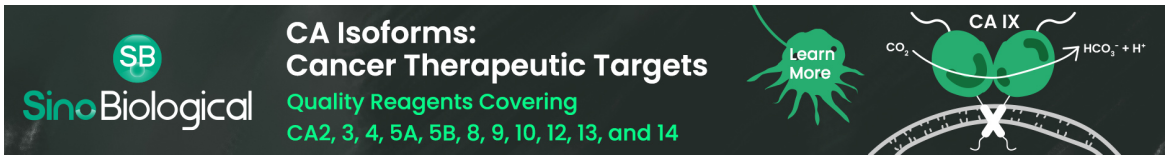

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Pneumolysin-Induced Lung Injury Is Independent of Leukocyte Trafficking into the Alveolar Space[1]

Ulrich A. Maus,[2]* Mrigank Srivastava,* James C. Paton,† Matthias Mack,‡ M. Brett Everhart,§ Timothy S. Blackwell,§ John W. Christman,§ Detlef Schlöndorff,‡ Werner Seeger,* and Jürgen Lohmeyer*

Pneumolysin (PLY) is a major virulence factor released by *Streptococcus pneumoniae* and has been implicated in the pathogenesis of pneumococcal pneumonia. In this study, we evaluated the contribution of newly recruited neutrophils and monocytes and resident alveolar macrophages to the pathogenesis of PLY-induced lung injury. Mice received either adhesion-blocking Abs to inhibit alveolar leukocyte trafficking or liposomal clodronate to deplete alveolar macrophages before intratracheal application of native PLY or its noncytotoxic derivative PdB. We found that treatment with PLY but not PdB resulted in increased lung vascular permeability. In addition, PLY also induced a decrease in the resident alveolar macrophage population, and the recruitment of peripheral blood neutrophils and monocytes into the alveolar space. Blockade of PLY-induced alveolar leukocyte trafficking by pretreatment of mice with anti-CD18 plus anti-CD49d Abs or depletion of circulating neutrophils did not attenuate the increase in lung permeability observed in response to intratracheal PLY. In addition, depletion of resident alveolar macrophages with clodronated liposomes did not reduce alveolar injury developing in response to PLY. PLY-induced lung injury was associated with only a small increase in bronchoalveolar lavage concentrations of cytokines. These data indicate that PLY-induced lung injury results from direct pneumotoxic effects on the alveolar-capillary barrier and is independent of both resident and recruited phagocytic cells. ***The Journal of Immunology,*** **2004, 173: 1307–1312.**

Streptococcus pneumoniae (pneumococcus) is the most prevalent pathogen involved in community-acquired pneumonia, septic meningitis, and otitis media. Invasive pneumococcal disease appears to depend on both pneumococcal cell wall components including peptidoglycan and lipoteichoic acid, as well as a multitude of virulence factors that include pneumolysin (PLY)[3] (1, 2). PLY is an intracellular 53-kDa protein belonging to the family of thiol-activated, cholesterol-binding toxins and is released by pneumococcal autolysis (3). It is produced by all known *S. pneumoniae* isolates, and beside its complement-depleting activities, PLY is well known to exert direct cytotoxic effects on host cells by oligomerizing into ring-shaped transmembrane pores encompassing 30–50 monomers (4). Infection models with PLY-deficient pneumococci revealed reduced acute lung injury scores, bacterial growth, reduced lung neutrophil trafficking, and a prolonged survival, when compared with wild-type pneumococci, which indicates that PLY has a major contribution to the pathogenesis of pneumococcal disease (5–7).

