MAJOR ARTICLE

Interferon-γ and Granulocyte-Macrophage Colony-Stimulating Factor Augment the Activity of Polymorphonuclear Leukocytes against Medically Important Zygomycetes

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Zygomycetes cause serious invasive infections, predominantly in immunocompromised and diabetic patients with poor prognoses and limited therapeutic options. We compared the antifungal function of human polymorphonuclear leukocytes (PMNLs) against hyphae of *Rhizopus oryzae* and *R. microsporus*, the most frequently isolated zygomycetes, with that against the less frequently isolated *Absidia corymbifera*. We then evaluated the effects of interferon (IFN)–γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), alone or combined, on PMNL antifungal function against these zygomycetes. Both PMNL oxidative burst in response to hyphae and PMNL-induced hyphal damage were significantly lower in response to *Rhizopus* species than in response to *A. corymbifera*. Incubation of PMNLs with IFN-γ and GM-CSF alone or combined for 22 h increased the PMNL-induced hyphal damage of all 3 species. The treatment of PMNLs with the combination of IFN-γ and GM-CSF significantly increased the release of tumor necrosis factor–α in response to *R. microsporus* and *A. corymbifera* hyphae. IFN-γ significantly reduced interleukin-8 release in response to all zygomycetes. Although *Rhizopus* species demonstrate a decreased susceptibility to the antifungal activity of human PMNLs, in comparison with *A. corymbifera*, IFN-γ and GM-CSF augment the hyphal damage of all 3 zygomycetes, suggesting a role for IFN-γ and GM-CSF in the management of invasive zygomycosis.

Zygomycetes are filamentous fungi that cause life-threatening infections in immunocompromised patients and those with diabetes mellitus [1–4]. Deferoxamine, cystic fibrosis, and malnutrition also have been observed as less frequent underlying diseases in patients with zygomycosis. Zygomycetes cause rhinocerebral, pulmonary, disseminated, cutaneous, and gastrointestinal forms of infection, as a result of angioinvasion, thrombosis, infarction, and necrosis of involved tissues [1, 2, 5].

Members of the genus *Rhizopus*—in particular, *R. oryzae* and *R. microsporus*—are the most common organisms isolated from patients with zygomycosis. By comparison, other species of the class Zygomycetes, such as *Absidia corymbifera*, rarely cause deeply invasive infections [6–8]. The biological basis for these differences in frequency of infection is unknown but may involve differences in host response to different genera or differences in zygomycete response to different hosts. Early studies of human host defenses against *R. oryzae* underscored the importance of intact innate immuni-

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ty, including functional polymorphonuclear leukocytes (PMNLs) [9–13]. Both oxidative and nonoxidative mechanisms appear to be critical in the process of antifungal PMNL activity [14–17]. Neutropenia or functional deficiency of PMNLs are the main risk factors for the development of zygomycosis [1–4].

Cytokines are a critical component of functional innate antifungal host defenses. In particular, interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), 2 clinically important therapeutic agents [18], augment the antifungal activity of PMNLs against *Aspergillus fumigatus* [19–21]. However, zygomycetes are members of a different taxonomic class of fungi, with many pathogenic features that are distinct from those of *Aspergillus* species, and little is known about the immunomodulatory activity of cytokines in augmenting host defenses against zygomycetes. In addition, GM-CSF stimulates the production of phagocytes and inhibits PMNL apoptosis [22–25], thus increasing the number of functional PMNLs [23]. Interleukin-8 (IL-8) and tumor necrosis factor–α (TNF-α) are well-known cytokines that strongly prime the oxidative burst of PMNLs in response to *N*-formyl peptides and, thus, could potentially play a critical role in fungal killing [26]. IL-8 also plays a critical role as a chemokine in recruiting PMNLs to foci of infection [27]. However, little is known about the immunoregulation of TNF-α and IL-8 in the innate host response to zygomycetes.

We therefore evaluated the antifungal activities of PMNLs against the most frequently isolated zygomycetes, *R. oryzae* and *R. microsporus,* alone or after treatment with IFN-γ and GM-CSF and compared these functions with those against the relatively uncommon species, *A. corymbifera.* Among the antifungal PMNL activities that we investigated were oxidative burst, hyphal damage, and release of IL-8 and TNF-α in response to hyphae of the 3 zygomycetes.

