Early Neutrophil Recruitment and Aggregation in the Murine Lung Inhibit Germination of *Aspergillus fumigatus* Conidia

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Several types of polymorphonuclear neutrophil (PMN) deficiency are a predisposing condition for fatal *Aspergillus fumigatus* infection. In order to study the defensive role of PMNs in the lungs, with particular reference to PMN recruitment and antimicrobial oxidant activity, responses to pulmonary instillation of *A. fumigatus* conidia were examined. Responses in BALB/c and C57BL/6 mice were compared with those in CXCXR2−/− and gp91phox−/− mice, which are known to have delayed recruitment of PMN to the lungs in response to inflammatory stimuli and inactive NADPH oxidase, respectively. In BALB/c mice, PMNs were recruited to the lungs and formed oxidase-active aggregates with conidia, which inhibited germination. In C57BL/6, gp91phox−/−, and CXCXR2−/− mice, PMN recruitment was slower and there was increased germination compared to that in BALB/c mice at 6 and 12 h. In gp91phox−/− mice, germination was extensive in PMN aggregates but negligible in alveolar macrophages (AM). Lung sections taken at 6 and 48 h from BALB/c mice showed PMN accumulation at peribronchiolar sites but no germinating conidia. Those from C57BL/6 and CXCXR2−/− mice showed germinating conidia at 6 h but not at 48 h and few inflammatory cells. In contrast, those from gp91phox−/− mice showed germination at 6 h with more-extensive hyphal proliferation and tissue invasion at 48 h. These results indicate that when the lungs are exposed to large numbers of conidia, in addition to the phagocytic activity of AM, early PMN recruitment and formation of oxidative-active aggregates are essential in preventing germination of *A. fumigatus* conidia.

Despite life-long exposure to *Aspergillus fumigatus*, very low morbidity is seen in immunocompetent individuals, indicating that a rapid and effective resistance to this organism has evolved. Individuals with normal immune systems rarely develop invasive pulmonary aspergillosis (IPA) even when exposed to high environmental concentrations of *A. fumigatus* conidia arising from, for example, disturbance of moldy wood chip piles, sludge, or compost (20) but may develop allergic bronchopulmonary aspergillosis or extrinsic allergic alveolitis (13). However, in recent decades there has been an increasing number of invasive and often fatal *A. fumigatus* infections in patients with immunosuppressive disorders (5, 16, 39), and *A. fumigatus* is now recognized as the leading airborne fungal pathogen in immunocompromised individuals (12, 35).

Resident alveolar macrophages (AM) phagocyte and subsequently destroy inhaled conidia (8, 38). However, the importance of polymorphonuclear neutrophils (PMN) in innate immunity to aspergillosis is indicated by the observation that patients and mice with PMN deficiency due to a variety of causes are susceptible to IPA (9, 30, 34, 37). The current study with mice was undertaken since there is limited information on PMN recruitment in response to inhalation of conidia and the mechanism by which PMN protect against aspergillosis.

Our hypothesis was that following exposure of the lung to conidia, early recruitment of PMN inhibits germination by the release of oxygen radicals from PMN due to their NADPH oxidase activity. To test our hypothesis, we compared the pulmonary immune response to *A. fumigatus* conidia in BALB/c and C57BL/6 mice, which are generally resistant to IPA, with that in CXCXR2−/− and gp91phox−/− mice, both of which have been reported to be susceptible to fatal IPA (18, 22, 32). In CXCXR2−/− mice, there is a delayed recruitment of PMN in response to inflammatory stimuli, which can be linked to the failure of their PMN to migrate towards the PMN-recruiting chemokines MIP-2 and KC (1, 18, 19). In gp91phox−/− mice, which are susceptible to IPA following low doses of conidia (22), there is deficient phagocyte NADPH oxidation (27) and a prolonged inflammatory response (22), as in patients with chronic granulomatous disease who have increased susceptibility to IPA (33). Results are presented that support our hypothesis and show that in normal BALB/c mice following respiratory challenge with *A. fumigatus* conidia, there is rapid sequestration of conidia within PMN aggregates that produce reactive oxygen species by NADPH oxidase, arresting conidial germination. A similar response is seen in C57BL/6 mice, though recruitment of PMN is slower than that seen in BALB/c mice, allowing some early germination to occur. This might explain why, following immunosuppression, C57BL/6 mice showed a higher mortality when exposed to *A. fumigatus* conidia than did BALB/c mice (34). Germination also occurs in CXCXR2−/− mice due to a 3-h delay in PMN recruitment and aggregation and is extensive in gp91phox−/− mice lacking NADPH oxidase activity. These results indicate that early PMN recruitment and aggregation of PMN with conidia arrest fungal germination and that destruction of hyphae by PMN (30) is probably of secondary importance in protecting the lung when it is exposed to large numbers of conidia.
**MATERIALS AND METHODS**

