Cytokine- and T Helper–Dependent Lung Mucosal Immunity in Mice with Invasive Pulmonary Aspergillosis

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The role of cytokine- and T helper (Th)–dependent lung mucosal antifungal immunity in murine invasive pulmonary aspergillosis (IPA) was investigated. Intact or leukopenic DBA/2 mice were resistant or highly susceptible, respectively, to infection caused by multiple intranasal injections of viable Aspergillus fumigatus conidia. Resistance was associated with unimpaired innate antifungal activity of pulmonary phagocytic cells, concomitant with high-level production of tumor necrosis factor (TNF)-α and interleukin (IL)-12 and the presence of interstitial lymphocytes producing interferon-γ and IL-2. Conversely, production of TNF-α and IL-12 was down-regulated in highly susceptible mice, which also had defective innate antifungal immunity and high-level production of IL-4 and IL-10 by lung lymphocytes. Resistance was increased in susceptible mice upon local IL-4 or IL-10 neutralization or IL-12 administration. These results indicate that, similar to observations in mice with disseminated aspergillosis, innate and Th1-dependent immunity play an essential role in host defense against IPA.

Invasive pulmonary aspergillosis (IPA), characterized by hyphal invasion and destruction of pulmonary tissue, is the most common manifestation of Aspergillus fumigatus infection in immunocompromised persons [1–3]. Neutropenia and administration of broad-spectrum antibiotics and corticosteroids are major predisposing conditions to this form of aspergillosis [3–5]. Effector mechanisms of the innate immune system are major host defenses against IPA [6]. Resident alveolar macrophages ingest and kill resting conidia, mainly through non-oxidative mechanisms, while neutrophils use oxygen-dependent mechanisms to attack hyphae germinating from conidia that escape macrophage surveillance [7–9]. The effectiveness of this system is evident from the observation that challenge, even with large numbers of conidia, fails to cause disease in immunocompetent animals [10]. Major risk factors for disease are defects in phagocyte respiratory burst (e.g., those occurring in chronic granulomatous disease) [11–14], cortisone-induced suppression of macrophage conidioidal activity [15, 16], and natural [17] or chemotherapy-induced neutropenia [3].

Increased risk of a chronic form of IPA, independent of neutropenia and corticosteroid therapy, occurs in persons with human immunodeficiency virus (HIV) infection [2, 18], who also show defective effector activity of neutrophils against A. fumigatus [19]. The recent suggestion that interleukin (IL)-10, produced at increased levels during HIV infection [20], may have a pathogenetic role in invasive aspergillosis [21] indicates that a dysregulated production of T helper (Th) cytokines may contribute to the pathogenesis of IPA. Th1 (interferon [IFN]-γ) and Th2 (IL-4 and IL-10) cytokines have opposite effects on antifungal effector functions of phagocytes [22].

In a murine model of invasive aspergillosis, Th1 and Th2 cytokines were produced differently in mice resistant or susceptible to infection [23, 24]. Development of protective acquired immunity was associated with the activation of CD4+ Th1 cells producing IFN-γ and macrophages producing IL-12. In contrast, production of IL-4 and IL-10 by CD4+ Th2 cells was associated with disease progression, such that IL-4 neutralization resulted in the induction of protective antifungal immunity. In addition to IL-4 neutralization, IFN-γ administration had a curative effect in this infection [24]. Treatment with IFN-γ rescued infected animals from death and was associated with early increased expression of both IFN-γ– and IL-12–specific messages. Conversely, IFN-γ neutralization resulted in increased pathology and, concomitantly, increased expression of the IL-10 message. These results are reminiscent of those obtained in a murine model of disseminated candidiasis in which resistance and susceptibility to infection were causally related to the dissimilar expansion of functionally distinct CD4+ Th cell subsets [25–27]. Th1 cells confer protection in genetically resistant mice and in susceptible mice treated with IL-4 or IL-10 antagonists. In contrast, Th2 responses are associated with disease progression and onset of nonprotective responses, as
observed in mice treated with IFN-γ or IL-12-neutralizing antibodies.

To define the importance of the different Th cell subsets and cytokines in IPA, we developed a murine model of IPA in which we assessed production of inflammatory and Th cytokines and the effects of cytokine administration or neutralization during the course of infection.