MATERIALS AND METHODS

PMNL isolation. PMNLs were isolated from heparinized whole blood of healthy young adult volunteers [19]. Blood was immediately allowed to settle with 3% dextran T500 (Pharmacia Biotech AB) in a 2:1 volume/volume proportion. PMNLs were separated by centrifugation over a ficoll (Lymphocyte Separation Medium, Gibco BRL Life Technologies) cushion. Contaminating erythrocytes were hypotonically lysed, and the PMNL suspension was washed in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} ($HBSS^-$; Gibco). The cells were resuspended in $HBSS^-$ and counted on a hemocytometer.

Cytokine treatment. PMNLs were incubated at a concentration of 5×10^6 cells/mL in RPMI 1640 supplemented with 10% pooled human serum (in the case of 2-h incubation) or 10% autologous serum (in the case of 22-h incubation) in the presence or absence of 100 ng/mL IFN-γ (Boehringer-Ingelheim) and/or 100 ng/mL GM-CSF (Schering-Plough; donated by Tore Abrahamsen, University of Oslo, Oslo, Norway), at 37°C and 5% CO_2. At the end of the 22-h incubation, PMNLs were counted using a hemocytometer, and the viability was assessed with trypan blue staining, ensuring >95% viability.

Fungi. Three clinical isolates of zygomycetes were used in these studies. *R. oryzae* (RO 27) was isolated from a cut-down wound site (Fungus Testing Laboratory, University of Texas Health Sciences Center, San Antonio), *R. microsporus* var. *rhizopodiformis* (AZN 1185) was isolated from an invasive infection, and *A. corymbifera* (AZN 4095 and CBS 271.65) was a clinical isolate from an undetermined site of infection. The 2 latter isolates were donated by Paul Verweij (University Medical Center St. Radboud, Neijmegen, The Netherlands).

Fungi from frozen stocks were inoculated on potato dextrose agar (Merck Darmstadt) plates and grown for 4 days. Sporangiospores were then harvested by scraping the surfaces of the plates, suspended in $HBSS^-$, and filtered through sterile gauze. After centrifugation at 400 *g* for 10 min, supernatant was aspirated, the pellet was resuspended in normal saline, and cells were counted on a hemocytometer. They were kept at 4°C for no longer than 3 weeks.

Hyphae were generated for each of the fungi by placing 200 μL of a suspension containing 7.5×10^5 (for superoxide anion [O_2^-] production assays) or 7.5×10^4 (for hyphal damage and cytokine release assays) sporangiospores/mL in yeast nitrogen base (YNB; Difco Laboratories) medium in each well of a 96–flat-bottom well cell-culture plate (Corning). The plate was then incubated at 25°C (*Rhizopus* species) or 32°C (*A. corymbifera*), for 14 h. These hyphae were used as stimuli in all of the experiments.

O_2^- quantification. The oxidative burst evidenced by the production of O_2^- was measured as reduction of cytochrome *c* from horse heart (Sigma Chemical Co.) [28]. Hyphae were prepared as described above. Once the hyphal network was established, in the case of O_2^- assays with serum-opsonized hyphae, the plate was centrifuged at 400 *g* at 25°C for 30 min. YNB was then replaced by 50% human serum in $HBSS^-$, and the plate was rotated at 37°C for 30 min, for opsonization. Because zygomycete hyphae were loosely attached to the bottom of the wells, plates were always centrifuged before aspiration of supernatant during washing. The plate was washed 3 times, and PMNLs were added at an effector cell:target (E:T) ratio of 1:1 to a final volume of 200 μL of $HBSS^-$ containing 60 μmol/L cytochrome *c* with serum-opsonized or nonopsonized hyphae. Basal O_2^- production was assessed in the absence of stimuli. After incubation at 37°C on a rotator for 1 h, 100 μL was transferred to another plate, and absorbance was read in a spectrophotometer at 550 nm, with a reference of 690 nm. The extinction coefficient of cytochrome *c* at 550 nm was taken as 29.5×10^4 L/mol·cm.