**Preparation of A. fumigatus conidia.** A clinical isolate (ATCC 13073 [American Type Culture Collection]) of *A. fumigatus* was reconstituted according to the supplier’s protocol. A conogenic strain expressing green fluorescent protein (GFP), kindly provided by Margo Moore (Simon Fraser University, British Columbia, Canada), was also used; it constitutively expresses GFP in resting conidia, swollen conidia, and hyphal forms due to control by the gfp promoter from *A. nidulans* (40). Conidia were grown at 37°C for 5 days on a Saouraud dextrose agar slant, collected in Hank's balanced salt solution (HBSS, product no. 10-1354; Cambrex Bio Science, Walkersville, MD) with 0.1% Tween 20 by gentle rocking, filtered through several thicknesses of Kimwipes to remove hyphal forms, counted with a hemocytometer, and then adjusted to the required inoculum strength with HBSS. Freshly harvested resting conidia were used for inoculations.

**Animal handling.** BALB/c and C57BL/6 mice were used as controls for knock-out mice lacking the CXCRI2 receptor and the gp91phox NADPH oxidase subunit gene, respectively. Breded BALB/c mice with the chemokine receptor CXCRI2 knocked out [C129S26/B6]-CXCRI2(Abrm)] were produced by backcrossing mice carrying the knockouts to BALB/c mice for 8 generations, and breeder mice with a null allele corresponding to the X-linked gp91phox component of NADPH oxidase (B6.129S-Cygbtm1Dts) were produced by backcrossing carrier females with C57BL/6 males for 13 generations. Breeding pairs of these mice were then obtained from the Jackson Laboratory (Bar Harbor, Maine) and reared in the Animal Resource Center at Montana State University. All animals were kept in specific-pathogen-free housing, and all manipulations were approved by the institutional internal review board for IACUC. To study chemokine-mediated chemotaxis of neutrophils, neutrophils were isolated from wild-type and gp91phox−/− mice as previously described with mice lacking the CXCR2 receptor and the gp91phox component of NADPH oxidase (41). Analyses for superoxide could not be conducted simultaneously for all animal groups due to differences in animal availability, and some variation between analyses was noted due to the use of reagents from different lot sources. Therefore, PMN from BALB/c mice (which were included in each analysis) were used as a reference control, and their superoxide production was defined as 100% (0.44 ± 0.04 nmol superoxide/106 PMN/min at 25°C). Values for other animal groups were then expressed as a percentage of the value for BALB/c mice for each experiment.

**Measurement of GFP fluorescence and conidial germination in BALB/c and vitro.** BALB/c, C57BL/6, and gp91phox−/− mice were inoculated with 3 × 106 conidia intratracheally or intrapharyngeally 24 h before recruitment was elicited in the lungs of mice by exposing them to nebulized *A. fumigatus* strain ATCC 13073 and the human clinical isolate (ATCC 13073 [American Type Culture Collection]) of *A. fumigatus* used in these and subsequent studies. A one-way analysis of variance with a Tukey posttest was used to determine the significance of differences when three or more groups were compared (see Fig. 2, 7B, and 8B).

