CD11b-DTR mouse induces the depletion of granulocyte-1 (Gr-1)^{hi} and Gr-1^{lo} PBMs in the context of ongoing LPS-induced pulmonary inflammation. *Left panel* of each represents FSC/SSC plots and gating strategy for mononuclear cells. *Right panels* show CD11b versus Gr-1 staining of mononuclear cells in peripheral blood of mice showing an absence of CD11b^{hi} Gr-1^{hi} and Gr-1^{lo} monocytes in mice receiving DT versus PBS. Representative flow plots of n = 5 mice. Double-positive cells reduced from 9.24 to 1.73%. (*B*) In CD11b-DTR mice receiving intratracheal LPS, two doses of 10 ng/g DT led to reduced histological features of ALI and reduced infiltration of F4/80-positive (*brown staining*) PBMs with retained alveolar macrophages. (*C*) At 48 hours after LPS, there was a marked reduction in neutrophil numbers in bronchoalveolar lavage fluid (BALF) (n = 7 mice per group) in DT-treated CD11b-DTR mice compared with control mice. Data are presented as mean and 95% confidence interval (CI). $P = 0.0003$ by t test. (*D*) Accompanying the reduced neutrophil influx, there was reduced total protein in BALF in DT-treated CD11b-DTR mice compared with control mice, reflecting a reduction in epithelial-endothelial injury (n = 7 mice per group). Data are presented as mean and 95% CI. $P = 0.029$ by t test. (*E*) Stromal-derived factor-1 (SDF-1) was significantly decreased in BALF from CD11b-DTR mice receiving DT compared with control mice (n = 7 mice per group). Data are presented as mean and 95% CI. $P = 0.0145$ by t test. (*F*) IL-10 was significantly increased in BALF from CD11b-DTR mice receiving DT compared with control mice (n = 7 mice per group). Data are presented as mean and 95% CI. $P = 0.013$ by t test. (*G*) *Top panel*: Whole body reflectance imaging of mice after intravenous-labeled albumin showing reduced airway accumulation of albumin in the LPS-DT animal on the *right* in comparison to the PBS-DT animal on the *left* (representative animal from n = 3 per group that were imaged). *Bottom panel*: Cathepsin activity (measured 24 h after administration of Prosense 750) is also reduced in murine lungs after DT treatment (comparing LPS-DT mouse on the *right* with LPS-PBS mouse on the *left*). (*H*) In mice receiving 100 μg of LPS intratracheally followed by DT, at 48 hours there is significant improvement in oxygenation (n = 4 mice per group). Data are presented as mean and 95% CI. $P = 0.028$ by Mann Whitney test.

recruitment (Figures 4B and 4C). Histological assessment showed reduced lung injury and inflammatory infiltrates (Figure 4B). Blinded scoring of histology showed a significant reduction in injury score (12.30 ± 1.2 vs. 7.0 ± 0.17; $P < 0.001$ by Student's t test). F4/80 staining of lungs showed reduced pulmonary ingress of monocytes with retained AMs (Figure 4B), and BALF analysis showed significantly reduced neutrophil numbers (Figure 4C) and total protein (as a measure of alveolar-endothelial disruption) (Figure 4D). There was no difference in the concentration of the key neutrophil chemokine KC in BALF at 48 hours by ELISA (data not shown). However, the recently characterized neutrophil chemokine SDF-1 was significantly reduced in mice that had received monocyte depletion by administration of DT (Figure 4E). Furthermore, lavage cytokines from these animals showed an antiinflammatory phenotype with increased IL-10 in monocyte-ablated animals (Figure 4F). Noninvasive whole-body optical imaging of treated mice also demonstrated a striking reduction in pulmonary vascular leak and protease activity (Figure 4G). Oxygenation of mice was assessed by performing arterial blood gas measurements in mice receiving 100 μg of LPS followed by monocyte depletion. This demonstrated significantly improved oxygenation (Figure 4H). Pulmonary vascular permeability and lung function were also improved (Figure E2).