Materials and Methods

Mice. We used female inbred DBA/2 (H-2b) mice (Charles River, Calco, Italy) and tumor necrosis factor (TNF)/LT-α/− and wild type TNF/LT-α/+ mice on a mixed 129SV × C57BL/6 background [28, 29], of both sexes, bred under specific pathogen-free conditions. All mice were 8- to 10-weeks old.

Microorganism, culture conditions, and infections. We used an A. fumigatus strain from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia. The microorganism was grown on Sabouraud dextrose agar (Difco, Detroit) supplemented with chloramphenicol for 4 days at room temperature. Abundant conidia were elaborated under these conditions. Conidia were harvested by washing the slant culture with 5 mL of 0.025% Tween 20 in normal saline and gently scraping the conidia from the mycelium with a plastic pipette (or by shaking vigorously). Cell debris was allowed to settle by gravity, and the suspension was decanted into 50-mL plastic conical tubes. Conidia were extensively washed with saline, then counted and diluted to the desired concentrations. Conidia were >95% viable (determined by serial dilution and plating of the inoculum on Sabouraud dextrose agar). For primary intranasal infection, mice were lightly anesthetized with inhaled diethyl ether before instillation (on 3 consecutive days) with a suspension of conidia in 20 mL of sterile saline slowly applied to the nostrils by micropipette with a sterile disposable tip. Animals were held upright until the suspension was decanted into 50-mL plastic conical tubes. Conidia were extensively washed with saline, then counted and diluted to the desired concentrations. Conidia were >95% viable (determined by serial dilution and plating of the inoculum on Sabouraud dextrose agar). For primary intranasal infection, mice were lightly anesthetized with inhaled diethyl ether before instillation (on 3 consecutive days) with a suspension of 2 × 107 conidia in 20 mL of sterile saline slowly applied to the nostrils by micropipette with a sterile disposable tip. Animals were held upright until the suspension was completely inhaled and normal breathing resumed. For secondary infections, 14 days after primary challenge, mice were injected intravenously via the lateral tail vein with 107 conidia in 0.5 mL of sterile saline or intranasally with 2 × 107 conidia for 3 consecutive days. Mice succumbing to fungal challenge were routinely necropsied for histopathologic confirmation of invasive aspergillosis. For histology, tissues were excised and immediately fixed in formalin. Sections (3–4 μm) of paraffin-embedded tissues were stained with periodic acid–Schiff. The Gomori methenamine silver-staining procedure was always used to confirm the presence of fungal cells.

Chitin assay. We used the chitin assay to quantify fungal growth in organs as described [30, 31]. In brief, organ samples were homogenized in 5 mL of 0.9% NaCl, centrifuged, resuspended in 4 mL of 3% sodium lauryl sulfate (Sigma, St. Louis), and heated to 100°C for 15 min. After cooling, the pellet was washed once with distilled water, resuspended in 3 mL of 120% (wt/vol) KOH solution, and heated to 130°C for 1 h. After 8 mL of ice-cold 75% ethanol was added, tubes were kept at 4°C for 15 min, and 0.3 mL of Celite suspension (Celite 545; Sigma) was added. After centrifugation, the pellet was washed with cold ethanol (40% [vol/vol]) and cold distilled water, and 0.5 mL of NaNO2 (5% [wt/vol]) and 0.5 mL of KHSO4 (95% [wt/vol]) were added to the pellet. After centrifugation, volumes of supernatant were mixed with ammonium sulfamate (NH4SO4·NH2·H2O, 12.5% [wt/vol]) and then with MBTH (3-methyl-benzo-2-thiazolone hydrazide HCl monohydrate; Sigma). After heating for 3 min and cooling, FeCl3·6H2O (0.83%) was added; the tubes were left standing for 30 min, and the optical density at 650 nm was read with a spectrophotometer. The chitin content of the organs was expressed as micrograms of glucosamine per organ.