**RESULTS**

PMN recruitment and aggregation around *A. fumigatus* conidia lead to inhibition of germination. In BALB/c mice, PMN recruitment was observed in BALF following pulmonary instillation of resting conidia. For example, 12 h following intrapharyngeal instillation of 106 conidia, 30% ± 2% (n = 6 mice) of leukocytes were PMN, compared to <1% in mock-inoculated mice. With 3 × 106 conidia, the corresponding value was 77% ± 6% (n = 6 mice). Following recruitment, PMN formed aggregates around conidia (Fig. 1), and subsequently very few hyphal forms were seen, suggesting that the aggregates may have inhibited germination. PMN aggregates were observed following inoculation of mice with conidia obtained from both *A. fumigatus* strain ATCC 13073 and the same strain modified to express GFP (41). Aggregates were observed when the lavage fluid was RPMI 1640 medium,
HBSS alone, or HBSS containing 3 mM EDTA (used routinely in our studies). Fluorescent conidia in a PMN aggregate from a wet mount are shown in Fig. 1A. Since the three-dimensional structure of PMN aggregates in wet mounts made photography and enumeration of conidia difficult, cytospin samples were routinely prepared from 100 μL BALF to better visualize the aggregates. The identity of PMN in aggregates around conidia was evident by use of transmitted-light differential interference contrast imaging but was also confirmed by Wright staining (Fig. 1B and C), in which conidia stain blue, or by a blue fluorescent Hoescht nuclear stain. It was found that PMN aggregation was not due to lavage per se, since PMN recruited to the lung following inhalation of LPS did not form aggregates but did so subsequently when exposed to conidia in vitro (Fig. 1D). Another indication that the aggregates were not due to an artifact was that when the number of aggregates in the BALF were related to the number of PMN in each aggregate, time-
dependent increases in both were noted during the first 24 h after instillation of 3 × 10^7 conidia into the lungs, but by 48 h these values had fallen dramatically (Fig. 1E).

To find out if PMN aggregates were a generalized response to airborne particulates similar in size to A. fumigatus conidia (2.5- to 3-μm diameter), experiments were carried out using 3 × 10^6 Rhizopus oryzae spores (6- to 8-μm diameter) or polystyrene beads (3-μm diameter). Nine hours after instillation into BALB/c mice, the spores and beads were also enclosed in PMN aggregates, suggesting a similar response. As was the case with A. fumigatus, germination of R. oryzae spores appeared to be inhibited by PMN aggregates (data not shown).

Delayed PMN recruitment and aggregation is associated with increased germination of A. fumigatus conidia in vivo. To test whether the PMN response that occurred in C57BL/6 mice was similar to that observed in BALB/c mice, PMN recruitment levels were compared for these two strains of mice 6 and 12 h after intrapulmonary inoculation with 3 × 10^6 resting A. fumigatus conidia expressing GFP. At the same time, PMN recruitment was examined in gp91phox−/− mice (for which C57BL/6 mice are the control; Jackson Laboratory mouse data sheet), which have been shown to be susceptible to aspergilllosis (27). At 6 h in C57BL/6 and gp91phox−/− mice, the numbers of PMN in 5-ml samples of BALF were 7% and 9%, respectively, of the number found in BALB/c mice (Fig. 2A). Due to the delayed recruitment of PMN, there were also fewer PMN aggregates containing conidia by 6 h in C57BL/6 and gp91phox−/− mice than in BALB/c mice (data not shown). Following additional PMN recruitment at 12 h, there were no longer lower numbers of PMN in the BALF of C57BL/6 and gp91phox−/− than in BALB/c mice (Fig. 2B). PMN aggregates with the fungus were observed in all groups at 12 h (data not shown). This observation showed that PMN were successfully recruited in the C57BL/6 and gp91phox−/− mice after an initial delay and then underwent aggregation with the fungus. No significant differences between the numbers of AM in the animal groups were found at 6 h. In BALB/c mice, AM numbers were not increased at 12 h, indicating the lack of AM recruitment despite the importance of conidial engagement by AM (8, 25). A surprising finding was that at 12 h, AM numbers in both C57BL/6 and gp91phox−/− mice were reduced relative to those in BALB/c mice (P < 0.05).

Coincident with the delayed PMN recruitment in the C57BL/6 and gp91phox−/− mice relative to that in BALB/c mice, there was an increase in germinating conidia (Fig. 2C). By 6 h, a significant increase in germination was observed in C57BL/6 mice relative to that in BALB/c mice (P < 0.05) and in gp91phox−/− mice relative to that in both BALB/c and C57BL/6 mice (P < 0.001). At 12 h, there were approximately three- and sevenfold more germinated conidia in the BALF from C57BL/6 and gp91phox−/− mice, respectively, than in the BALF from BALB/c mice (P < 0.01). The number of hyphal forms of the fungus in gp91phox−/− mice was again significantly higher than that in C57BL/6 mice (P < 0.001). The delayed recruitment of PMN was thus associated with increased germination, but the results with gp91phox−/− mice indicated the further importance of NADPH oxidase in preventing germination.