Hyphal damage assay. PMNL-induced hyphal damage was assessed by use of a modification of the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]2H-tetrazolium-5-carboxanilide sodium salt; Sigma) plus coenzyme Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) assay [29]. Hyphae were generated as described above. Once the hyphal network was established, the plates were centrifuged at 400 *g* at 25°C for 30 min. YNB in each plate was then replaced by 200 μL of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, and PMNLs were added at the corresponding E:T ratios. After incubation at 37°C with 5% CO_2 for 2 or 22 h, the plates were centrifuged at 400 *g* at 10°C for 30 min. One hundred ninety microliters of RPMI 1640 was then replaced with H_2O, and the plates were centrifuged again, to lyse the cells without aspiration of hyphae. After a second wash performed in this manner, H_2O in each plate was replaced by 150 μL of PBS (Biochrom KG) containing 25 mg/mL XTT and 40 μg/mL coenzyme Q_0. Aspiration of the wells was performed very gently with a multichannel pipette. Because of the possibility of aspiration of hyphae, wells were set in octuplicate. After incubation with XTT, the wells of the plate were observed under the microscope, to ensure that hyphae were not accidentally aspirated from the wells. After incubation with XTT at 37°C with 5% CO_2 for 1 h, 100 μL from each plate was transferred to a new plate, and the change in color (absorbance) was assessed spectrophotometrically at 450 nm by use of a 690-nm reference. Antihyphal activity was calculated according to the following formula: percentage of hyphal damage $= (1 - X/C) \times 100$, where X is the absorbance of experimental wells and C is the absorbance of control wells with hyphae only.

Cytokine release from PMNLs. Release of IL-8 and TNF-α from IFN-γ– and GM-CSF–treated PMNLs was measured by incubating PMNLs for 4 h in microtiter plate wells containing 200 μL of $HBSS^-$ with 1.5×10^5 PMNLs/well. PMNLs were added to wells containing hyphae of the 3 zygomycetes (generated from sporangiospores, as described above) at an E:T ratio of 10:1. For baseline release of IL-8 and TNF-α, freshly isolated PMNLs were also incubated for 2 or 22 h without IFN-γ and GM-CSF. Untreated and treated PMNLs were added at the same E:T ratio to microtiter plate wells containing fungal hyphae and were suspended in $HBSS^-$ at 37°C for 4 h. At the end of the incubation period, supernatants were stored at −20°C until testing for IL-8 and TNF-α concentrations. TNF-α and IL-8 production was assessed using Quantikine ELISAs (R&D Systems). The sensitivities of the TNF-α and IL-8 assays were <4 and <10 pg/mL, respectively.

Statistical analysis. Each experiment was performed with the cells of 1 donor and by use of quadruplicate or octuplicate wells for each condition. The mean value of these replicate wells was taken as the value for this particular donor/experiment. The means of the replicate wells of each experiment were then used in the data analysis to calculate the mean ± SE of all the experiments at the same conditions. The statistics program Instat (version 3.0; Graphpad) was used for analysis. Differences in PMNL response to the zygomycetes were evaluated by repeated-measures analysis of variance (ANOVA) with Bonferroni posttest. Differences in antifungal effects between the cytokine-treated PMNLs and the untreated controls were performed using repeated-measures ANOVA with Dunnett's posttest for multiple comparisons. A 2-sided $P < .05$ indicated statistical significance.

RESULTS

O_2^- production. We evaluated the oxidative burst of PMNLs immediately after their isolation, by use of serum-opsonized or nonopsonized hyphae as stimuli (figure 1). Both serum-opsonized and nonopsonized hyphae of *R. oryzae, R. microsporus,* and *A. corymbifera* stimulated PMNLs to produce increased amounts of O_2^-, compared with production by unstimulated PMNLs ($P < .05$). Nonopsonized hyphae of *A. corymbifera* stimulated PMNLs to release significantly more O_2^- than did nonopsonized hyphae of either *Rhizopus* species ($P < .05$). This difference tended to be significant ($P < .1$) in the case of serum-opsonized hyphae.

Opsonized hyphae of *R. oryzae* and *R. microsporus* stimulated PMNLs to produce higher amounts of O_2^- than did nonopsonized hyphae of these fungi ($P < .001$). A trend of higher O_2^- production in response to serum-opsonized *A. corymbifera* hyphae than in response to nonopsonized hyphae also was evident.