In vivo analysis and treatments. For differential cell counts, cytospin preparations of collagenase-treated lung cells were stained with May-Grünewald Giemsa reagents (Sigma) before analysis. To improve distinction between neutrophils and eosinophils, a modified Diff-Quik whole blood stain procedure was used. For immunosuppression, mice were injected intraperitoneally with 150 mg/kg of cyclophosphamide (Sigma) 1 day before challenge or subcutaneously with 125 mg/kg/injection of cortisone acetate (Sigma) 3 days before, the day of, and 2 and 4 days after challenge. Long-lasting neutrophil depletion was obtained, as described [32], by intraperitoneal administration of anti-Ly6G (RB6-8C5) affinity purified monoclonal antibody (MAb; gift of R. Coffman, DNAX, Palo Alto, CA) at a dose of 100 μg/injection, the day before and 2 days after infection. For cytokine-anticytokine treatments, doses and time of administration were as described [33–35]. Soluble IL-4 receptor (sIL-4R; Behringwerke, Marburg, Germany) and recombinant (r) mouse IL-12 (Genetics Institute, Cambridge, MA) were given subcutaneously or intranasally at 10 μg/injection or 100 ng/injection, respectively, the day of the first fungal challenge and 1, 2, and 3 days later. Affinity purified anti-mouse IL-10 MAb (from the SXC-1 hybridoma; American Type Tissue Collection, Rockville, MD) was given subcutaneously, the day of the first fungal challenge and 1 day later (100 μg/injection). Control mice received vehicle alone or isotype-matched antibodies (Zymed Laboratories, South San Francisco). Endotoxin was removed from all solutions with Detoxi-gel (Pierce Chemical, Rockford, IL).

Collection of bronchoalveolar fluid (BAL). Lungs were lavaged thoroughly with 0.5 mL of PBS injected via the trachea. The lavage fluid was centrifuged, and the supernatant was removed and immediately stored at −80°C until analysis of cytokine contents.

Isolation and culture of interstitial lung lymphocytes and phagocytes. Intestinal lung lymphocytes were isolated as described [36]. The pulmonary vasculature was perfused with 5 mL of PBS containing 100 U/mL heparin to eliminate peripheral blood cells, and the lungs were removed, minced, and incubated for 90 min at 37°C in digestion buffer containing 0.7 μg/mL collagenase (Sigma)

| Table 1. Course of invasive pulmonary aspergillosis in intact or immunocompromised DBA/2 mice. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| treatment | median survival (days) | no. of dead mice/ no. infected |
|None | >60 | 4/18 |
|Cyclophosphamide | 5 | 18/18 |
|Cortisone acetate | 6 | 12/12 |
|Anti-Ly6G MAb | 5 | 12/12 |

* Mice were given multiple intranasal injections of viable Aspergillus fumigatus conidia (2 × 105 on days 0, 1, and 2. Treatments were as follows: cyclophosphamide (150 mg/kg) intraperitoneally (ip) on day before 1st challenge; cortisone acetate (125 mg/kg/ip) 3 days before, day of 1st fungal inoculation, and 2 and 4 days later; anti-Ly6G monoclonal antibody (MAb) (100 μg/mouse/ip), 1 day before 1st fungal challenge and 2 days later.
Table 2. Course of local or systemic Aspergillus fumigatus reinfection in DBA/2 mice with primary invasive pulmonary aspergillosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary infectiona</th>
<th>Secondary infectionb</th>
<th>MST</th>
<th>D/T</th>
<th>Chitin contentc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 × 10^4 in</td>
<td>—</td>
<td>&gt;60</td>
<td>2/12</td>
<td>22.69</td>
</tr>
<tr>
<td>2</td>
<td>8 × 10^4 in</td>
<td>8 × 10^5 in</td>
<td>&gt;60</td>
<td>0/6</td>
<td>0.19d</td>
</tr>
<tr>
<td>3</td>
<td>8 × 10^4 in</td>
<td>1 × 10^5 iv</td>
<td>6</td>
<td>6/6</td>
<td>2.62^d</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>1 × 10^4 iv</td>
<td>3</td>
<td>6/6</td>
<td>4.38</td>
</tr>
</tbody>
</table>

NOTE. MST = median survival time (days); D/T = no. of dead mice/no. infected.

a Mice were given multiple intranasal (in) injections of viable A. fumigatus conidia (2 × 10^7) on days 0, 1, and 2.

b Mice were reinjected as in (a) or with 1 intravenous (iv) injection of 10^7 A. fumigatus conidia.

c Chitin contents in lungs (groups 1 and 2) and kidneys (groups 3 and 4) expressed as μg of glucosamine/organ.

d P < 0.05 (groups 2 vs. 1 and 3 vs. 4).

and 30 μg/mL bovine pancreatic DNase I (Sigma). Large particulate matter was removed by passing the cell suspension through a small loose nylon-wool plug, and lymphocytes were enriched over a single-step Ficoll gradient. Cells were resuspended in RPMI containing 10% fetal calf serum (FCS), 2-mercaptoethanol (50 μM), sodium pyruvate (1 mM), HEPES (10 mM), and gentamicin (50 μg/mL) and plated at a concentration of 2 × 10^5/mL in 96-well microtiter plates in the presence of 10 μg/mL concanavalin A (Sigma). After culture for 48 h, supernatants were harvested, and cytokine production was determined by specific ELISAs. Purified lung macrophages and neutrophils to be assessed for antifungal activity were obtained by 2-h plastic adherence or by positive selection with the anti-granulocyte MAbs, RB6-8C5, respectively, as described [32].