FIG. 1. PMN aggregates containing A. fumigatus conidia in BALF from BALB/c mice. (A to C) Photomicrographs of PMN aggregates in BALF 12 h after intrapulmonary inoculation with 3 × 10^6 resting conidia of A. fumigatus. (D) Aggregate of LPS-elicited mouse alveolar PMN with conidia produced by in vitro incubation. (B and C) The Wright-stained cytospin preparation shows PMN aggregates containing both resting and swollen conidia stained blue (red arrows) (magnifications, ×630 [B] and ×1,000 [C]). (A and D) The wet mounts show PMN aggregates containing GFP-labeled conidia at ×630. The scale bars represent 10 μm. (E) Aggregate numbers and sizes 0 to 48 h after instillation of 3 × 10^7 conidia into the lungs of BALB/c mice.
To further examine the effect of delayed PMN recruitment on germination of conidia, a more detailed time course study was conducted with BALB/c and CXCR2<sup>−/−</sup> mice (for which BALB/c mice are the accepted control). Delayed PMN recruitment in CXCR2<sup>−/−</sup> mice relative to that in BALB/c mice was observed at 3 and 6 h (<i>P < 0.05</i>), but after 9 h, PMN numbers in CXCR2<sup>−/−</sup> mice were indistinguishable from those in BALB/c mice (Fig. 3A), and aggregation of many of the PMN with conidia had occurred. To determine how much of the PMN response to inoculation was due to the vehicle, the effect of mock inoculation with HBSS alone was examined after 24 h. In BALB/c mice, 183-fold (<i>P < 0.001</i>) fewer PMN were found in the BALF after mock inoculation than following exposure to conidia. In CXCR2<sup>−/−</sup> mice, the corresponding difference was a 232-fold (<i>P < 0.05</i>) lower number. These results indicate that the vehicle was not an important contributor to the PMN response.

In contrast to PMN recruitment to the lungs, which was found in both BALB/c and CXCR2<sup>−/−</sup> mice in response to inoculation with conidia, recruitment of AM was not evident until 48 h, when there were 2.7- and 3.5-fold more AM than at time zero in BALB/c and CXCR2<sup>−/−</sup> mice, respectively (<i>P < 0.005</i>) (Fig. 3B). The total number of AM in the BALF was lower in CXCR2<sup>−/−</sup> mice than in BALB/c mice at 6 to 12 h, but the difference was significant only at 6 h (<i>P = 0.01</i>).

In addition to cell differential measurements, the actual numbers of free conidia and conidia associated with either PMN or AM in the BALF of BALB/c and CXCR2<sup>−/−</sup> mice were measured (Fig. 4A and B, respectively). The association of PMN with conidia was significantly greater in BALB/c than in CXCR2<sup>−/−</sup> mice at 3, 6, 9, and 24 h (<i>P < 0.05</i> for each time point examined). No statistical differences were found for the proportion of conidia associated with AM in BALB/c versus CXCR2<sup>−/−</sup> mice at any time point. At 3 h after inoculation in inoculation was due to the vehicle, the effect of mock inoculation with HBSS alone was examined after 24 h. In BALB/c mice, 183-fold (<i>P < 0.001</i>) fewer PMN were found in the BALF after mock inoculation than following exposure to conidia. In CXCR2<sup>−/−</sup> mice, the corresponding difference was a 232-fold (<i>P < 0.05</i>) lower number. These results indicate that the vehicle was not an important contributor to the PMN response.

