

# Essential Role for Neutrophils but not Alveolar Macrophages at Early Time Points following *Aspergillus fumigatus* Infection

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Alveolar macrophages and neutrophils mediate innate immune defense against the opportunistic fungal pathogen *Aspergillus fumigatus* and are believed to be essential for host survival following inhalation of fungal spores (conidia). Although alveolar macrophages are postulated to kill inhaled conidia and neutrophils are believed to act against hyphae, the relative contribution of alveolar macrophages and neutrophils to early defense against *A. fumigatus* remain incompletely defined. To more precisely characterize the contributions of alveolar macrophages and neutrophils in antifungal host defense, we selectively depleted each cell population at different times following pulmonary challenge with conidia. Mice depleted of alveolar macrophages prior to pulmonary *A. fumigatus* infection recruited neutrophils normally and restricted hyphal tissue invasion. In contrast, neutrophil depletion prior to or within 3 h after infection was associated with high mortality. Neutrophil depletion at later time points, however, was associated with nearly normal survival rates. Our studies suggest that neutrophils, but not alveolar macrophages, provide essential anticonidial defense and that a brief period of influx into the respiratory tree is sufficient to prevent conidial germination and invasive disease.

Invasive aspergillosis is a major cause of infectious morbidity and mortality in immunocompromised patients [1–4]. Development of invasive aspergillosis signifies host failure to kill inhaled spores (conidia) and to prevent hyphal tissue invasion. Although loss of or deficiency in myeloid function is common among high-risk persons, the precise in vivo role of and temporal requirements for specific myeloid cells in host defense against *Aspergillus fumigatus*, the most common cause of invasive aspergillosis, remain controversial. The tim-

ing between conidial exposure and myeloid dysfunction or injury remains poorly understood but likely influences the outcome of infection.

Although neutropenia is an important risk factor for invasive aspergillosis [5, 6], animal and in vitro studies support a key role for alveolar macrophages in the early stages of host defense [7]. This tenet is based in part on the finding that corticosteroids impair macrophage killing of conidia [8, 9] and enable hyphal escape from intracellular compartments [10, 11]. Corticosteroid-treated mice are more susceptible to resting than to swollen, germinating conidia [7], findings that were interpreted to signify that macrophages form a primary defense against inhaled conidia. Conversely, induction of neutropenia with nitrogen mustard renders mice more susceptible to germinating than to resting conidia, supporting the notion that neutrophils act primarily against products of germination [7]. In addition, neutrophil contact with hyphae induces degranulation and the respiratory burst [12].

Animal models of invasive aspergillosis traditionally

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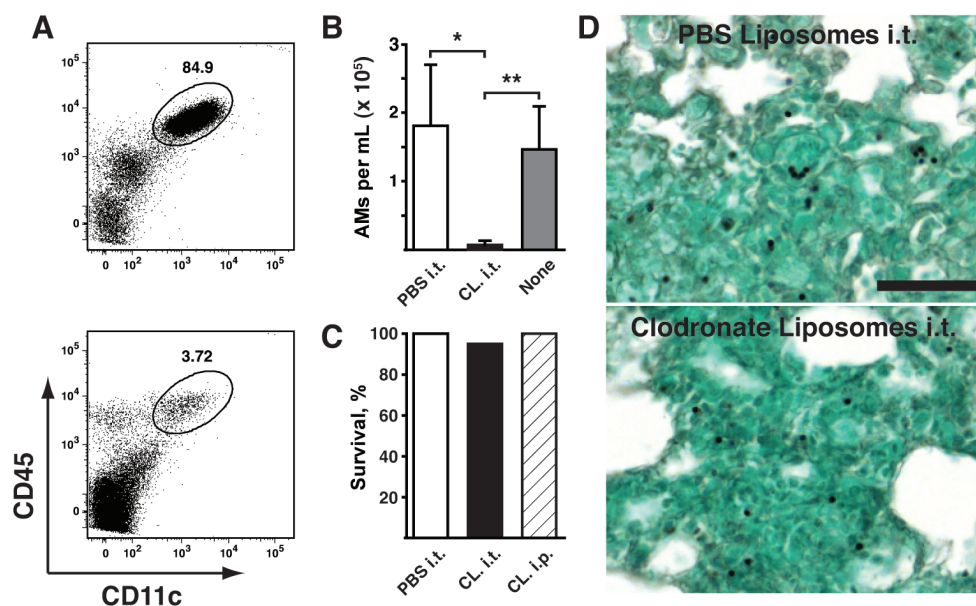
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**Figure 1.** Alveolar macrophage (AM) depletion and murine susceptibility to *Aspergillus fumigatus*. *A*, Flow cytometric analysis of bronchoalveolar lavage fluid collected after intratracheal (i.t.) administration of phosphate-buffered saline (PBS) (*top panel*) or clodronate (CL) liposomes (*bottom panel*) 4 and 3 days earlier. Ungated bronchoalveolar lavage samples were stained for CD45 and CD11c; gates representing AMs (CD45<sup>hi</sup>, CD11c<sup>hi</sup>) are illustrated, and the event frequencies are indicated. *B*, The bar graphs show the bronchoalveolar lavage fluid AM concentration ( $\pm$  standard deviation) in mice (5–8 per group) that received PBS (*white bar*), CL (*black bar*), or no (*gray bar*) liposomes. Bronchoalveolar lavage fluid samples had red blood cells lysed, were adjusted to a 0.3-mL volume, were enumerated, and were analyzed by flow cytometry as in (*A*). One of 2 representative experiments is shown. \* $P = .0036$  and \*\* $P = .010$ , by Mann-Whitney log-rank sum test and Bonferroni correction. *C*, 21-Day survival of mice that received PBS (*white bar*,  $n = 10$ ) or CL liposomes via the i.t. (*black bar*,  $n = 20$ ) or intraperitoneal (i.p.) (*hatched bar*,  $n = 10$ ) route prior to i.t. infection with  $2.5 \times 10^7$  to  $4 \times 10^7$  *A. fumigatus* conidia. The data are pooled from multiple experiments. No significant difference was found between experimental groups by Fisher's exact test. *D*, Representative methenamine silver-stained lung sections from mice treated with i.t. PBS (*top panel*) or CL (*bottom panel*) liposomes on day 3 after infection. Two experiments with 3 mice per group were performed. The scale bar represents 50  $\mu$ m.