Others have demonstrated that purified PLY elicits a variety of proinflammatory responses in neutrophils and mononuclear phagocytes, including the release of proinflammatory cytokines, the activation-dependent up-regulation of β_2 integrins, as well as increased Ca^{2+} uptake (8–11). Recently, it has been observed that PLY induces activation of mononuclear phagocytes by signaling via TLR4 (12). This dual function of PLY as a cytolysin to promote pathogen invasion and as a stimulus for modulation of innate host defense mechanisms, has provoked the speculation that proinflammatory mediators released by PLY-activated mononuclear phagocytes may contribute to alveolar-endothelial injury (5, 13). If so, resident alveolar macrophages, as the primary innate host defense barrier of the lung, would be a likely target cell involved in the lung inflammatory response to PLY challenge. Moreover, recruitment of neutrophils into the alveolar compartment in response to PLY challenge might enhance the extent of organ injury provoked by this bacterial agent. Little is known about the proinflammatory interactions of the purified toxin with the innate host defense system in vivo, including the interaction of PLY with both resident and alveolar recruited leukocytes.

We show that purified PLY, but not its noncytolytic derivative PdB, provoked severe vascular leakage and lung edema when administered intratracheally in intact mice. In parallel, profound effects of PLY on lung leukocyte populations were noted, including alveolar neutrophil recruitment and depletion of the alveolar macrophage pool followed by its repopulation by recruited monocytes to the alveolar space. However, none of the observed leukocyte responses contributed to the vascular leakage that developed in PLY-challenged mice. We conclude that the acute lung injury observed in PLY-challenged mouse lungs is due to a direct cytotoxic effect of PLY on cells of the alveolar-endothelial barrier. Interventions that depleted resident alveolar macrophages or prevented recruitment

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[3] Abbreviations used in this paper: PLY, pneumolysin; BAL, bronchoalveolar lavage; HU, hemolytic unit.

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of peripheral blood leukocytes had no effect on the permeability changes seen in PLY-treated mice.

Materials and Methods

Animals

Female BALB/c mice (weight, 18–21 g) were purchased from Charles River Laboratories (Sulzfeld, Germany).

Reagents

Native PLY and its noncytotoxic derivative PdB (PLY with a Trp^{433}-Phe mutation) were purified from recombinant *Escherichia coli* as previously described (14). Proteins were >95% pure, as judged by SDS-PAGE and Coomassie blue staining, with specific activities of $\sim 8.25 \times 10^5$ hemolytic units (HU)/mg of protein for PLY and $< 5 \times 10^3$ HU/mg for PdB. The preparation of function-blocking hamster anti-mouse CD18 mAb (clone 2E6; isotype IgG2a) was recently described (15). Rat anti-mouse CD49d (VLA-4; clone PS/2; isotype IgG2b) was purchased from Serotec (Munich, Germany). Rat monoclonal anti-murine Gr-1 (clone RB6-8C5; rat isotype IgG2b) was purchased from BD Biosciences (Heidelberg, Germany). Abs used in this study were recently evaluated in experimental inflammatory mouse models (15–17). Ab as well as PLY and PdB preparations were ensured to be free of endotoxin as determined by *Limulus* amebocyte lysate assays (detection limit, <10 pg/ml; Coatest; Chromogenix, Mölndal, Sweden). For inhibition experiments, mice received function-blocking Abs (anti-CD18 or anti-CD49d or combinations of anti-CD18 plus anti-CD49d) at 50 μg each per mouse via lateral tail vein injections 15 min before intratracheal PLY applications. Neutropenia was induced in mice by i.p. injection of anti-Gr-1 Ab 24 h before and subsequent to PLY applications, as recently described in detail (17). For the 48- and 72-h time points, blocking Ab applications were given concomitantly with and at 24-h intervals subsequent to PLY instillations.

Preparation of liposome-encapsulated dichloromethylene-diphosphonate

Liposomal encapsulation of clodronate was done as previously reported (18, 19). Briefly, 8 mg of cholesterol was added to 86 mg of egg phosphatidylcholine, and the chloroform phase was evaporated under helium until a white film remained. Further removal of the chloroform phase was performed under low vacuum in a Savant (Holbrook, NY) SpeedVac concentrator. The clodronate solution was made by dissolving 1.2 g of dichloromethylene diphosphonic acid in 5 ml of sterile PBS. Five milliliters of the clodronate solution was added to the liposomes and mixed thoroughly. Empty liposomes were made by the addition of sterile PBS alone. This solution was sonicated and ultracentrifuged at $10{,}000 \times g$ for 1 h at 4°C. The liposomal pellets were then removed and resuspended in PBS, followed by ultracentrifugation at $10{,}000 \times g$ for 1 h at 4°C. Subsequently, liposomes were resuspended in 5 ml of sterile PBS, stored at 4°C, and used within 48 h. The final concentration of the liposomal clodronate suspension was 5 mg/ml.