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of cells by centrifugation to characterize the inflammatory response and, in particular, to identify either PMN- or AM-specific recruiting or stimulatory molecules. In BALB/c and CXCR2\(^{−/−}\) mice immediately after inoculation with \(A.\) \textit{fumigatus} conidia, measured chemokine and cytokine levels were low (Fig. 5): 142 ± 35 pg/ml for TNF-\(\alpha\) and <10 pg/ml for IL-6 and four other cytokines, IL-10, IL-12, MCP-1, and IFN-\(\gamma\). In BALB/c mice, 3 h after exposure to conidia there were 3.5- and 47-fold increases in the concentrations of TNF-\(\alpha\) and IL-6, respectively, relative to those at time zero (\(P < 0.05\)) and similar concentrations were found in CXCR2\(^{−/−}\) mice at 3 h. However, at 6, 12, 24, and 48 h, TNF-\(\alpha\) and IL-6 levels were higher in CXCR2\(^{−/−}\) mice than in BALB/c mice, with increases in TNF-\(\alpha\) being the most marked (\(P < 0.05\)). Concentrations of the other four cytokines were low or undetectable in both BALB/c and CXCR2\(^{−/−}\) mice from 0 to 12 h. However, in CXCR2\(^{−/−}\) mice between 12 and 24 h, the levels of IFN-\(\gamma\), IL-10, and IL-12, but not MCP-1, increased significantly (\(P < 0.05\)) and at 24 h were significantly higher than in BALB/c mice. At 48 h, only the levels of IL-12, TNF-\(\alpha\), and IL-6 were significantly higher in CXCR2\(^{−/−}\) mice than in BALB/c mice. CXCR2\(^{−/−}\) mice thus showed overall greater cytokine increases than BALB/c mice.

**NADPH oxidase-deficient PMN do not inhibit germination of conidia.** Defects in NADPH oxidase profoundly predispose humans and experimental animals to invasive aspergillosis (9, 22). The fates of conidia in mice with active NADPH oxidase and in gp91\(^{phox−/−}\) mice were therefore compared in more detail. Typical PMN aggregates and AM from BALB/c and gp91\(^{phox−/−}\) mice 12 h after inoculation with \(3 \times 10^6\) conidia expressing GFP are shown in Fig. 6. Although all of the conidia used in this set of experiments were found to constituutively and uniformly express GFP when collected from the Sabouraud dextrose agar slant, their association with PMN aggregates led to a variable loss of GFP fluorescence. Consequently, PMN aggregates from BALB/c mice contained both fluorescent and nonfluorescent conidia (Fig. 6A) but very rarely germinated conidia expressing GFP. In contrast, the PMN aggregates from gp91\(^{phox−/−}\) mice contained fluorescent conidia with hyphal formation that was often extensive (Fig. 6B). AM from both BALB/c and gp91\(^{phox−/−}\) mice contained conidia (Fig. 6C and D, respectively), sometimes with 12 or more conidia in a single AM, though about 98% of the AM did not contain any conidia. As with the PMN aggregates in BALB/c mice, some conidia in the AM of NADPH oxidase-competent mice had lost fluorescence. Since loss of conidial fluorescence occurred in BALB/c but not in gp91\(^{phox−/−}\) mice and germination occurred in PMN aggregates of gp91\(^{phox−/−}\) but not in BALB/c mice, these differences were attributed to differences in NADPH oxidase activity and the ability to generate oxygen radicals. To test this hypothesis, conidia expressing GFP were exposed in vitro to either hydrogen peroxide or hypochlorous acid, which are potent antimicrobials produced by PMN with competent NADPH oxidase (10). GFP fluorescence in resting conidia was reduced in a concentration-dependent manner by both hydrogen peroxide and sodium hypochlorite (which dissociates to form hypochlorous acid), but the latter was approximately 1,000 times more potent and had a steeper concentration-response curve (Fig. 7A). Fifty percent inhibitory concentrations were not compared because of the steepness of the curve.
for sodium hypochlorite and the lack of experimental points to define it. However, in response to both oxidants, the loss of fluorescence was associated with the loss of the ability to germinate. GFP fluorescence as a marker for fungal viability has previously been used for evaluating the responses of spores of 

Aureobasidium pullulans to oxidants and other biocides (41).

A quantitative comparison of conidial fluorescence levels in PMN aggregates and in AM at 12 h in gp91phox−/−, C57BL/6, and BALB/c mice was made, and data based on complete loss of fluorescence are shown in Fig. 7B. Loss of fluorescence was found for conidia associated with PMN aggregates in both BALB/c and C57BL/6 mice and was greater than the loss of fluorescence in conidia associated with AM. However, in gp91phox−/− mice, conidia engaged by either PMN aggregates or AM showed no significant loss of fluorescence. In BALB/c mice, there was minimal germination of conidia in PMN aggregates but some germinated conidia were found in PMN aggregates from C57BL/6 mice. The even greater amount of germination in PMN aggregates from gp91phox−/− mice suggested that aggregation of PMN without a functioning NADPH oxidase fails to prevent conidial germination and hyphal proliferation. In contrast, germination was rarely observed in the AM of gp91phox−/−, C57BL/6, or BALB/c mice.