rely on the administration of cytotoxic agents and corticosteroids. Whereas this strategy is successful in recapitulating many aspects of human disease, extrapolating the role of individual cell types in host defense is difficult because these regimens exert pharmacologic effects on multiple cell types [13]. For example, corticosteroids impair not only macrophage [10] but also neutrophil function by inducing defects in trafficking, adherence, and the release of antimicrobial effector molecules [14].

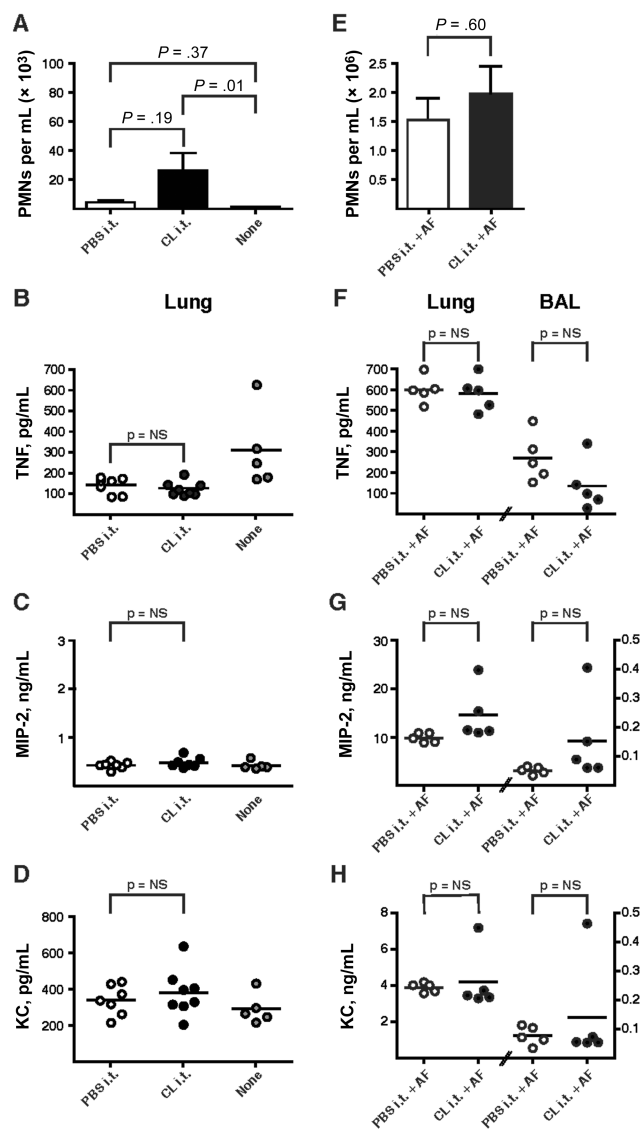
Recent approaches to examine the role of innate immune cells or effector systems against *A. fumigatus* rely on knockout mice [15–17] and antibodies that deplete leukocyte subsets [18, 19] or interfere with their trafficking [18, 20]. Mice deficient in the chemokine receptor CXCR2 or that are defective in CXCR2 signaling develop invasive aspergillosis [17, 18]. Delayed airway neutrophil influx in CXCR2<sup>−/−</sup> mice leads to conidial germination, a result that supports an early role for neutrophils in host defense [17]. Conidial exposure induces alveolar macrophages to transcribe CXCR2 ligands (ie, macrophage inflammatory protein-2 [murine chemokine CXCL2; MIP-2] and keratinocyte-derived chemokine [murine chemokine CXCL1; KC]) in vivo and secrete these chemokines in

vitro, consistent with a key role for alveolar macrophages in the orchestration of neutrophil recruitment [21–23].

In this study, we examined the temporal requirements for alveolar macrophages and neutrophils following murine pulmonary *A. fumigatus* infection with use of approaches that target these cells with specificity and temporal control. Our results suggest that alveolar macrophages are dispensable for survival. Alveolar macrophage-depleted mice were capable of limiting hyphal tissue invasion, likely because of preserved lung inflammatory responses and neutrophil airway recruitment. In contrast, mice developed invasive aspergillosis if neutrophil depletion occurred prior to or within  $\sim 3$  h after infection. Neutrophil depletion at later time points was not associated with mortality, even when conidial inocula were standardized for different experimental conditions. These results support an

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

**Figure 2.** Effect of clodronate liposome administration on lung dendritic cell, macrophage, monocyte, and neutrophil populations.