DT Administration in CD11b-DTR Mice Attenuates Neutrophil Recruitment in a Model of Live Bacterial Infection

To evaluate if neutrophil recruitment was dependent on monocytes in a model of direct bacterial instillation, we delivered 10^5 colony-forming units of *Pseudomonas aeruginosa* followed by DT administration in the CD11b-DTR mouse. In this model, mice were lavaged 18 hours after bacterial instillation. There was a significant ($P = 0.0002$) reduction in alveolar neutrophil numbers but no significant difference in recoverable bacteria in BALF (Figure E4).

Targeted Depletion of CCR2^{hi} (Gr-1^{hi}) PBMs Results in Attenuation of Neutrophil Numbers in BAL at 48 Hours

sLC in wild-type mice and DT in the CD11b-DTR resulted in Gr-1^{hi} and Gr-1^{lo} PBM depletion. To test whether the effect was specific to particular PBM subtypes, we used low doses of an anti-CCR2 monoclonal antibody (MC21) to specifically deplete CCR2^{hi} Gr-1^{hi} PBMs (26). Preliminary studies confirmed that this dose of MC21 did not lead to peripheral blood neutrophil depletion in naive mice (n = 3 mice per group receiving 20 μg of MC21 or isotype control and neutrophil percentage in whole blood was analyzed the following day; $P = 0.7$ using Mann-Whitney test). MC21 mAb (20 μg/mouse) was administered 6 hours after intratracheal LPS and then repeated 24 hours to maintain Gr-1^{hi} monocyte depletion (Figures 5A–5C). MC21 administration to LPS-treated mice resulted in a significant attenuation of neutrophil numbers in BALF (Figure 5D) and reduced myeloperoxidase (MPO, a neutrophil marker) in whole lung homogenates (Figure 5E) in comparison to isotype-treated control mice. In MC21-treated mice, there was a trend for a reduction in total BALF protein (Figure 5F). SDF-1 and pulmonary vascular permeability (Figure E2) were significantly reduced in MC21-treated mice.

PBM Depletion Reduces Pulmonary Vascular Permeability and Improves Indices of Ventilation

All of the PBM depletion methods significantly reduced pulmonary vascular leak measured by Evans blue (28) (Figure E2).

Mice were subjected to whole-body plethysmography to record an index of ventilation. DT in the CD11b-DTR significantly improved an index of ventilation (Figure E2).

PBM Depletion Reduces Pulmonary Interstitial Neutrophils

Having demonstrated the reduced alveolar neutrophil influx after PBM depletion, we wished to determine if the pulmonary interstitial pool of neutrophils was reduced. Using methods recently described (38), we identified neutrophils in lung homogenates by dual staining for Ly6G and CD11b (Figure E6). Fluorescently labeled antibody against granulocyte-1 (Gr-1) was injected intravenously and served as a marker for intravascular neutrophils (CD11b$^+$, Ly6G$^+$, and Gr-1$^+$). DT administration in CD11b-DTR mice and sLC in wild-type mice significantly reduced the total percentage of pulmonary interstitial neutrophils (Figure E6), suggesting a role for PBMs in transendothelial migration as recently described (39).

Adoptive Intravenous Transfer of Mononuclear Cells but Not Macrophages Restores LPS-mediated Lung Inflammation

To confirm that selective depletion of PBMs was responsible for the attenuation of neutrophil numbers in BAL, adoptive transfer experiments were performed. CD11b-DTR mice received intratracheal LPS at time 0 and intraperitoneal DT at 6 and 24 hours after LPS but also received adoptive transfer of various primary cells (obtained from syngeneic wild-type mice). Compartmental (pulmonary) and intravenous repletion studies were performed.