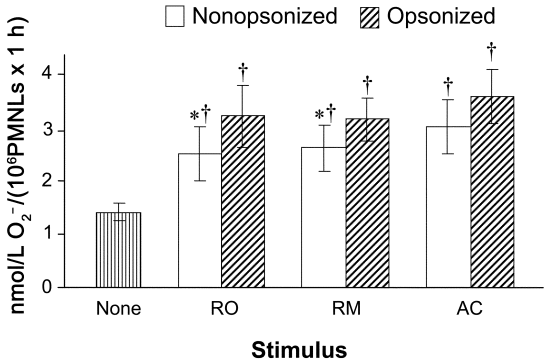

Figure 1. Superoxide anion (O_2^-) production by freshly isolated human polymorphonuclear leukocytes (PMNLs) challenged with no stimulus *(vertically striped bars)* or with nonopsonized *(white bars)* or serum-opsonized *(diagonally striped bars)* hyphae of *Rhizopus oryzae* (RO), *R. microsporus* (RM), and *Absidia corymbifera* (AC). PMNLs were added at an effector cell:target ratio of 1:1. Columns represent the means ± SEs of values derived from 7 experiments. Asterisks (*) indicate significant differences between nonopsonized *Rhizopus* species and *A. corymbifera,* evaluated by repeated-measures analysis of variance (ANOVA) with Bonferroni posttest. Daggers (†) indicate significant differences between all 3 species and nonstimulated PMNLs, evaluated by repeated-measures ANOVA with Dunnett's posttest for multiple comparisons ($P < .05$).

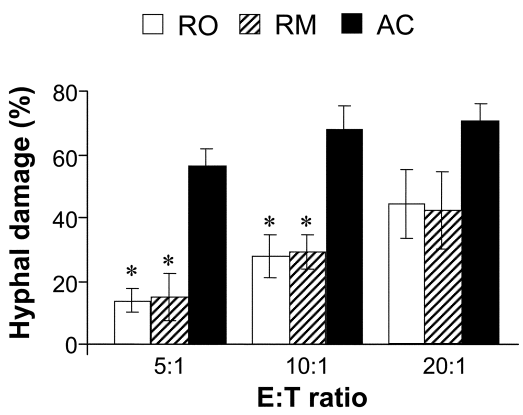

Figure 2. Percentage of damage induced by freshly isolated human polymorphonuclear leukocytes (PMNLs) on hyphae of *Rhizopus oryzae* (RO; *white bars*), *R. microsporus* (RM; *striped bars*), and *Absidia corymbifera* (AC; *black bars*). PMNLs were added at effector cell:target (E:T) ratios of 5:1, 10:1, and 20:1. Columns represent means ± SEs of values derived from 8 experiments. Differences between *Rhizopus* species and *A. corymbifera* in percentage hyphal damage at each E:T ratio were evaluated by repeated-measures analysis of variance with a Bonferroni posttest for multiple comparisons. *$P < .05$.

PMNL-induced hyphal damage. PMNLs induced hyphal damage of each of the 3 species in a E:T ratio–dependent manner, showing a significant linear trend ($P < .005$) (figure 2). PMNLs damaged hyphae of *A. corymbifera* to a significantly higher degree than they damaged hyphae of *Rhizopus* species, at E:T ratios of 5:1 and 10:1 ($P < .001$). A similar trend was observed at an E:T ratio of 20:1 ($P = .087$).

Effects of cytokines on PMNL oxidative burst. After incubation for 2 h, IFN-γ and GM-CSF alone or combined significantly increased O_2^- production by PMNLs in response to serum-opsonized hyphae of *A. corymbifera* ($P < .01$) (figure 3). In addition, GM-CSF and its combination with IFN-γ augmented PMNL oxidative burst in response to nonopsonized *A. corymbifera* hyphae ($P < .01$). However, only GM-CSF significantly increased the PMNL oxidative burst in response to nonopsonized hyphae of *R. oryzae* at 2 h ($P < .01$), and no cytokine affected the oxidative burst in response to serum-opsonized hyphae of *R. oryzae* at 2 h. In the case of *R. microsporus*, both GM-CSF and its combination with IFN-γ augmented the oxidative burst in response to nonopsonized hyphae ($P < .01$), whereas only GM-CSF showed an enhancing effect in response to serum-opsonized hyphae ($P < .05$). These effects were not evident after incubation for 22 h, since treated PMNLs released amounts of O_2^- similar to those released by untreated PMNLs.

Effects of cytokines on hyphal damage. PMNLs that had been treated with IFN-γ, GM-CSF, or their combination for 22 h exhibited a significantly increased capacity to induce hyphal damage of all 3 zygomycetes (figure 4). Whereas untreated PMNLs damaged 43.2% ± 8.4% of hyphae of *A. corymbifera*, PMNLs treated with IFN-γ, GM-CSF, or their combination damaged 78.4% ± 2.0%, 78.4% ± 2.0%, and 76.1% ± 3.8% of hyphae, respectively ($P < .01$). Further, whereas untreated PMNLs damaged 13.2% ± 10.1% of hyphae of *R. oryzae*, those treated with IFN-γ, GM-CSF, or their combination damaged 40.1% ± 6.9%, 40.6% ± 9.8%, and 47.4% ± 6.4% of hyphae, respectively ($P < .01$). Similar augmentation of hyphal damage was shown by cytokine-pretreated PMNLs against *R. microsporus* hyphae ($P < .05$).