**Figure 3.** Alveolar macrophage depletion and inflammatory responses in naive or infected mice. *A–D*, bronchoalveolar lavage (BAL) sample neutrophil concentration (*A*) and lung tumor necrosis factor (TNF) (*B*), macrophage inflammatory protein–2 (MIP-2) (*C*), and keratinocyte-derived chemokine (KC) (*D*) levels in mice after intratracheal (i.t.) administration of phosphate-buffered saline (PBS) (white), clodronate (CL) (black), or no liposomes (gray) 4 and 3 days earlier. *A*, BAL fluid samples were enumerated and analyzed by flow cytometry to compute neutrophil concentrations. *B–D*, Lung homogenates were analyzed for cytokine and chemokine levels by enzyme-linked immunosorbent assay. *E–H*, BAL neutrophil concentration (*E*) and BAL and lung TNF (*F*), MIP-2 (*G*), and KC (*H*) levels were measured in mice that received PBS (white) or CL (black) liposomes 4 and 3 days prior to infection with  $2.6 \times 10^7$  *Aspergillus fumigatus* conidia. The BAL fluid and lung tissues were collected for analysis 24 h after infection. *A–H*, 5–8 mice per group. One of 3 independent experiments is shown. *P* values are shown for 2-group comparisons by Mann-Whitney rank-sum test corrected for multiple comparisons by the Bonferroni method. NS, not significant.

early, nonredundant role for neutrophils, but not alveolar macrophages, in conidial defense.

## MATERIALS AND METHODS

**Reagents.** Dichloromethylenebisphosphonate (clodronate) was a gift of Roche Diagnostics GmbH and was incorporated into liposomes as described elsewhere [24]. Mice received 100  $\mu$ L phosphate-buffered saline (PBS) or clodronate liposomes delivered intratracheally. The monoclonal antibody RB6-8C5 (rat IgG<sub>2b</sub>) was purified by protein G chromatography and quantified by Bradford assay with use of bovine serum albumin as a standard [25]. For depletion studies, mice received 0.1 mg of RB6-8C5 in PBS via intraperitoneal injection.

**Animal care and *Aspergillus* infection.** C57BL/6 mice were purchased from The Jackson Laboratory. All in vivo studies were approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. For survival studies, mice were maintained on Sulfatrim (Research Diets). *A. fumigatus* strain AF293 conidial suspensions were administered to anesthetized mice via the intratracheal route as described elsewhere [21].

**Organ harvest, enzyme-linked immunosorbent assay (ELISA), and flow cytometry.** Following euthanasia, 3 mL of bronchoalveolar lavage (BAL) fluid was collected in PBS and 5% fetal calf serum. The lungs were perfused with PBS and homogenized in 4 mL of PBS containing 0.025% Tween 20, for colony forming unit (CFU) determination. For ELISA studies, the BAL fluid and lung homogenates were clarified by centrifugation and analyzed for tumor necrosis factor (TNF), interferon (IFN)- $\gamma$  (BD Biosciences), MIP-2, and KC (R&D Systems) according to the manufacturers' instructions.

For flow cytometric analysis, finely minced lung tissue was digested at 37°C in PBS and 5% fetal calf serum containing 3 mg/mL collagenase IV (Worthington Biochemical Corporation) and 20 U/mL DNase I (Roche) for 60 min, was ejected 5 times through a 20-gauge syringe, had red blood cells lysed, and was passed through a 100- $\mu$ m filter.

BAL and lung cells were enumerated using a Z2 Coulter Counter (7–15  $\mu$ m window; Beckman Coulter) and were stained with the following antibodies: anti-Ly6C-FITC (clone AL-21), anti-Ly6G-PE (clone A18), anti-CD11b-PERCP-Cy5.5, anti-CD11c-APC, anti-CD45-APC-Cy7 (BD Biosciences), anti-7/4-Alexa 647, anti-F4/80-FITC, and mouse anti-rat IgG2b-FITC (AbD Serotec). Alveolar macrophages were identified as SSC<sup>high</sup>CD45<sup>+</sup>CD11c<sup>+</sup> BAL cells, and neutrophils were identified as CD45<sup>+</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> cells. In specific experiments, 7/4 and Ly6G expression was used to define neutrophils. Flow cytometric data was collected on a BD LSR II flow cytometer and was analyzed with FlowJo, version 8.7.1 (Treestar).

**Histology.** Mice were euthanized either 3 days after infection or following the final manipulation (ie, RB6-8C5 admin-

**Figure 4.** Effect of clodronate liposome administration on lung and broncheolar lavage fluid interferon- $\gamma$  levels.

istration). Perfused lungs were fixed in 10% formalin, embedded in paraffin, cut in 8- $\mu$ m slices, stained with Gomori methenamine silver, and analyzed by microscopy.

**Statistics.** Statistical analyses were performed using Stata, version 10.0 (StataCorp). For 2-group comparisons, the Mann-Whitney *U* test was used. For 3-group comparisons, the Kruskal-Wallis 1-way analysis of variance was calculated and, in cases of statistical significance, pairwise comparisons were analyzed using the Mann-Whitney test. For multiple comparisons, the *P* values were corrected by the Bonferroni method.

Survival data were plotted by the Kaplan-Meier method and analyzed using log-rank testing. Fisher's exact method was used to analyze differences in 21-day survival among experimental groups. Statistical significance was defined by a *P* value of  $<.05$ .

## RESULTS

**Alveolar macrophage depletion and susceptibility to *A. fumigatus*.** To examine the role of alveolar macrophages in defense against *A. fumigatus* infection, C57BL/6 mice underwent alveolar macrophage depletion via intratracheal instillation of clodronate liposomes [24, 26]. Alveolar macrophage depletion was assessed by flow cytometric analysis (figure 1A) and enumeration of BAL cells (figure 1B). Consistent with prior studies [26, 27], intratracheal administration of clodronate liposomes on consecutive days led to  $>90\%$  alveolar macrophage depletion 3 days later (figure 1B). Depletion of pulmonary-resident CD103<sup>+</sup> dendritic cells [28], CD11b<sup>+</sup> dendritic cells, or CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>−</sup> monocytes was not observed (figure 2). Alveolar macrophage depletion was not observed in mice treated with PBS liposomes (figure 1B) or in mice treated intraperitoneally with clodronate liposomes (data not shown), a route of administration that targets splenic macrophage populations [24].