Compartmental pulmonary delivery of BMDMs to the LPS-treated lung after DT treatment had no affect on BAL neutrophil count at 48 hours (data not shown). Two infusions of intravenous primary murine BMDMs (5×10^6) were then given to DT-treated mice with established LPS injury, which also failed to rescue neutrophil numbers in BALF (815 ± 201 neutrophils/μl BALF in wild-type mice receiving LPS and DT vs. 213 ± 46 neutrophils/μl BALF in DT-treated CD11b-DTR mice with LPS-induced ALI and macrophage infusion; $P = 0.034$ by Student's t test; n = 5 per group). In contrast, intravenous adoptive transfer of freshly isolated wild-type MNCs (5×10^6) resulted in an increase of neutrophil numbers in BALF (Figure 6A) and a highly significant rescue of neutrophil numbers measured by flow cytometric Ly6G staining of single-cell suspensions of whole murine lungs (overall $P < 0.0001$) (Figure 6B). Lymphocytes isolated by negative magnetic selection from MNCs acted as cellular controls. Infusion of lymphocytes did not restore BALF or blood neutrophilia. Infused, labeled MNCs trafficked to the inflamed lung, to the liver, and to the spleen. In contrast, infused, labeled lymphocytes trafficked to liver and spleen but not to the inflamed lung (Figure E6).

Monocyte infiltration and dendritic cell (DC) maturation in the murine LPS–inflamed lung has been recently characterized, and MHC II^{hi} CD11c^{hi} CD11b^{hi} myeloid infiltrating DCs are potent producers of neutrophil chemokines (40). We therefore investigated expression of CD11b on monocytoid subsets in lung tissue. There was a significant increase in CD11b expression on lung CD11c^{hi}, MHC II^{hi} cells in monocyte-depleted CD11b-DTR LPS-treated mice receiving MNC infusions (Figure 7), with partial rescue toward levels observed in LPS-treated wild-type mice, in marked contrast to those receiving lymphocytes.