The augmenting effects of IFN-γ and GM-CSF were time dependent, requiring prolonged incubation of PMNLs with the cytokines. Thus, PMNLs that had been pretreated with IFN-γ, GM-CSF, or their combination for only 2 h did not show a significant increase in induction of hyphal damage for any of the 3 species, with the exception of IFN-γ–pretreated PMNLs and subsequent challenge with *R. microsporus* hyphae ($P = .027$; data not shown).

Effects of cytokines on TNF-α and IL-8 release from PMNLs.

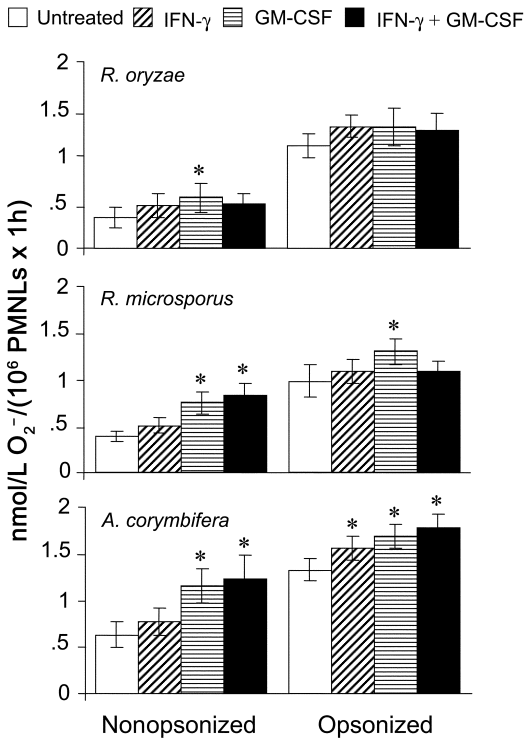

Figure 3. Superoxide anion (O_2^-) production by human polymorphonuclear leukocytes (PMNLs) incubated for 2 h without cytokines *(white bars)* or with 100 ng/mL interferon-γ (IFN-γ; *diagonally striped bars*), 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; *horizontally striped bars*), or both *(black bars)* and then challenged by nonopsonized or serum-opsonized hyphae of *Rhizopus oryzae (upper panel), R. microsporus (middle panel),* or *Absidia corymbifera (lower panel).* PMNLs were added at an effector cell:target ratio of 1:1. Columns represent means ± SEs of data derived from 8 experiments. Differences between cytokine-treated PMNLs and untreated controls were evaluated by repeated-measures analysis of variance with Dunnett's posttest for multiple comparisons. *$P < .05$.

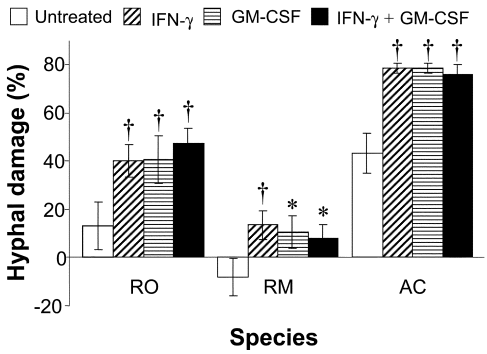

Figure 4. Percentage of hyphal damage induced by human polymorphonuclear leukocytes (PMNLs) incubated for 22 h without cytokines *(white bars)* or with 100 ng/mL interferon-γ (IFN-γ; *diagonally striped bars*), 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; *horizontally striped bars*), or both *(black bars).* PMNLs were added to hyphae of *Rhizopus oryzae* (RO), *R. microsporus* (RM), and *Absidia corymbifera* (AC) at an effector cell:target ratio of 5:1. Columns represent means ± SEs of data derived from 8 experiments. Differences between cytokine-pretreated and untreated PMNLs were evaluated by repeated-measures analysis of variance with Dunnett's posttest for multiple comparisons. $^{*}P < .05$; $^{\dagger}P < .01$.