Alveolar macrophage depletion did not result in murine susceptibility to intratracheal challenge with  $2.5 \times 10^7$  to  $4 \times 10^7$  conidia. The 21-day survival rate approached 100% in 3 experiments pooled for analysis (figure 1C). Similar results were observed in mice treated intraperitoneally with clodronate or intratracheally with PBS liposomes. Histologic analysis of Gomori methenamine silver-stained lung sections showed that alveolar macrophage depletion did not interfere with host ability to limit conidial germination (figure 1D). Abundant conidia were visualized within the parenchyma, surrounded by neutrophil-rich infiltrates. Hyphae invading the lung parenchyma

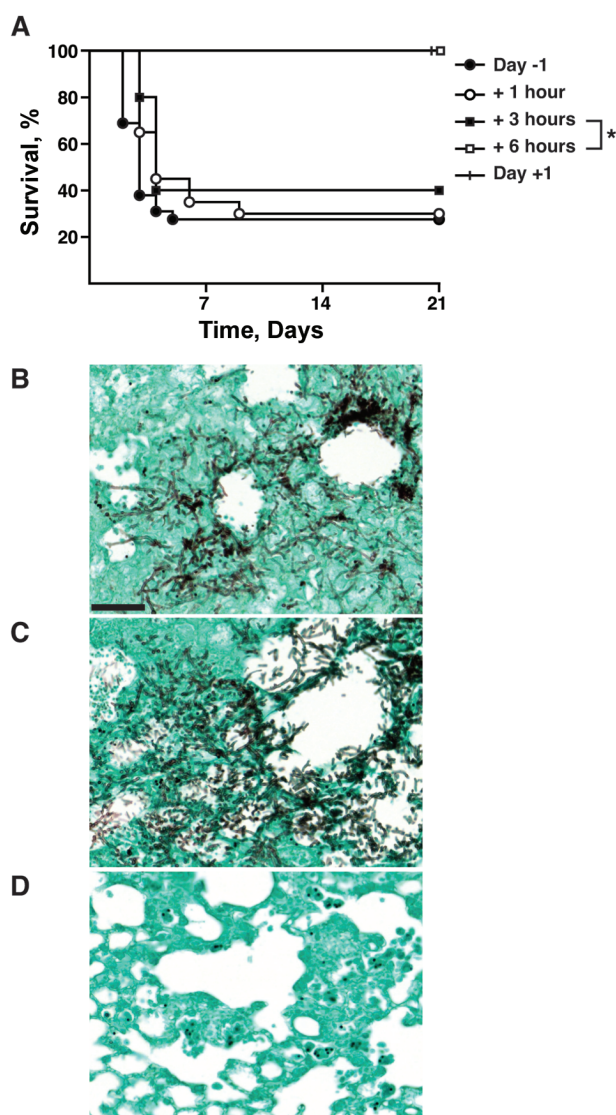
were not observed (figure 1D), though in rare instances, individual hyphae within large airways were noted (data not shown). Thus, C57BL/6 mice control conidial germination and survive *A. fumigatus* challenge in the absence of alveolar macrophages.

**Effect of alveolar macrophage depletion on inflammatory responses.** Because the alveolar macrophage depletion strategy per se may induce inflammation, BAL and lung neutrophil counts and inflammatory cytokine and chemokine levels were examined in mice treated with clodronate, PBS, or no liposomes. Neutrophil counts ( $\pm$  standard deviation) in BAL fluid samples were marginally higher in mice treated with clodronate liposomes ( $26.6 \times 10^3 \pm 34.0 \times 10^3$  neutrophils/mL), compared with mice treated with PBS liposomes ( $4.6 \times 10^3 \pm 3.8 \times 10^3$  neutrophils/mL) or no liposomes ( $1.3 \times 10^3 \pm 0.5 \times 10^3$  neutrophils/mL) (figure 3A), although statistical significance was reached only when comparing the clodronate and no liposome groups ( $P = .01$ , by Mann-Whitney test). The neutrophil airway influx associated with clodronate liposome treatment only constituted  $\sim 1\%$  of the influx associated with *A. fumigatus* infection (figure 3E).

TNF, MIP-2, KC, and IFN- $\gamma$  were chosen for analysis because of their role in *A. fumigatus* host defense [18, 29–31]. Intratracheal administration of clodronate or PBS liposomes did not trigger TNF, MIP-2, KC, or IFN- $\gamma$  release in the lungs of naive mice (figures 3B–3D 4). In all groups, TNF, MIP-2, and KC levels in the airway were below the limit of detection (15 pg/mL; data not shown). Thus, the alveolar macrophage depletion strategy is highly unlikely to exert proinflammatory effects that influence the experimental outcome.