Treatment of animals

Intratracheal application of PLY or its noncytotoxic derivative PdB was done following recently described protocols (15, 20). Briefly, mice were anesthetized with tetrazoline hydrochloride and ketamine, and tracheae were exposed by surgical resection. Intratracheal instillations of PLY or PdB were performed under stereomicroscopic control (MS 5; Leica, Wetzlar, Germany) using a 29-gauge Abbocath (Abbott, Wiesbaden, Germany), which was inserted into tracheae. After instillations, the neck wound was closed with sterile sutures. For depletion of resident alveolar macrophages, a single dose of liposomal clodronate (100 μl) was administered to mice by intratracheal instillation. Control mice received empty (PBS-containing) liposomes, as recently described (18, 20). Twenty-four hours after the pretreatment of mice with liposomal clodronate to deplete the resident alveolar macrophage pool (>86%) (18, 20), mice received intratracheal applications of native PLY for various time points.

Collection of serum and bronchoalveolar lavage (BAL)

Mice were sacrificed with an overdose of isoflurane (Forene; Abbott, Wiesbaden, Germany). Collection of serum and BAL for the isolation of resident alveolar macrophages and alveolar recruited leukocytes from PLY- or PdB-treated animals was performed as recently described in detail (18, 20). Quantification of leukocyte subpopulations contained in BAL fluid was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations, using overall morphologic criteria, including differences in cell size and shape of nuclei and subsequent multiplication of those values by the respective absolute BAL cell counts, as recently described (18).

In vivo lung permeability assay

For evaluation of PLY-induced lung permeability, mice received an i.v. injection of FITC-labeled human albumin (1 mg/mouse in 100 μl of PBS; Sigma-Aldrich, Deisenhofen, Germany) 1 h before death, as recently described (15, 18). Undiluted BAL fluid samples and serum samples (diluted 1/10 and 1/100 in PBS; pH 7.4) were placed in a 96-well microtiter plate, and fluorescence intensities were measured using a fluorescence spectrometer (FL 880 microplate fluorescence reader; Bio-Tek, Winooski, VT) operating at 488-nm absorbance and 525 ± 20-nm emission wavelengths, respectively. The lung permeability index is defined as the ratio of fluorescence signals of undiluted BAL fluid samples to fluorescence signals of 1/10-diluted serum samples (15, 18).

Statistics

All data are given as mean ± SD. Differences between controls and treatment groups were calculated by one-factor ANOVA with posthoc tests by Dunnett. Pairwise contrast (PLY vs PdB timewise) was performed by confidence intervals provided by the two-way ANOVA analysis. Statistical significances between various treatment groups were calculated with SPSS for Windows software program (SPSS, Chicago, IL) and assumed to be statistically significant when p values were <0.05.

Results

Effect of native PLY vs PdB on lung permeability in intact mice

PLY is known to exert strong cytotoxic effects on isolated pulmonary epithelial and endothelial cells, leading to a loss of lung barrier function (13), which per se has a major impact on pulmonary gas exchange in inflamed lungs. Initial dose-response experiments using various PLY concentrations (10–50 ng of PLY/mouse, corresponding to ~8.3–41.5 HU/mouse) revealed mortality rates of >20% when PLY was applied at >40 ng of PLY/mouse (data not shown). Therefore, in the current study, mice received either PLY or PdB applications at doses of 40 ng/mouse (corresponding to ~33.2 HU/mouse), where mortality was only occasionally observed.

Application of PLY but not PdB into the lungs of intact mice increased lung permeability with maximum values observed at 6 h posttreatment; permeability remained elevated 48 h after PLY treatment (Fig. 1).