PMN aggregates use reactive oxygen species to inhibit the germination of conidia. To further examine the importance of oxidant production within PMN aggregates, NBT was added prior to cytospin preparation to BALF samples obtained from BALB/c, CXCR2−/−, C57BL/6, and gp91phox−/− mice 9 h after inoculation with conidia. Oxidant production was then assessed by the levels of reduction of the soluble yellow NBT to insoluble blue formazan (11). Only low background levels of formazan deposition were found on phagocytes not in association with conidia, and very rarely was formazan deposition found around conidia that appeared to be within AM and then only in modest amounts. In contrast, there was marked formazan deposition on PMN-conidium aggregates obtained from BALB/c, CXCR2−/−, C57BL/6, and gp91phox−/− mice (Fig. 8A, panels 1, 2, 3, and 4, respectively). However, when BALF phagocytes from BALB/c mice were stimulated with 1 μM PMA, all PMN showed formazan staining, although only background levels could be detected in the AM, whether or not they contained conidia (data not shown). Thus, BALB/c,
CXCR2−/−, and C57BL/6 mice produce PMN aggregates capable of generating reactive oxidants, but despite this, germinated conidia were found in the C57BL/6 and CXCR2−/− mice, presumably due to delayed PMN recruitment and aggregation. PMN aggregates from BALF of mice inoculated with R. oryzae spores and polystyrene beads also showed formazan deposition (data not shown).

To determine whether human peripheral PMN respond to A. fumigatus conidia in a manner similar to that observed for murine alveolar PMN, human peripheral blood was collected from donors and PMN were prepared and incubated with A. fumigatus conidia. It was found that oxidase-active aggregates around conidia were produced in vitro by human PMN (data not shown).

Since experiments using NBT reduction did not allow quantitative comparisons of oxidant-generating capacities, cytochrome c reduction by LPS-recruited PMN from BALB/c, CXCR2−/−, C57BL/6, and gp91phox−/− mice was also studied. BALF collected from BALB/c, C57BL/6, and gp91phox−/− mice 9 h following aerosol exposure to LPS contained cell concentrations and PMN:AM ratios essentially the same as those seen 9 h following inoculation with conidia. However, using a double-blind examination of cytospin slides (n = 14) under ×200 magnification (which did not allow fungal elements to be identified), no PMN aggregates were found in BALF following inhalation of LPS but PMN aggregates were consistently found following inhalation of conidia. PMN were recruited by LPS less reliably in CXCR2−/− mice than in BALB/c mice (data not shown). LPS-recruited phagocytes from BALB/c, C57BL/6, and CXCR2−/− mice exposed to PMA showed no significant differences in superoxide production as assessed by cytochrome c reduction, whereas negligible amounts of superoxide were found for LPS-recruited phagocytes from mice lacking the gp91phox gene required for oxidase activity (Fig. 8B). AM from all animal groups were found by this method to produce insignificant levels of superoxide, consistent with previous data (25).

Lung tissues show infiltration by germinating conidia when NADPH oxidase is inactive. Lung sections from BALB/c mice collected 6 and 48 h after inoculation with A. fumigatus conidia showed leukocyte accumulation at the peribronchiolar sites of
FIG. 8. Nitroblue tetrazolium staining of PMN aggregates and superoxide generation by LPS-recruited PMN. (A) PMN aggregates with formazan deposition indicating areas of NADPH oxidase activity. The PMN aggregates are from the BALF of BALB/c (panel 1), CXCR2−/− (panel 2), C57BL/6 (panel 3), and gp91phox−/− (panel 4) mice 9 h following inoculation with $3 \times 10^6$ resting conidia. In aggregates from the gp91phox−/− mice, the only visible staining is associated with germinating conidia. The scale bars represent 10 μm. (B) Superoxide generation by LPS-recruited PMN from BALB/c, CXCR2−/−, C57BL/6, and gp91phox−/− mice assessed by PMA-stimulated cytochrome c reduction. The average results for at least five animals per group are expressed as a percentage of the activity observed in BALB/c animals. The average activity of BALB/c cells was found to be 0.44 nmol superoxide/10⁶ PMN/min at 25°C. The error bars show ±SEM.
conidial deposition, though germinated forms were not found at either time in GMS-stained sections (Fig. 9A and B, respectively). In CXCR2−/− and C57BL/6 mice 6 h after inoculation, germinating conidia were present in alveoli but there were very few inflammatory cells (Fig. 9C and E, respectively). At 48 h, the lungs of CXCR2−/− mice did not show tissue invasion (Fig. 9D). At 48 h in C57BL/6 animals, some ungerminated conidia were present but hyphae were absent, presumably due to removal by phagocytes (Fig. 9F). Cellular infiltrates were found to consist of inflammatory cells, as determined by hematoxylin and eosin staining (data not shown). In contrast, lung sections prepared after inoculation of gp91phox−/− mice showed some germinating conidia as early as 6 h, and by 48 h, extensive hyphal proliferation with tissue invasion was evident (Fig. 9G and H, respectively).