**Alveolar macrophage depletion and preservation of inflammatory responses after infection.** Because alveolar macrophages secrete inflammatory mediators in response to *A. fumigatus* challenge [21–23, 32, 33], the effect of alveolar macrophage depletion on airway neutrophil recruitment and lung and BAL cytokine and chemokine levels was examined 24 h after infection, a time point that is associated with peak TNF levels [34]. Alveolar macrophage depletion did not reduce airway neutrophil recruitment (figure 3E). In addition, alveolar macrophage depletion was associated with preserved TNF, MIP-2, and KC release in the lungs and airways of infected animals (figure 3F–3H). Although lung MIP-2 levels were higher in infected mice treated with clodronate liposomes, this finding failed to achieve statistical significance after correction for multiple comparisons ( $P = .054$ ). These results suggest that alve-

**Figure 5.** RB6-8C5 administration and depletion of lung neutrophils and monocytes.



**Figure 6.** Relationship between the onset of neutrophil depletion, murine mortality, and hyphal tissue invasion. *A*, Survival of mice infected with  $2 \times 10^7$  to  $4 \times 10^7$  *Aspergillus fumigatus* conidia and given RB6-8C5 either 1 day prior to, or +1 h, +3 h, +6 h, or +1 day after infection. The graph includes pooled data from 2–4 experiments per group (10–29 mice per group). \* $P = .0045$  by log-rank test between the +3 h and +6 h groups. *B–D*, Fungal tissue invasion in mice that received RB6-8C5 on day 1 before infection (*B*) or +1 h (*C*) or 1 day (*D*) after infection with  $1.1 \times 10^7$  *A. fumigatus* conidia. Lungs were harvested 3 days after the last manipulation (ie, after infection [*B* and *C*] or after RB6-8C5 administration [*D*]). Gomori methenamine silver staining demonstrates hyphal lesions in (*B* and *C*) and the presence of conidia in (*D*). Representative examples are shown from 2 experiments with 3 mice per group. The scale bar represents 50  $\mu\text{m}$ .

olar macrophage depletion neither reduces lung or airway TNE, MIP-2, and KC production nor impairs neutrophil airway influx 24 h after infection.

In summary, these data indicate that alveolar macrophages are dispensable for control of *A. fumigatus* infection in otherwise

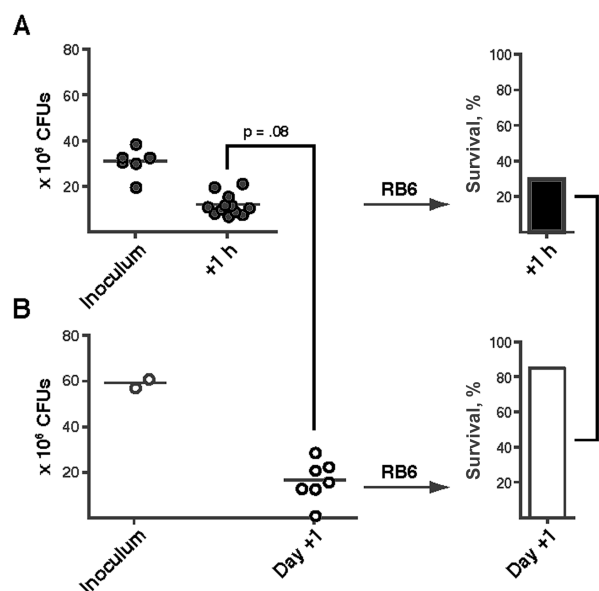
immunocompetent mice. Alveolar macrophage depletion is overcome because of preserved inflammatory responses and neutrophil airway influx, resulting in host ability to prevent the formation of tissue-invasive hyphae.

**Timing of neutrophil depletion and the outcome of *A. fumigatus* infection.** Because alveolar macrophages appear redundant for defense against *A. fumigatus* infection, the timing of neutrophil depletion on the development of invasive aspergillosis and survival was examined. Although invasive aspergillosis develops in mice that are neutropenic at the time of infection [7, 18, 19], the temporal requirement for neutrophils in host defense remains unknown. We observed that C57BL/6 mice reared in pathogen-free conditions and infected with  $1 \times 10^7$  to  $4 \times 10^7$  conidia had reduced CFUs in lung tissue by 90%–95% by 48 h after infection, consistent with results published elsewhere [7, 11]. However, we routinely cultured  $10^4$ – $10^5$  CFUs from lung samples at 30 days after infection (data not shown), suggesting that infected mice may remain vulnerable to neutrophil depletion for prolonged periods.

To determine the effect of neutrophil depletion at specific time points in relation to infection, mice were treated with a single 0.1 mg intraperitoneal dose of monoclonal antibody RB6-8C5 (anti-Gr-1; anti-Ly6G/Ly6C). This method of transient neutrophil depletion was chosen because it is well-characterized and specific compared with other methods (eg, administration of cyclophosphamide [19] or irradiation). RB6-8C5-induced neutropenia enables the establishment of invasive aspergillosis in the lungs of infected mice [18], and tissue damage is exacerbated by neutrophil recovery [19]. The dose used for experiments led to >99% neutrophil depletion in the circulation (data not shown) and in single cell lung suspensions but to no significant depletion in CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> monocytes (Gr-1<sup>+</sup> or inflammatory monocytes; figure 5). C57BL/6 mice administered RB6-8C5 24 h prior to infection were highly susceptible to intratracheal *A. fumigatus* challenge, with an observed mortality rate of 72% (figure 6A).

RB6-8C5-mediated neutrophil depletion initiated 1 or 3 h after infection resulted in a similar mortality to that observed among mice treated with RB6-8C5 24 h before infection (figure 6A). In contrast, no deaths were observed among mice that received RB6-8C5 at 6 or 24 h after infection (figure 6A) or at later time points (data not shown). Thus, infected mice remain sensitive to neutrophil depletion for 3 h after infection.