Effect of PLY vs PdB on alveolar neutrophil recruitment and the resident alveolar macrophage pool

Intratracheal application of PLY elicited a strong neutrophilic alveolitis, peaking 24 h posttreatment. Elevated polymorphonuclear neutrophil values were observed until 72 h posttreatment, with a decline toward baseline values thereafter. In contrast, in mice receiving the noncytotoxic derivative, PdB, a weak but significant neutrophilic alveolitis was observed only at 12 h posttreatment. These data suggest that the cytolysin activity of PLY elicited the profound lung inflammatory response (Fig. 2). In parallel, PLY but not PdB induced a decline in alveolar macrophage numbers with maximum alveolar macrophage depletion observed at 6–12 h posttreatment (Fig. 3*A*). A significant proportion of alveolar macrophages from PLY-treated mice stained positive with propidium iodide in vitro, demonstrating the pore-forming impact of the toxin on resident alveolar macrophages in vivo (data not shown). Interestingly, the observed drop in alveolar macrophage numbers was followed by a delayed recruitment of peripheral blood monocytes into the alveolar airspace, leading to an expansion of the alveolar macrophage pool at 72 h ($p < 0.05$) and 120 h posttreatment (Fig. 3*A*).

PLY-induced alveolar monocyte trafficking was found to be significantly inhibited by pretreatment of mice with the function-blocking anti-CCR2 Ab MC21 (Fig. 3*B*). This suggests that a

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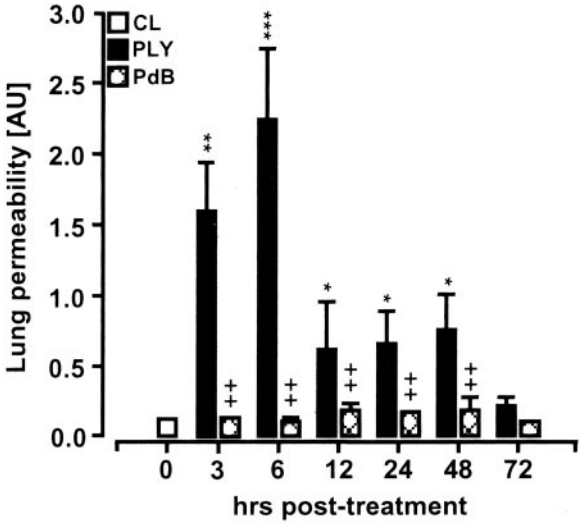

FIGURE 1. Effect of intratracheal PLY or PdB application on the lung permeability in intact mice. Mice were either left untreated (□; 0-h time point) or were treated with PLY (40 ng/mouse; ■) or PdB (40 ng/mouse; ⊠) for various time points. One hour before death, mice received an i.v. injection of FITC-labeled albumin (1 mg/mouse) and at the indicated time points, mice were subjected to BAL and collection of serum. ***, **, and *, $p < 0.001$, $p < 0.01$, and $p < 0.05$ vs control, respectively. ++, $p < 0.01$ vs PLY-alone-treated mice. The lung permeability values are shown as mean ± SD of at least five animals per time point and treatment group. AU, Arbitrary unit.

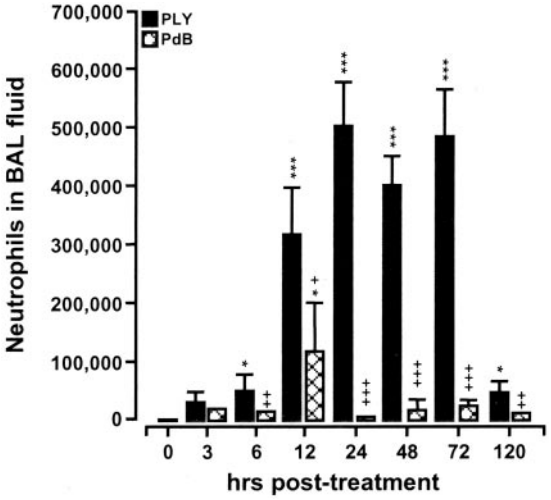

FIGURE 2. Effect of PLY vs PdB on alveolar neutrophil recruitment. Mice were either left untreated (0 h) or received intratracheal applications of PLY (40 ng/mouse; ■) or its noncytotoxic derivative, PdB (40 ng/mouse; ⊠). At the indicated time points, mice were subjected to BAL and determination of BAL fluid differentials as described in *Materials and Methods*. *** and *, $p < 0.001$ and $p < 0.05$ vs control, respectively. +++, ++, and +, Significantly reduced ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively) vs PLY-alone-challenged mice. The values are given as mean ± SD of five to seven experiments each per time point and stimulus.