After incubation of PMNLs with the combination of IFN-γ and GM-CSF for 2 h, TNF-α release from PMNLs in response to *R. microsporus* and *A. corymbifera* hyphae was significantly increased, compared with that from untreated PMNLs (figure 5, *upper panel*). Whereas TNF-α release from untreated PMNLs in response to *R. microsporus* hyphae was 477 ± 89 pg/mL, TNF-α release from PMNLs treated with the combination of IFN-γ and GM-CSF was augmented to 1222 ± 236 pg/mL ($P < .05$). Similarly, when untreated PMNLs were challenged with *A. corymbifera* hyphae, TNF-α release was 392 ± 118 pg/mL, whereas, in the case of PMNLs treated with the combination of IFN-γ and GM-CSF, it increased to 849 ± 286 pg/mL ($P < .05$). A significant enhancing effect of the combination of IFN-γ and GM-CSF also was observed in response to *R. microsporus* after 22 h (TNF-α release from untreated PMNLs was 354 ± 78 pg/mL, vs. 905 ± 193 pg/mL from PMNLs treated with both cytokines; $P < .05$), but there was a trend of enhancement in response to *A. corymbifera* hyphae (figure 5, *lower panel*). Comparable amounts of TNF-α were measured in untreated PMNLs and in PMNLs treated with each cytokine alone after 2 or 22 h in response to *R. microsporus* and *A. corymbifera.* When PMNLs treated for 2 or 22 h with IFN-γ, GM-CSF, or their combination were challenged with *R. oryzae* hyphae, there was no effect on TNF-α release.

PMNLs treated with IFN-γ for 2 h had significantly decreased IL-8 release in response to *R. oryzae, R. microsporus,* and *A. corymbifera* hyphae, compared with that of untreated PMNLs ($P < .01$) (figure 6, *upper panel*). The suppression of IL-8 release in response to all 3 zygomycetes became even more pronounced after incubation of PMNLs with IFN-γ for 22 h ($P < .01$) (figure 6, *lower panel*). Treatment of PMNLs with GM-CSF or its combination with IFN-γ for 2 or 22 h did not produce a significant effect on IL-8 release in response to any of these 3 zygomycetes; the only exception was GM-CSF treatment of PMNLs for 2 h and stimulation by *A. corymbifera.*

DISCUSSION

In this study, we have found that human PMNLs have a reduced capacity to mount an oxidative burst in response to both *Rhizopus* species and to induce hyphal damage of these zygomycetes, in comparison with its response to *A. corymbifera.* However, IFN-γ and GM-CSF augmented PMNL-induced hyphal damage of all 3 zygomycetes, in a time-dependent manner. Furthermore, treatment of PMNLs with the combination of cytokines enhanced the release of TNF-α in response to *R. microsporus* and *A. corymbifera* but not in response to *R. oryzae* hyphae. By comparison, IFN-γ inhibited IL-8 release in response to hyphae of the 3 zygomycetes. To our knowledge, this is the first time that intergenus differences in host response to zygomycetes have been reported and that the effects of IFN-γ and GM-CSF on PMNL response to these fungi have been described.

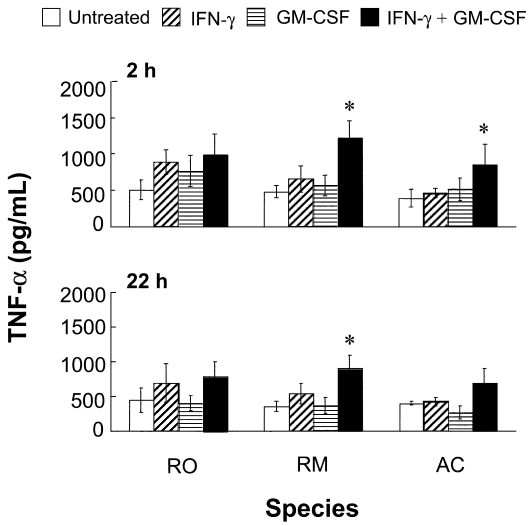

Figure 5. Release of tumor necrosis factor (TNF)–α by human polymorphonuclear leukocytes (PMNLs) treated without cytokines *(white bars)* or with interferon-γ (IFN-γ; *diagonally striped bars*), granulocyte-macrophage colony-stimulating factor (GM-CSF; *horizontally striped bars*), or both *(black bars),* as assessed by ELISA. Supernatants were assayed after incubation of PMNLs with or without 100 ng/mL IFN-γ and/or 100 ng/mL GM-CSF at 37°C for 2 h *(upper panel)* or 22 h *(lower panel)* and subsequent incubation of PMNLs with *Rhizopus oryzae* (RO), *R. microsporus* (RM), and *Absidia corymbifera* (AC) for 4 h. Columns represent means ± SEs of data derived from 5 experiments. Differences between cytokine-pretreated and untreated PMNLs were evaluated by repeated-measures analysis of variance with Dunnett's posttest for multiple comparisons. $^{*}P < .05$.