Examination of Gomori methenamine silver-stained lung sections revealed that murine susceptibility to RB6-8C5 administration correlated with the presence of tissue-invasive hyphae. Mice treated with RB6-8C5 either 24 h before (figure 6B) or 1 h after (figure 6C) conidial challenge exhibited hyphal lesions in airways and terminal air spaces with similar patterns of hyphal tissue invasion. In contrast, mice treated with RB6-8C5 at 24 h after infection demonstrated numerous conidia



**Figure 7.** Outcome of infection with standardization of the lung fungal burden at the onset of neutrophil depletion. Mice were infected with a target of  $3 \times 10^7$  conidia (A) or  $6 \times 10^7$  conidia (B) and were treated with RB6-8C5 at 1 h (A) or 24 h (B) after infection. The concentration of the inoculum was determined by plating serial dilutions of the conidial suspensions. For each experiment, 2–5 mice were sacrificed at the time of RB6-8C5 administration 1 h (A) or 24 h (B) after infection to determine lung colony-forming units (CFUs). The remaining mice (5–10 mice per experiment) were monitored for survival for 21 days. The 21-day survival data was pooled (20 mice per group) from 3 (A) or 2 (B) independent experiments. \* $P = .001$ , by Fisher's exact test.

within the lungs without evidence of hyphal tissue invasion (figure 6D).

A trivial explanation for differences in survival among experimental groups is a difference in fungal burden at the onset of neutrophil depletion. To exclude this possibility, the inoculum was adjusted to achieve the same lung fungal burden at the time of RB6-8C5 administration (figure 7A and 7B). Despite a trend toward higher CFU counts in lung samples of mice that received RB6-8C5 at 24 h after infection ( $P = .08$ , by Mann-Whitney rank-sum test), this group had a greater 21-day survival rate than did mice that received RB6-8C5 at 1 h after infection ( $P = .001$ , by Fisher's exact test). This result indicates that differences in survival are not attributable to differences in lung fungal burden at the time of neutrophil depletion.

**Survival and presence of neutrophils in alveolar macrophage-depleted mice.** To examine the effects of combining neutrophil and macrophage depletion, mice were treated with RB6-8C5 as well as clodronate liposomes via the intratracheal or intraperitoneal route. Mice treated intratracheally with clodronate liposomes and RB6-8C5 demonstrated a similar susceptibility to *A. fumigatus* infection as did mice treated with

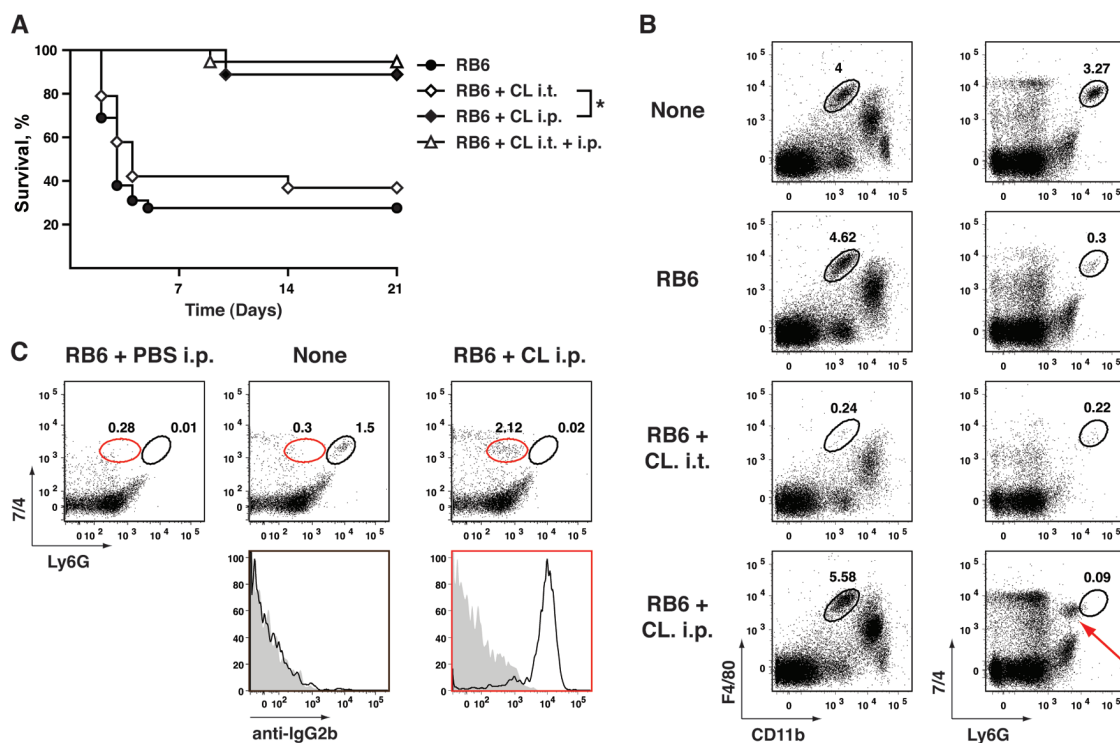
RB6-8C5 alone (figure 8A), consistent with a predominant role for neutrophils over alveolar macrophages in host defense in the experimental model examined.

As a control, 2 groups of mice received intraperitoneal clodronate liposomes (with or without intratracheal clodronate liposomes) and were subsequently treated with RB6-8C5. Intraperitoneal administration of clodronate liposomes rendered both groups resistant to conidial challenge (figure 8A). Single cell lung suspensions from these mice were analyzed to examine the cell populations affected by intratracheal or intraperitoneal administration of clodronate liposomes and RB6-8C5. In this experiment, 7/4 and Ly6G were used to define neutrophils to avoid the use of antibodies with the same isotype (IgG2b) as RB6-8C5. As expected, RB6-8C5 depleted circulating neutrophils (data not shown) and neutrophils in perfused lungs (figure 8B, top row vs. second row) but not CD11b<sup>+</sup> F4/80<sup>+</sup> macrophages. Intratracheal administration of clodronate liposomes followed by RB6-8C5 led to depletion of lung macrophage and neutrophil populations (figure 8B, third row). Intraperitoneal administration of clodronate liposomes followed by RB6-8C5 did not deplete lung macrophages (figure 8B, bottom row) and reduced the cell frequency in the neutrophil gate. In addition, a 7/4<sup>+</sup>Ly6G<sup>int</sup> cell population was observed (figure 8B, red arrow).