CCL2-CCR2-dependent de novo recruitment of circulating monocytes into the lungs of mice was largely responsible for the macrophage repopulation of the alveolar compartment (Fig. 3*B*). At the same time, neutrophilic alveolitis developing in PLY-challenged mice was not affected by MC21 pretreatment (Fig. 3*C*), indicating that neutrophil recruitment is not dependent upon the recruitment of peripheral blood monocytes to the alveolar space. Notably, the drop in alveolar macrophage numbers was associated with an increase in BAL fluid TNF-α and MIP-2 concentrations (TNF-α, 0 pg/ml (control), 6 ± 8 pg/ml (6 h), 20 ± 40 pg/ml (12 h), 1 ± 2 pg/ml (24 h); MIP-2, 45 ± 37 pg/ml (control), 146 ± 84 pg/ml (6 h), 69 ± 42 pg/ml (12 h), 12 ± 3 pg/ml (24 h); $n = 5$).

Effect of anti-CD18 or anti-CD49d Ab treatment or transient neutropenia on PLY-induced neutrophilic alveolitis and lung permeability

Neutrophil trafficking to the lung is mediated by CD18-dependent and -independent recruitment pathways that depend upon the stimulus applied (16, 22). Pneumococcal infection elicits primarily CD18-independent neutrophil trafficking toward the lung, with the β_1 integrin CD49d possibly acting as alternatively engaged adhesion molecule (23, 24). To evaluate the role of newly recruited neutrophils and monocytes in PLY-induced alveolar-endothelial injury, mice were pretreated with either function blocking anti-CD18 Abs or combinations of function blocking anti-CD18 plus anti-CD49d Abs or anti-Gr-1 Abs to deplete circulating neutrophils. Fig. 4*A* shows that PLY-induced neutrophil trafficking to the lung was strongly and significantly inhibited upon pretreatment of mice with anti-CD18 Abs. This inhibitory effect was further increased when anti-CD18 and anti-CD49d Ab applications were combined, with the strongest inhibitory effects resulting in >85% reduction of alveolar polymorphonuclear neutrophil numbers at 12–72 h post-PLY challenge. Similarly, induction of transient neutropenia significantly abrogated alveolar neutrophil recruitment in response to PLY, with a ~90% reduction observed at 12–72 h post-PLY challenge (Fig. 4*A*). Importantly, both adhesion-blocking strategies or induction of transient neutropenia to inhibit alveolar leukocyte trafficking in response to PLY application failed to significantly attenuate PLY-induced lung permeability, suggesting that alveolar recruited neutrophils did not contribute to increased lung permeability observed in PLY-challenged mouse lungs (Fig. 4*B*).

Effect of liposomal clodronate-mediated resident alveolar macrophage depletion on PLY-induced acute lung injury

We recently demonstrated that depletion of alveolar macrophages by liposomal clodronate significantly reduced acute lung injury and neutrophilic alveolitis in a mouse model of endotoxin-induced acute lung inflammation (18). In contrast, in the present study, we observed that liposomal clodronate-induced depletion of alveolar macrophages did not attenuate PLY-induced alveolar endothelial injury or alveolar neutrophil accumulation within the lungs of intact mice (Fig. 5), suggesting that resident alveolar macrophages do not contribute to increased lung permeability and are not involved in alveolar neutrophil trafficking in PLY-challenged mouse lungs.