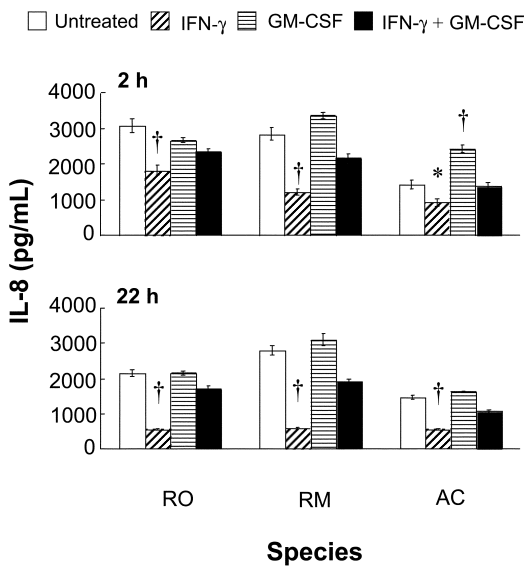

Figure 6. Release of interleukin-8 (IL-8) by human polymorphonuclear leukocytes (PMNLs) treated without cytokines *(white bars)* or with interferon-γ (IFN-γ; *diagonally striped bars*), granulocyte-macrophage colony-stimulating factor (GM-CSF; *horizontally striped bars*), or both *(black bars),* as assessed by ELISA. Supernatants were assayed after incubation of PMNLs with or without 100 ng/mL IFN-γ and/or 100 ng/mL GM-CSF at 37°C for 2 h *(upper panel)* or 22 h *(lower panel)* and subsequent incubation of PMNLs with *Rhizopus oryzae* (RO), *R. microsporus* (RM), and *Absidia corymbifera* (AC) for 4 h. Columns represent means ± SEs of data derived from 5 experiments. Differences between cytokine-pretreated and untreated PMNLs were evaluated by repeated-measures analysis of variance with Dunnett's posttest for multiple comparisons. $^{*}P < .05$; $^{\dagger}P < .01$.

Despite the long-standing clinical interest in zygomycetes as causative agents of serious and frequently fatal infections in immunocompromised and diabetic patients, little has been accomplished in recent years to improve the understanding of host defenses against zygomycetes and the role of cytokines. Indeed, the antifungal activity of PMNLs and macrophages, as well as the mechanisms involved, were investigated in pioneering studies 2 decades ago [9–13]. With the exception of 1 study demonstrating the ex vivo effects of granulocyte colony-stimulating factor in up-regulating PMNL-induced hyphal damage of *R. arrhizus* (synonymous with *R. oryzae*) [30], no studies have examined the effects of any cytokines on multiple species of zygomycetes.

The finding that both *Rhizopus* species stimulated PMNL oxidative burst less often than did *A. corymbifera* and suffered less hyphal damage from PMNLs may be related to the relatively high frequency of *Rhizopus* species and the comparatively lower frequency of *A. corymbifera* as causative agents of zygomycosis [1–5]. These data suggest that reduced susceptibility to innate host defense by members of the genus *Rhizopus* may contribute to their greater prevalence as pathogens in immunocompromised patients. Differences in cell-wall constituents and ligands on the surface of the hyphae of the different genera of zygomycetes may account for the differences in the stimulation of PMNLs and the susceptibility to their antifungal activity. An alternative explanation that may account for such differences is that organism-related factors (e.g., diffusible components of the fungal surface or production of toxins by *Rhizopus* hyphae) compromise any effects of PMNLs challenged with that fungus. That soluble factors from *R. oryzae* may enhance the PMNL respiratory burst was suggested in the study by Liles et al. [30]. Similar to hyphae of other filamentous fungi, such as *A. fumigatus, Fusarium* species, and *Scedosporium* species [12, 31, 32], hyphae of zygomycetes induced an increase in O_2^- production, compared with nonstimulated PMNLs, and the antihyphal activity induced by PMNLs was E:T ratio dependent.