Neutrophils that bind RB6-8C5 are rapidly removed from the circulation and other organs, including the lungs [35]. After administration of <sup>125</sup>I-labelled RB6-8C5, radiolabeled neutrophils are sequestered in the spleen and liver [35], suggesting that the reticuloendothelial system mediates RB6-8C5-dependent neutrophil clearance. To determine whether systemic administration of clodronate liposomes influences neutrophil clearance, mice were treated intraperitoneally with clodronate liposomes followed by RB6-8C5, and single cell lung suspensions were stained with FITC-labeled anti-rat IgG2b to detect bound RB6-8C5.

Administration of PBS liposomes did not interfere with RB6-8C5-dependent neutrophil clearance in the lung (figure 8C, black gate), and 7/4<sup>+</sup>Ly6G<sup>int</sup> cells were not observed (figure 8C, red gate). In contrast, mice treated intraperitoneally with clodronate liposomes accumulated 7/4<sup>+</sup>Ly6G<sup>int</sup> cells with bound anti-rat IgG2b antibody (figure 8C). This result indicates that 7/4<sup>+</sup>Ly6G<sup>int</sup> cells represent neutrophils with bound RB6-8C5. Thus, mice treated intraperitoneally with clodronate liposomes followed by RB6-8C5 are resistant to intratracheal *A. fumigatus* challenge because of defective neutrophil depletion rather than direct effects of intraperitoneal clodronate liposome administration.

**Impact of neutrophil depletion after infection in alveolar macrophage-depleted mice.** Mice that undergo neutrophil depletion within 3 h after infection are as susceptible to invasive aspergillosis as neutropenic mice at the time of infection (fig-



**Figure 8.** Murine survival attributable to failure of neutrophil depletion on systemic administration of clodronate (CL) liposomes. **A**, Survival rate of mice administered no liposomes, or intratracheal (i.t.) and/or intraperitoneal (i.p.) CL liposomes 4 and 3 days before infection with  $2.5 \times 10^7$  to  $4.1 \times 10^7$  conidia. All mice received RB6-8C5 one day before infection. Data were pooled from 1–2 experiments per group (9–19 mice per group).  $*P = .012$ , by log-rank test. **B**, Flow cytometric analysis of lung suspensions from mice that received RB6-8C5 (on day 1) and CL liposomes via the i.t. or i.p. route, as indicated (on day –4 and day –3). CD45<sup>+</sup> lung leukocytes were analyzed for F4/80, CD11b (left column), 7/4, and Ly6G expression (right column), and the frequency of lung macrophages (left column) and neutrophils (right column) are indicated. The red arrow indicates a 7/4<sup>int</sup> Ly6G<sup>int</sup> cell population that stains with a lower Ly6G intensity than do cells in the neutrophil gate. **C**, Flow cytometric analysis of single-cell lung suspensions from mice that received RB6-8C5 in combination with i.p. PBS or CL liposomes, as indicated. CD45<sup>+</sup> lung leukocytes were stained with 7/4, Ly6G, and FITC-labeled mouse anti-rat IgG2b or a FITC-labeled isotype control antibody. The black gate indicates neutrophils; the red gate indicates neutrophils coated with monoclonal antibody RB6-8C5. The histograms in the bottom row demonstrate FITC–anti-rat IgG2b (black lines) or isotype control antibody (gray) binding to cells found in the black (left) or red (right) gate.

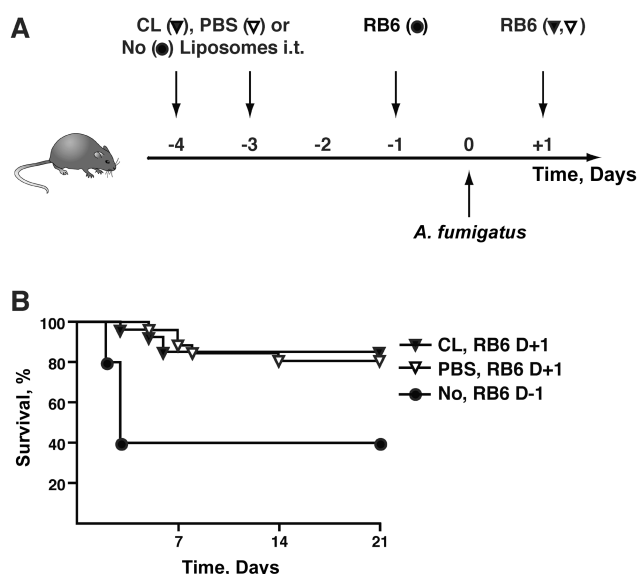
ure 6). To determine whether the presence of alveolar macrophages can alter murine susceptibility to *A. fumigatus* in the context of neutrophil depletion, murine mortality was compared in alveolar macrophage-depleted or alveolar macrophage-sufficient mice that underwent neutrophil depletion 24 h after infection (figure 9A). There was no difference in survival rate among experimental groups ( $P = .75$ , by log-rank test), consistent with the notion that loss of alveolar macrophages does not render mice more susceptible to neutrophil depletion after infection.