DISCUSSION

Studies using rodent models of ALI demonstrate that the early influx of neutrophils is primarily directed by cytokine gradients
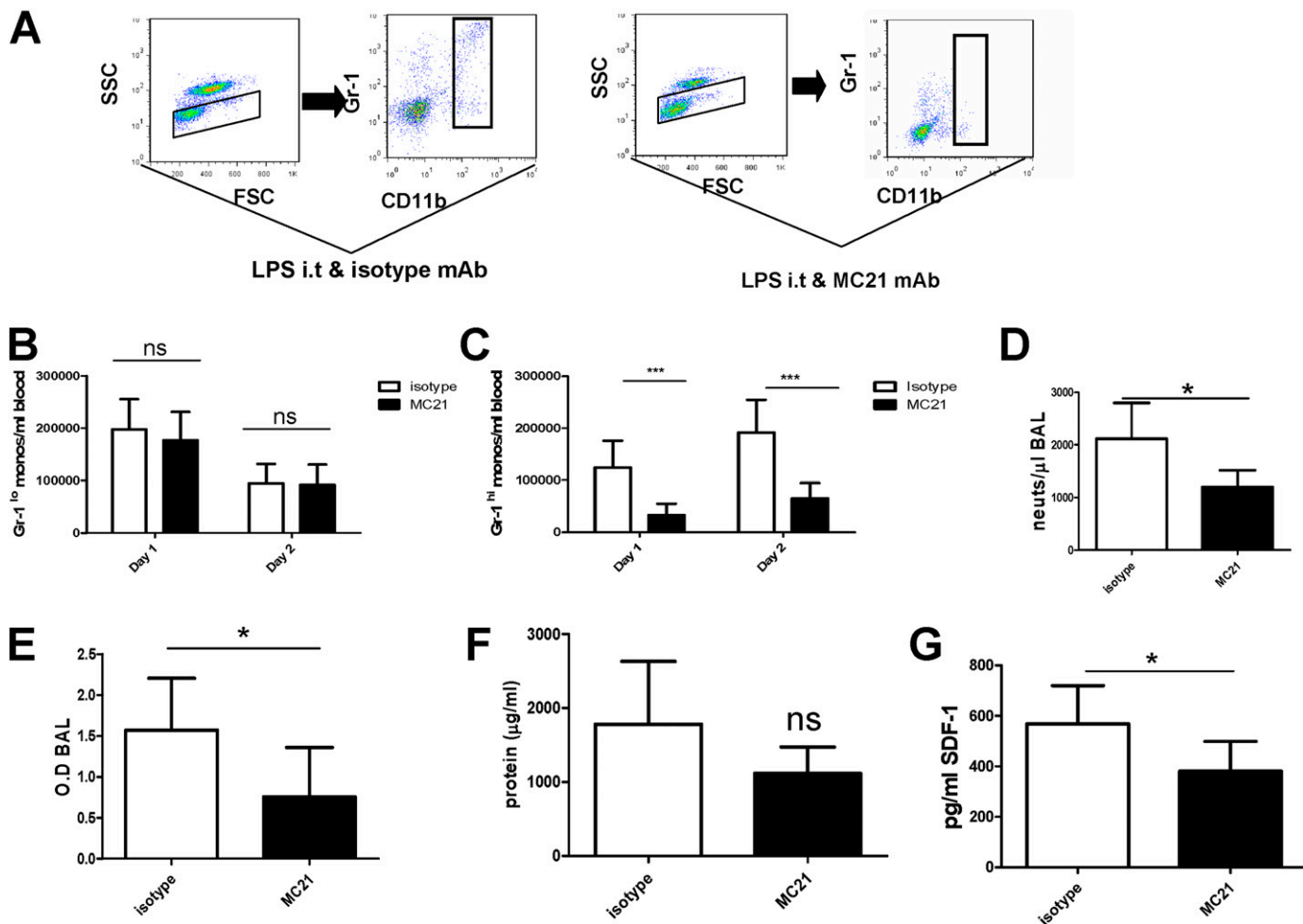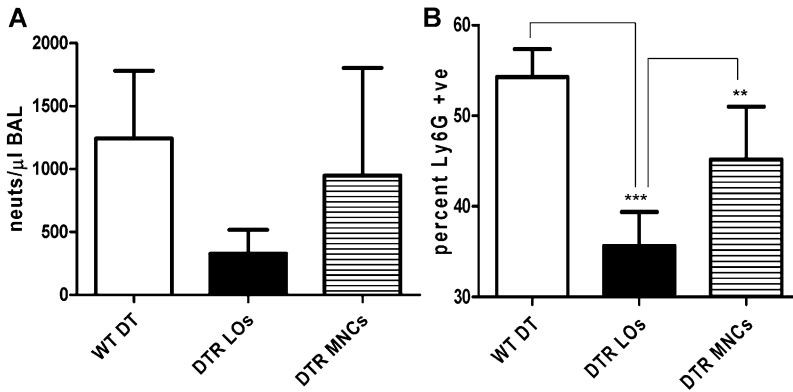
Figure 5. Specific granulocyte-1 (Gr-1)[hi] CCR2^{hi} monocyte depletion results in a reduction in neutrophil numbers in bronchoalveolar lavage fluid (BALF) at 48 hours after experimental acute lung injury. (*A*) Representative flow plots of murine whole blood 24 hours after MC21 monoclonal antibody (mAb) administration shows specific depletion of Gr-1^{hi} PBMs in the setting of LPS-induced acute lung injury. The *left panel* for each condition represents FSC/SSC plots and gating strategy for mononuclear cells. *Right panels* show CD11b versus Gr-1 staining of mononuclear cells in peripheral blood of mice showing the absence of CD11b^{hi} Gr-1^{hi} monocytes in mice receiving MC21 versus isotype control. Double positives reduced from 7.14 to 0.42%. (*B*) Quantification of Gr-1^{lo} monocytes in whole blood 24 (Day 1) or 48 hours (Day 2) after LPS shows no difference in animals receiving MC21 versus isotype control mice. Data are presented as mean and 95% confidence interval (CI) (n = 10 per group). ns = not significant (*P* = 0.633) by repeated measures two-way ANOVA. (*C*) Quantification of Gr-1^{hi} monocytes in whole blood 24 (Day 1) or 48 h (Day 2) after LPS are significantly decreased with MC21 administration, as quantified by flow cytometry. Data are presented as mean and 95% CI (n = 10 per group). *P* < 0.0001 by repeated measures two-way ANOVA. (*D*) Forty-eight hours after intratracheal LPS, neutrophils are reduced in BALF from mice treated with MC21 mAb compared with isotype-treated control mice (n = 10 per group). Data are presented as mean and 95% CI. *P* = 0.011 by *t* test. (*E*) Myeloperoxidase (MPO) is reduced in whole lung homogenates from LPS-treated mice receiving MC21 mAb, compared with isotype-treated control mice expressed as optical density (450 nm) of MPO substrate (n = 10 mice per group). Data are presented as mean and 95% CI. *P* = 0.0327 by *t* test. (*F*) Accompanying the reduced neutrophil influx, there was a nonsignificant trend for reduction in total protein in BALF in treated mice (n = 5 mice per group). Data are presented as mean and 95% CI. *P* = 0.07 by Student's *t* test. (*G*) SDF-1 was significantly reduced in BALF in monocyte-depleted mice (n = 6 per group). Data are presented as mean and 95% CI. *P* = 0.0379 by *t* test.