**DISCUSSION**

PMN are important for resistance to *A. fumigatus*, since IPA occurs in humans and other animals when there is neutropenia.
due to a variety of causes (23). In the present study, after inoculation of BALB/c mice with as few as 10⁴ conidia per animal, PMN were recruited to the lungs, suggesting that they had a role in the innate response to the fungus. The rate of recruitment was important, since significantly greater early germination was observed in the C57BL/6 and CXCR2−/− mouse strains that showed slower PMN recruitment than in BALB/c mice. These results suggest that early PMN recruitment reduces germination of conidia.

To better understand the relationship between phagocyte types in the lung, the responses of AM and PMN to A. fumigatus conidia were compared. AM responded first to the conidia since they are resident cells in the lung, but the observation that some early germination of free conidia occurred in C57BL/6 and CXCR2−/− mice before the arrival of significant numbers of PMN indicates that AM alone could not effectively prevent germination. In BALB/c mice, the numbers of AM were not increased by recruitment until after 24 h, and this correlated with delayed increases in the macrophage-recruiting chemokine MCP-1 and the macrophage-activating cytokines IFN-γ and IL-12. PMN were recruited much earlier than peripheral monocyte-derived AM, which may in part be due to the early increases in TNF-α levels shown in the BALF, which can induce MIP-2 secretion by AM (24), which then signals via CXCR2 for PMN recruitment. Rapid increases in IL-6 levels could also support early PMN recruitment by stimulating granulocyte production in the bone marrow. PMN recruitment resulted in more interactions of conidia with PMN than with AM. Differences in TNF-α and IL-6 levels in BALB/c and CXCR2−/− mice may be due to a greater recruitment signal in the CXCR2−/− mice to compensate for their delayed neutrophil recruitment. Nevertheless, increased levels of TNF-α in the BALF of CXCR2−/− mice relative to those in BALB/c mice following instillation of conidia into the lungs was not expected, since the opposite has been reported using different proinflammatory stimuli in CXCR2−/− mice (3), immunoneutralized CXCR2 mice (6), and rabbits treated with a CXCR2 antagonist (26). Our results for phagocyte recruitment are in agreement with a previous study by Duong et al. utilizing a similar dose of A. fumigatus conidia in BALB/c mice which showed that PMN increased in the BALF by 4 h but that AM increases were not noted until after 48 h (4). That previous study also illustrates the extent to which cortisone-induced immunosuppression, a well-established predisposing condition for IPA, slows PMN recruitment following exposure to A. fumigatus conidia, so favoring early germination.