## DISCUSSION

The objectives of this study were to examine the essential role and temporal requirements for alveolar macrophages and neutrophils in host defense against *A. fumigatus*. An important finding is the redundancy of alveolar macrophages for murine survival, neutrophil recruitment, and inflammatory responses in a pulmonary infection model. This conclusion is based on

a compartmentalized macrophage depletion strategy rather than systemic impairment with a broadly immunosuppressive agent. A second finding relates to the brief period of host susceptibility to neutrophil depletion after infection. Neutrophil influx to the lungs in the first 6 h after infection is sufficient to protect mice from invasive disease.

The finding that alveolar macrophage-depleted mice sustain neutrophil recruitment and the synthesis of inflammatory mediators may appear surprising. However, this finding has been reported in other pulmonary infection models, such as those for infection due to *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [36, 37]. Preserved neutrophil recruitment may reflect a role for epithelial cells and endothelial cells in phagocytic and inflammatory responses against *A. fumigatus* [38–40]. In addition, natural killer cells and CCR6<sup>+</sup> myeloid dendritic cells participate in inflammatory responses in neutrophil-depleted mice [20, 41]. The saturable uptake of conidia by alveolar macrophages in vitro [11] and the dispersal of conidia observed in



**Figure 9.** Susceptibility of alveolar macrophage-depleted mice to *Aspergillus fumigatus* with neutrophil depletion 24 h after infection. *A*, Experimental scheme that depicts the alveolar macrophage and neutrophil depletion strategy in 3 groups of mice. Mice were treated with phosphate-buffered saline (PBS) (white inverted triangles; 19 mice) or clodronate (CL) (black inverted triangles; 18 mice) liposomes on day  $-4$  and day  $-3$  before infection with  $1.7 \times 10^7$  to  $2 \times 10^7$  *A. fumigatus* conidia and were given monoclonal antibody RB6-8C5 24 h later. A third group of mice (black circles) received no liposomes and were treated with RB6-8C5 1 day prior to infection (10 mice). *B*, Survival of experimental groups described in (*A*). The survival data is pooled from 2 experiments with 5–10 mice per experimental group. No statistically significant difference in survival was noted between mice that received CL or PBS liposomes prior to infection.  $P < .05$  between the RB6 day  $-1$  group and groups administered CL or PBS liposomes and the RB6 day 1 group by log-rank test.

lung sections suggest that it is highly unlikely that the small number of alveolar macrophages that escape in vivo depletion can compensate for the profound loss of these cells in the generation of inflammatory responses. More likely, alveolar macrophage depletion may result in a transient reduction in the synthesis of inflammatory mediators in the period immediately after infection ( $<24$  h). However, any alveolar macrophage-dependent reduction is readily compensated within a time frame that permits intact neutrophil airway recruitment and control of conidial germination.

Although these studies argue against an essential, nonredundant role for alveolar macrophages against *A. fumigatus*, this conclusion does not preclude a role for these cells in optimal responses against *A. fumigatus*. Because these studies did not employ broadly immunosuppressive or myelotoxic strategies to render mice susceptible to low conidial inocula (at the cost of damaging multiple leukocyte subsets), we do not exclude the possibility that alveolar macrophages have the capacity to clear small inocula without participation of other cell subsets.

Consistent with this possibility, there is evidence that mice administered cytotoxic chemotherapy may become susceptible to targeted macrophage depletion [42]. Thus, in patients with significant myeloid injury, the slow turnover and relative resistance of alveolar macrophages to irradiation [43] may enable these cells to maintain a cellular defense against inhaled conidia after the production, trafficking, or function of short-lived leukocytes (eg, neutrophils) has been impaired.

The requirement for neutrophils in the period immediately after infection period is consistent with an essential role for these cells in preventing conidial germination at the earliest stage after infection [17]. Our results suggest that there is a critical time window for neutrophil recruitment for protection to occur. Induction of neutropenia beyond 6 h after infection did not result in murine mortality, even when inocula were standardized for the time point of depletion. Neutrophils inactivate conidia and prevent their germination by phagocytic and nonphagocytic mechanisms [17, 44, 45]. Although extravasated neutrophils undergo apoptosis rapidly, the rapid establishment of neutrophil-dependent extracellular defense mechanisms (eg, lactoferrin release, the formation of BAL aggregates, and DNA extracellular traps) may serve to control conidial germination and hyphal tissue invasion, even with subsequent interruption in neutrophil influx.

The results presented in this study lend a cautionary note to cell depletion strategies that target macrophages and neutrophils. We found that clodronate liposomes administered systemically eliminate phagocytic cells that promote the clearance of antibody-coated neutrophils. Neutrophil and macrophage depletion strategies are often combined in animal models of inflammatory and infectious disease [46, 47]. Therefore, it is imperative to verify that the depletion strategy of a particular cell type does not unintentionally interfere with depletion of a second cell subset.

Our findings relate to infection control practices in high-risk patient populations. A major clinical goal is to prevent opportunistic infections in these populations, in part through reduction in exposure and administration of antimicrobial prophylaxis. Acute infection in high-risk hosts is a likely route to the development of invasive aspergillosis. However, a second possibility is that exposure prior to the onset of immune suppression or myeloblation may enable conidia to persist long enough in a viable form to establish invasive disease in the context of incipient or progressive myeloid injury [48]. The results presented in this study are most consistent with the first possibility, because of the rapid resistance of mice to transient neutrophil depletion after infection, despite a significant viable burden of spores in the lung. Although a prior history of invasive aspergillosis has a deleterious effect on the outcome of allogeneic stem cell transplantation [49, 50], the window of susceptibility following conidial exposure to administration of

corticosteroids or cytotoxic agents remains unknown. Understanding both the specific requirements for and the critical timing of innate immune cell action in relation to exposure is highly relevant to the formulation of strategies to protect vulnerable hosts during periods of highest risk.

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