generated from resident lung leukocytes and epithelial cells (41–43). This directs intraalveolar movement of neutrophils from the marginated pool within the lung and from the initial wave of bone marrow–mobilized neutrophils in the circulation (24). However, as we and others have shown, this initial wave of neutrophil-specific murine cytokines (KC, MIP-2) declines rapidly (18), and the factors controlling the sustained response remain unclear. Recent work implicates pulmonary epithelial cell–produced SDF-1 in later phase neutrophil recruitment (5), but the role of the innate immune cellular response in regulating the ongoing neutrophil influx is not clearly defined. PBMs are increasingly being recognized in many organ systems as orchestrators of leukocyte recruitment (11, 22), prime

initiators of endothelial damage (44, 45), and pivotal contributors to fibrogenesis (32, 46). However, despite the significant body of work suggesting that monocytes contribute to ALI (11, 13, 16, 39, 44, 45, 47–49), no published reports exist of therapeutic monocyte depletion in established experimental ALI. We hypothesized in this study that infiltrating PBMs play a major role in regulating ongoing neutrophil recruitment and consequent vascular leak and that depletion of the PBM pool is a therapeutic target in the setting of established ALI.

Given the controversy regarding the role of PBMs in ALI, we used three independent techniques in a LPS-induced ALI model, specifically aimed at investigating the function of PBMs in regulating the persistent phase of neutrophil recruitment in

Figure 6. Monocyte repletion in CD11b-DTR mice results in a partial rescue of LPS-induced pulmonary neutrophilia. Wild-type (WT) and CD11b-DTR mice received 10 μg of intratracheal LPS followed by two intraperitoneal doses of 10 ng/g diphtheria toxin (DT). Mononuclear cells (MNCs) (or controls) were administered intravenously on two occasions (5 × 10^6 cells), and bronchoalveolar lavage fluid (BALF) was performed at 48 hours. (*A*) MNCs partially rescued neutrophil numbers in BALF at 48 hours, whereas lymphocytes (LOs) did not. Data are presented as mean and 95% confidence interval (n = 5 per group). Not significant by one-way ANOVA and Bonferroni's post test. Overall *P* value = 0.096. (*B*) Single cell digests of lungs of mice from the same experiment showed a reduction in the percentage of Ly6G cells, which was partially rescued by MNC infusion. Data are presented as mean and 95% confidence interval (n = 5 per group). Analyzed by one-way ANOVA and Bonferroni's post test. Overall *P* value < 0.0001.