In this study, we describe—for the first time, to our knowledge—a modification of the XTT assay previously used for the study of hyphal damage of other filamentous fungi [29, 31–33], to study PMNL-zygomycete interactions. Since hyphae of zygomycetes adhere to the plastic surface of wells very loosely, we centrifuged the plates before each aspiration and took care to leave a small amount of H_2O in the wells during the washes. With this modification, XTT, an easily performed nonradioisotopic assay, could be used for the study of hyphal damage of zygomycetes instead of a radioactive assay, which previous investigators were required to use [13].

IFN-γ and GM-CSF are among the cytokines most frequently used for prevention and treatment of invasive fungal infections [18]. This and the fact that they, in theory, have many favorable features for immune up-regulation against zygomycosis prompted us to investigate their activities against these organisms. Indeed, GM-CSF has been found to augment phagocytosis and oxidative burst and to increase the number and membrane expression of several classes of surface receptors on PMNLs, such as FMLP, CD11b, and C3bi [23, 34, 35]. Additionally, GM-CSF increases PMNL fungicidal activity against several pathogenic species of fungi [20, 21, 36–38] and protects mice in 2 models of deep candidiasis [39, 40]. Similarly, IFN-γ induces a Th1 response, which favors resistance to invasive fungal infections [18, 21, 41]. This cytokine primes O_2^- production of PMNLs in response to FMLP, in a time-dependent manner, by a mechanism that involves synthesis of proteins [42, 43]. Additionally, IFN-γ enhances PMNL fungistatic and fungicidal activities against certain fungal pathogens [21, 37, 38, 44–46]. Administration of this cytokine to experimental animals with fungal infections has yielded encouraging results [47–50]. In patients with chronic granulomatous disease, IFN-γ reduced the risk of infections by a mechanism independent from O_2^- production [51], and administration of IFN-γ to healthy volunteers improved their phagocytic host defense, indicating that this cytokine may be useful in the treatment of patients with other immune disorders [52].

We found a time dissociation between augmentation of O_2^- production and hyphal damage by treatment of PMNLs with IFN-γ and GM-CSF. Whereas the optimal time for increase of O_2^- production was 2 h, the optimal time for increase of hyphal damage was 22 h. This time dependency in response to stimuli, which has previously been noted, to a lesser extent, with other filamentous fungi [53, 54], may be due to (1) increased amounts of myeloperoxidase or other antifungal proteins, as a result of augmented signal transduction and synthesis of proteins induced by the cytokines; (2) posttranslational processing of these proteins; and/or (3) an increased rate of degranulation of PMNLs releasing antifungal proteins. These data also demonstrate a potentially critical role for nonoxidative mediators (e.g., antimicrobial peptides) in mediating the enhanced PMNL host response to *Rhizopus* and *Absidia* species. Indeed, although IFN-γ had no effect on O_2^- production in response to *Rhizopus* species, it substantially enhanced hyphal damage of all 3 zygomycetes.

The combination of IFN-γ and GM-CSF showed no better effects than IFN-γ or GM-CSF alone in enhancing hyphal damage or O_2^- production. However, the combination induced differentiation of PMNLs into major histocompatibility complex class II–expressing antigen-presenting cells [55–58], enabling them to participate in the subsequent T cell response. Although these results are derived from in vitro models, we suggest that the combination may have therapeutic potential that warrants further evaluation in animal models of zygomycosis.

Our study also demonstrated species-specific increases of TNF-α release as a result of treatment with the combination of IFN-γ and GM-CSF, in contrast to the decreased IL-8 release from IFN-γ–treated PMNLs. These data reflect different immunoregulatory mechanisms in host defenses against each of the 3 zygomycetes. Perhaps the down-regulation of IL-8 expression by IFN-γ–treated PMNLs is induced by a negative feedback pathway to modulate the inflammatory response against zygomycetes.

The data provided in this study contribute to a better understanding of the pathogenesis of zygomycosis. Because of the difficulty in treatment and the high mortality of patients with infections caused by zygomycetes, IFN-γ and GM-CSF may prove to be useful therapeutic adjuncts. Further investigations in immunocompetent and immunosuppressed animal models (e.g., corticosteroid-treated or NADPH-oxidase–knockout mice) and, ultimately, clinical trials are warranted to assess the utility of IFN-γ and GM-CSF as adjuncts to conventional antifungal chemotherapy.

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