Discussion

In the present study, we have shown that instillation of native PLY into the lungs of mice drastically increased lung permeability and induced a strong alveolar neutrophil recruitment. Moreover, we found that PLY depleted the alveolar macrophage pool, and this was followed by a delayed CCR2-dependent recruitment of peripheral blood monocytes. Strategies to inhibit alveolar leukocyte accumulation using adhesion-blocking Abs failed to attenuate PLY-induced lung injury. Experimental depletion of resident alveolar macrophages by liposomal clodronate did not reduce PLY-induced lung permeability increase and neutrophil influx. These data support the view that direct injurious effects of PLY on target cells of the alveolar compartment, rather than secondary leukocyte

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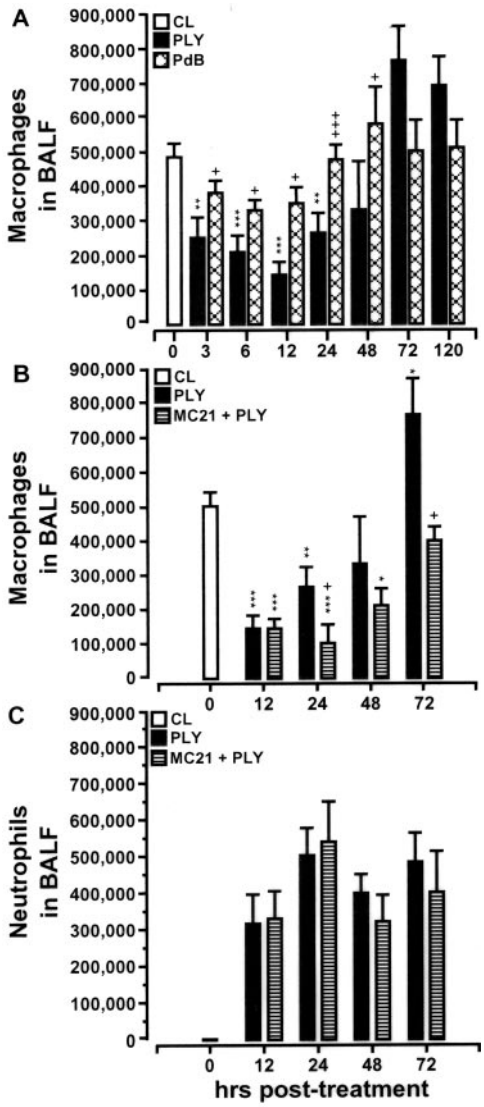

FIGURE 3. Effect of PLY on the resident alveolar macrophage pool in intact mice. Mice were either left untreated (CL; □) or were treated with PLY (40 ng/mouse; ■ in *A–C*) or its noncytotoxic derivative PdB (40 ng/mouse; ☒ in *A*) or were pretreated with the anti-CCR2 Ab MC21 before PLY application (▤ in *B* and *C*). At the indicated time points, mice were sacrificed and total numbers of alveolar macrophages/monocytes (*A* and *B*) or total numbers of alveolar recruited neutrophils (*C*) were determined. ***, **, and *, in *A* indicates $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, vs control. +++ and +, $p < 0.001$ and $p < 0.05$, respectively, vs PLY-treated mice. ***, **, and * in *B*, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, vs control macrophage numbers. + in *B*, Significantly reduced macrophage numbers ($p < 0.05$) vs PLY-alone-challenged mice. Values are given as mean ± SD of five experiments.