ALI. In each case, treatments were administered for two consecutive days to ensure sustained depletion of PBMs (34, 35). Caveats for each individual depletion technique exist, but the observation that all three depletion strategies significantly attenuated neutrophil numbers in BALF (Table 1) in established ALI supports the general supposition.
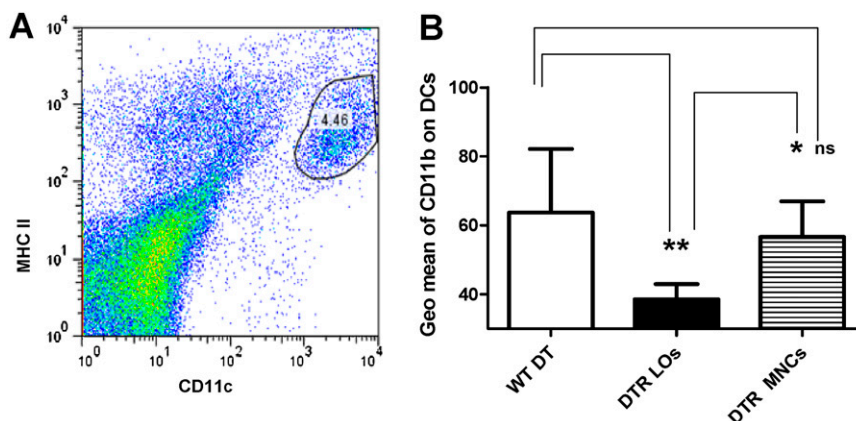
The first technique we used was sLC administration. sLC has been extensively used in the literature to specifically deplete cells of the MPS (25). However, there have been few attempts to deplete PBMs in models of ALI using sLC (49, 50). These prior studies used early time points and showed no significant effects on neutrophil recruitment to the acutely inflamed lung but did not address later phase responses. A recent study using sLC depletion supported a role for monocytes in the transendothelial migration of neutrophils in an ischemia-reperfusion lung injury model (39), supporting our findings.

The treatment protocol we used resulted in a 70% reduction of PBMs (Gr-1^hi and Gr-1^lo subsets) without affecting resting neutrophils or resident AMs. However, there was also substantial ablation of hepatic and splenic macrophage pools and a consequent increase in the circulating number of neutrophils in the ALI model (data not shown). sLC resulted in a significant decrease in LPS-mediated neutrophil count in BALF at 48 hours. This divergent phenotype of decreased tissue neutrophils in the presence of significantly elevated blood neutrophilia closely resembles previous studies in an antiglomerular basement membrane model of renal injury (51). It is possible that the increased numbers of circulating neutrophils was due to their inadequate clearance by liver and splenic macrophages, which are depleted with sLC. Irrespectively, our observations suggested a major

role for the PBM and/or its tissue descendents in the recruitment of neutrophils from the blood to the lung during the "persistent phase" of ALI.

We therefore used the transgenic CD11b-DTR mouse (18, 19), an inducible system with significantly less hepatic/splenic macrophage depletion (Figure 3G). In the setting of ALI, this transgenic system offers an effective means by which to specifically deplete Gr-1^hi and Gr-1^lo PBM subsets. Unlike other tissue-resident macrophages, AMs are resistant to depletion owing to their low or absent expression of CD11b (Figure 3) (22), and neutrophils are not affected (18, 52, 53). Using this method for therapeutic PBM depletion in established murine ALI, there was a significant reduction in pulmonary neutrophil numbers in BALF at 48 hours. This corresponded with histological evidence of reduced lung injury (Figure 4B) and a significant reduction in SDF-1 in BALF. In the peripheral blood there was a significant reduction in neutrophilia, suggesting that the mobilization of neutrophils from bone marrow was reduced (data not shown). Given that the pulmonary production of SDF-1 is now recognized as a potential regulator of ongoing neutrophil recruitment to the inflamed lung, these findings suggest PBMs may contribute directly or indirectly to pulmonary SDF-1 expression.

sLC in wild-type mice and DT administration in CD11b-DTR mice led to depletion of the Gr-1^hi and Gr-1^lo monocyte subsets. Gr-1^hi monocytes express CCR2 and have been described as the prime inflammatory monocyte subset (54). To determine if this subset contributed significantly to neutrophil influx, we used a low-dose antimurine CCR2 mAb that was unlikely to fully block the CCR2 axis (26) yet demonstrated direct ADCC of peripheral CCR2-expressing cells. This intervention resulted in