Despite the increased germination observed in the mice showing delayed recruitment, hyphal penetration of tissue was not found at 48 h in the lungs of either CXCR2−/− or C57BL/6 mice. Thus, in the absence of immunosuppression, delayed PMN recruitment and early germination alone did not lead to tissue invasion by hyphae, and no mortality was observed in the mice within the time frame of our experiment, though it is known that germinating conidia can elicit a greater inflammatory response than resting conidia (7). However, 35% mortality was observed by Schuh et al. in CXCR2−/− mice 48 h after intratracheal administration of 5 × 10⁴ conidia under anesthesia induced by intraperitoneal administration of ketamine (32) rather than the brief isoflurane inhalation used in the present study. Differences in anesthesia may therefore be important in determining outcome and may also contribute to differences between the present results and those reported by Mehrad et al., who used a polyclonal antibody to neutralize CXCR2 2 h before intraperitoneal administration of pentobarbital for intratracheal instillation of conidia (18). Despite using the same ATCC strain (ATCC 13073) and a similar dose of conidia to those used for our CXCR2−/− mice (1 × 10⁷ to 2 × 10⁷ versus 3 × 10⁷ used in our studies), Mehrad et al. observed inflammatory infiltrates containing branched hyphae in the lungs at 48 h (18). The CXCR2−/− mouse strain used in the current study is based on the BALB/c strain, whereas the CXCR2 neutralization (18) was carried out with C57BL/6 mice, which we report as showing delayed recruitment of PMN and significantly more early germination than occurs in BALB/c mice. This difference in C57BL/6 mice could therefore make them more susceptible to IPA following administration of the anti-CXCR2 antibody than the CXCR2−/− mice produced from BALB/c mice. This explanation may also account for the greater mortality in C57BL/6 mice than in BALB/c mice following neutrophil depletion (34).

PMN recruitment in response to the instillation of conidia was associated with the formation of aggregates with conidia. The number and size of these aggregates were not constant but increased as PMN were recruited to the lungs (Fig. 1 and 3). From 24 to 48 h, the number and size of the aggregates decreased proportionately, presumably due to a decreased number of conidia at the later time point. It has been reported that resting conidia are particularly resistant to PMN-derived antimicrobials (14, 15). Formation of PMN aggregates may be a mechanism to overcome this resistance, allowing PMN to generate sufficiently high local concentrations of antimicrobial factors such as oxidants to kill the sequestered conidia. Our observation that both resting and swollen conidia are found within PMN aggregates (as shown in Fig. 1B) indicates that both forms are engaged by PMN, although the swollen forms may be more susceptible to killing (7, 14, 15). Oxidase-active PMN aggregates also formed following inhalation of R. oryzae spores or polystyrene beads, and in vitro aggregation of human peripheral PMN with polystyrene beads has been previously described (36). This suggests that Aspergillus-specific surface molecules may not be the trigger for aggregation but that aggregation may be a conserved response vital for innate defense against many types of inhaled microorganisms and other particulate matter.

The fate of conidia in AM and PMN was examined using a strain of A. fumigatus that constitutively expresses GFP. The greater loss of fluorescence found in PMN aggregates than in AM for both BALB/c and C57BL/6 mice indicates that there is faster killing of conidia by PMN aggregates than by AM, supporting the proposal that early PMN recruitment and aggregation are important in preventing infection. In vitro, oxidant-mediated reduction of GFP fluorescence in conidia was found to correlate with the subsequent failure of conidia to form colonies, indicating that loss of fluorescence can be used as a marker of oxidant damage. In vivo, the 50% loss of conidial fluorescence observed in PMN aggregates from BALB/c and C57BL/6 mice 12 h after the administration of conidia was also associated with oxidant damage, since the majority of the conidia failed to germinate. In the absence of oxidants, no loss of fluorescence would thus be expected and germination...
should occur. This proved to be the case for conidia within PMN aggregates in gp91<sub>phox</sub><sup>+/−</sup> mice. In view of the faster recruitment of PMN in BALB/c mice relative to that in C57BL/6 mice, it was expected that there would be a greater loss of fluorescence in the BALB/c mice, but no difference was found using measurements based on total loss of fluorescence. If the loss of fluorescence could have been graded, then this might have been a more sensitive indicator for comparisons between the two strains.

Studies on the role of AM NADPH oxidase in killing <i>A. fumigatus</i> conidia have produced contradictory results (22, 25, 31). In the present comparison of BALB/c, C57BL/6, and gp91<sub>phox</sub><sup>+/−</sup> mice, formazan staining of AM containing conidia could not be distinguished from background staining. There was also only 10% less conidial fluorescence in AM from C57BL/6 mice than in AM from gp91<sub>phox</sub><sup>+/−</sup> mice. Together, these observations indicate that oxidants in AM either have a modest effect on conidia or that conidia may be inhibiting these observations. Indeed, there is inhibition of germination independent of oxidase activity within PMN aggregates contributes importantly to the lack of hyphal development and IPA in immunocompetent animals upon exposure to large numbers of conidia.

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