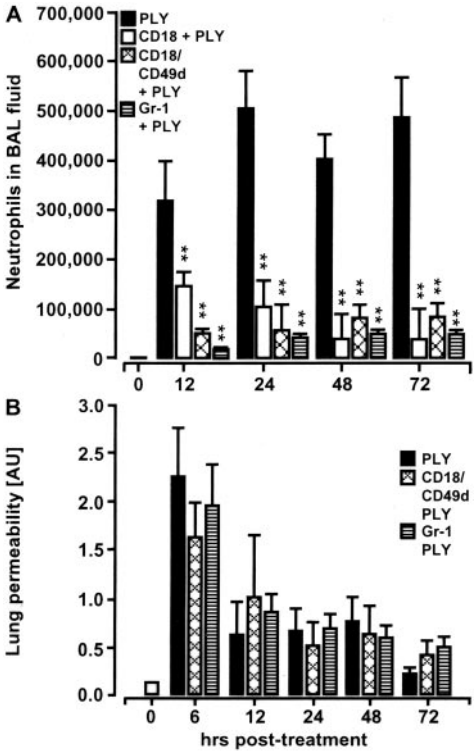

FIGURE 4. Effect of adhesion-blocking Abs or transient neutropenia on PLY-induced neutrophilic alveolitis and lung permeability increase in intact mice. *A*, Mice were either left untreated (0-h time point) or treated with PLY in the absence of function-blocking Abs (40 ng/mouse; ■) or received i.v. injections of function-blocking anti-CD18 Abs (□) or anti-CD18 plus anti-CD49d Abs (☒) or were pretreated with anti-Gr-1 to induce neutropenia (▤) followed by intratracheal application of PLY (40 ng/mouse). Mice were sacrificed at the indicated time points and subjected to BAL for determination of BAL fluid leukocyte differentials. *B*, Mice were either treated with PLY alone (■ in *B*) or were pretreated with anti-CD18 and anti-CD49d Abs (☒ in *B*) or were made transiently neutropenic (▤ in *B*) followed by PLY application. One hour before sacrifice, mice then received FITC-labeled albumin i.v. to determine lung vascular leakage. **, $p < 0.01$ vs PLY-alone treatment. Values are given as mean ± SD of five experiments. AU, Arbitrary unit.

recruitment, underlie vascular leakage and pulmonary edema formation in mice lungs attacked by this bacterial toxin.

PLY is one of the most important cytotoxic virulence factors released by virtually all clinical isolates of the pneumococcus and has been reported to mimic major histopathologic findings observed in pneumococcal pneumonia (3, 5). Direct cytotoxic effects on alveolar epithelial cells, separation of tight junctions between epithelial cells, and neutrophil accumulation are among the key inflammatory events observed in PLY-treated or pneumococcus-infected mice (5, 13, 25, 26). In addition, in vitro studies suggested that PLY stimulates the release of potent proinflammatory agonists such as TNF-α, IL-1β, and IL-8 by mononuclear phagocytes and neutrophils, which may amplify the lung inflammatory response to PLY challenge (8, 10, 11). A recent study supports this assumption by showing that PLY, beside its cytotoxic and complement-depleting activities, also appears to signal via the pattern recognition receptor TLR4 (12). In the present study, we addressed the question whether resident alveolar macrophages and/or newly recruited leukocytes contribute to vascular leakage and edema formation developing in response to PLY application in mice. We found a strong increase in lung vascular permeability in PLY-challenged mouse lungs, peaking as early as 6 h posttreatment. This vascular leakage preceded increases in the alveolar neutrophil and macrophage population that peaked at later time points (12–72 h). The finding that purified PLY diminished the resident alveolar macrophage pool with subsequent provocation of a delayed CCR2-dependent monocyte recruitment and re-expansion of the alveolar macrophage pool size is novel and demonstrates that inflammatory monocyte trafficking toward PLY-challenged mouse lungs depends virtually exclusively on the CCR2-CCL2 axis.

Neutrophilic alveolitis occurs in PLY-induced mouse lung inflammation (25), although a detailed time-response study of lung inflammatory variables developing in response to purified PLY application in intact mice has not yet been reported. Rijneveld et

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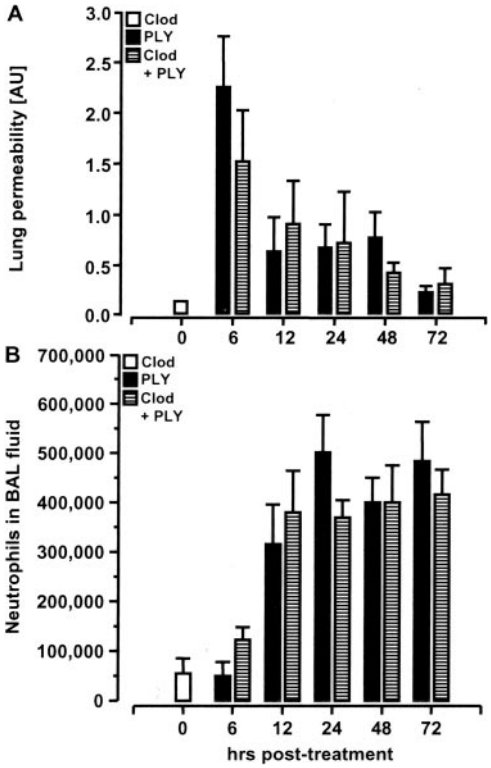