Figure 7. Monocyte repletion in CD11b-DTR mice results in a partial rescue of a LPS-induced population of pulmonary monocytoid cells expressing CD11b/MHC II. Wild-type (WT) and CD11b-DTR mice received 10 μg of intratracheal LPS followed by two intraperitoneal doses of 10 ng/g diphtheria toxin (DT) at 6 and 24 hours after LPS. Mononuclear cells (MNCs) (approximately 30% of which are monocytes) or lymphocytes (LOs) were administered intravenously on two occasions at 6 and 24 hours after LPS, and lungs were harvested at 48 hours. (*A*) Monocytoid cells resembling dendritic cells (DCs) were characterized in whole lung digests by CD11c and MHC II expression (*left panel gate*). (*B*) Geometric mean of CD11b expression on gated cells was partially but significantly rescued by intravenous MNC infusion. Data are presented as mean and 95% confidence interval. Analyzed by one-way ANOVA and Bonferroni's post test. Overall *P* value = 0.003 (n = 5 per group).

a 95% reduction in Gr-1^{hi} monocytes and led to a less striking reduction in pulmonary neutrophilia at 48 hours when compared with depletion of the Gr-1^{hi} and Gr-1^{lo} subsets. A caveat for this depletion methodology is that neutrophils do express CCR2. We showed in preliminary studies that low-dose MC21 did not deplete naive murine neutrophils; however, this does not preclude depletion during inflammation because neutrophil CCR2 may be up-regulated, thereby increasing susceptibility to ADCC.

The question of whether AMs contributed to the reduction in neutrophil numbers in BALF was addressed by performing local compartmental ablation of resident AMs. This resulted in a trend for an increase in neutrophil numbers (not statistically significant) in BALF at 48 hours in mice receiving 10 μg of LPS in comparison to control ALI mice (Figure E3). This is similar to findings describing that depletion of AMs increases neutrophil recruitment (55). Indeed, these studies further support our contention that the AM is not directing the ongoing influx of neutrophils; rather, circulating monocytes are responsible.

Although systemic macrophage reconstitution had no phenotypic effect, MNC repletion partially rescued neutrophil numbers in whole lung after LPS treatment (Figure 6). In that regard, recent descriptions of monocyte emigration and maturation to pulmonary DCs and macrophages in naive and inflammatory situations (15, 56) highlight the possibility of targeting PBMs and impinging on DC/macrophage maturation in acute inflammation within the lung. Indeed, MNC rescue of neutrophil emigration in lungs (after DT administration in LPS-induced ALI in the CD11b-DTR mice) was associated with an inflammatory DC phenotype in lung digests (Figure 7) (CD11c^{hi} MHC II^{hi}, CD11b^{hi}). We did not perform lineage tracing studies to prove conclusively that myeloid-derived monocytes directly contributed to this population, but this supposition is supported by recent studies (40).

MNCs have been administered as a therapy in murine models of lung injury, but up to now this strategy has been applied in the late stages of LPS-induced ALI. Indeed, Prota and colleagues (57) assessed therapeutic efficacy at 28 days after LPS administration and Yamada and colleagues at 1 week after LPS instillation (58). We speculate that the acute phase of inflammation driven by monocytes may be detrimental to the host and that late-phase mononuclear cell recruitment may be beneficial. In that regard, regulatory T lymphocytes (59) have been shown to be critical in the resolution of LPS-induced ALI through promoting neutrophil apoptosis. As such, the beneficial effects of MNC therapy in studies by Yamada and colleagues (58) and Prota and colleagues (57) may have been mediated via lymphocytes rather than monocytes.

We show data that PBMs facilitate pulmonary neutrophil recruitment in a model of live bacterial infection (Figure E4). However, this does not preclude potentially deleterious sequalae of PBM depletion in the setting of infection given the pleiotropic immunomodulatory functions of Gr1^{hi} and Gr-1^{lo} monocyte subsets (13, 14, 32, 34, 35).

In summary, we have demonstrated that PBM depletion attenuates ALI, associated with consistent and significant reduction in neutrophil numbers in BALF and consequent tissue injury. However, determining the exact point at which to target this inflammatory response for optimal therapeutic benefit remains challenging. Nevertheless, by depleting monocytes and hence their tissue descendents in ALI, we have been able to sustain an injury-attenuation effect in the "persistent" phase of ALI. This supports the further study and development of monocyte-targeted therapies for ALI/ARDS.

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