FIGURE 5. Effect of liposomal clodronate-induced alveolar macrophage depletion on PLY-induced lung permeability increase and neutrophilic alveolitis in intact mice. Mice were pretreated with liposomal clodronate alone for 24 h to deplete alveolar macrophages (□ at 0-h time point in *A* and *B*) or received intratracheal applications of PLY alone to be assessed after various time points (6–72 h) (40 ng/mouse; ■), or received liposomal clodronate for 24 h followed by intratracheal PLY challenge (6–72 h) (40 ng/mouse; ▤). One hour before sacrifice, PLY-alone-treated or clodronate-plus-PLY-treated mice received an i.v. injection of FITC-labeled albumin for the determination of lung permeability. At indicated time points, mice were subjected to BAL and collection of serum for analysis of lung permeability (*A*) or alveolar neutrophil accumulation (*B*). Values are given as mean ± SD of five experiments. Clod, Liposomal clodronate.

al. (25) suggested a possible contribution of the neutrophil chemoattractant MIP-2 to alveolar neutrophil recruitment in PLY-challenged mice, although the cellular source of the MIP-2 was not determined. Because both virulent pneumococci and purified PLY have been shown to increase cell surface expression of β_2 integrins on neutrophils, thereby promoting neutrophil adhesion to alveolar epithelial cells (8, 27, 28), we questioned whether adhesion-blocking strategies prevent alveolar neutrophil accumulation in PLY-challenged mice. Both adhesion-blocking anti-CD18 and anti-CD49d Abs presently used were recently shown to be highly effective in blocking both alveolar neutrophil and monocyte trafficking in a mouse model of LPS-induced lung inflammation and in transmigration assays in vitro (16, 21). We observed strong neutrophil blocking effects of anti-CD18 Abs in PLY-challenged lungs, with further efficacy upon anti-CD49d coapplication, supporting the role of β_1 and β_2 integrin pathways in PLY-induced alveolar leukocyte trafficking. Both combined Ab application to block alveolar neutrophil recruitment and induction of transient neutropenia to reduce the lung neutrophil burden did not significantly reduce the lung permeability increase in PLY-challenged mouse lungs, suggesting that neutrophil trafficking does not play a major role in the lung vascular leakage and alveolar edema upon PLY attack. This finding is opposite to recent observations of our group in a mouse model of LPS-induced acute lung inflammation, where blockade of alveolar neutrophil trafficking significantly reduced the vascular leakage (15, 18, 29). Thus, in contrast to other lung inflammatory mouse models, where neutrophils appear to contribute to lung injury, our data support the concept that PLY-induced lung injury is developing largely independently from recruited neutrophils.

Similarly, experimental depletion of alveolar macrophages by administration of liposomal clodronate before PLY application did not reduce the lung permeability increase or the concomitantly developing neutrophilic alveolitis under PLY attack. These findings suggest a negligible role for alveolar macrophages in both induction of vascular leakage and attraction of neutrophils to the alveolar space after challenge with PLY. This finding is again in striking contrast to the central role of alveolar macrophages in endotoxin-driven pulmonary inflammation (18, 19).

In conclusion, PLY challenge of intact mouse lungs provoked a strong vascular leakage, accompanied by enhanced neutrophil and monocyte recruitment into the alveolar compartment. However, maneuvers to deplete lung leukocyte populations did not inhibit the vascular leakage observed upon bacterial toxin application, suggesting that direct cytotoxic effects of PLY on alveolar target cells may represent the predominant underlying mechanism.

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