Cytokine and IgE assays. The levels of TNF-α, IFN-γ, and IL-2, -4, -5, -6, and -10 in BAL fluids and culture supernatants of activated cells were determined by cytokine-specific ELISAs, using pairs of anti-cytokine MAbs as described [23, 29, 34, 35, 37]. The antibody pairs used were as follows, listed by capture/biotinylated detection: TNF-α, MP6-XT22/MP6-XT3; IFN-γ, R4-6A2/XMG1.2; IL-2, JES6-1A12/JES6-5H4; IL-4, BVD4-1D11/BVD6-24G2; IL-5, TRFK-5/TRFK-4; IL-6, MP5-20F3/MP5-32c11; and IL-10, JES5-2A5/SXC-1 (PharMingen, San Diego). For IL-12 p70 measurement, a modified antibody-capture bioassay was used [38]. Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines (all from PharMingen, except IL-12, from Genetics Institute). A micro-ELISA procedure was used to quantitate total IgE in BAL fluid, as described [25].

Hyphal damage and conidialidal assays. We used a colorimetric MTT assay [39] to study Aspergillus hyphal damage by lung cells. Graded numbers (10^6, 5 × 10^6, and 10^7) of lung cells were added to 10^5 conidia that had been cultured at 37°C in 5% CO2 with FCS for 16–18 h in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). After culture, >95% of the conidia had germinated to hyphae (~150–200 μm long). After 2 h at 37°C with occasional shaking, the supernatants were aspirated, effector cells were lysed by the addition of sodium deoxycholate (0.5%), and hyphal viability was determined by MTT staining. For conidialidal activity, 10^5 conidia were mixed with 10^5, 5 × 10^5, and 10^4 lung cells for 3 h in 96-well flat-bottomed microtiter plates (Costar), and the number of colony-forming units (cfu) was determined as described [40]. In selected experiments, purified lung macrophages and neutrophils were assessed for conidialidal activity and hyphal damage. The percentage of cfu inhibition (mean ± SE) was determined as percentage of colony formation inhibition: I = [100 − (cfu experimental group/cfu control cultures)] × 100.

Statistical analysis. Survival data were analyzed using the Mann-Whitney U test; significance was defined as P < 0.05. Student’s t test was used to determine statistical significance between organ clearance and differential cell counts. In vivo groups consisted of 4–6 animals. The data reported were pooled from 3–5 experiments.

Results

Course of IPA in intact or immunocompromised mice. To develop a model of IPA, we used DBA/2 mice, which are highly susceptible to pulmonary and invasive aspergillosis [23, 41]. Because the establishment of primary pulmonary aspergillosis in mice is dependent on the dose of the fungus [10], intact or differently immunocompromised mice were given multiple intranasal administrations of 2 × 10^5 viable conidia and monitored for mortality and fungal growth in organs. The results (table 1) show that most intact mice survived infection, as opposed to mice treated with cyclophosphamide or cortisone acetate or neutrophil-depleting MAb, which all succumbed. On assaying surviving mice for resistance to subsequent intranasal or intravenous reinfection, we found that, although the survival time was not significantly modified, fungal growth was significantly impaired in the lungs and kidneys of mice reinfected intranasally or intravenously, respectively (table 2). Resistance to infection correlated with limited fungal growth in different organs, as observed in intact mice 1 day after the last fungal infection.

![Figure 1](image-url). Chitin content in organs of intact (open bars) or cyclophosphamide-treated (solid bars). DBA/2 mice inoculated intranasally with 2 × 10^5 viable conidia of Aspergillus fumigatus on days 0, 1, and 2. Cyclophosphamide (150 mg/kg) was given intraperitoneally 1 day before first fungal challenge. Chitin content was measured 1 day after final fungal challenge. *P < 0.05, cyclophosphamide-treated vs. intact infected mice.
Figure 2. Histology of lungs of mice with invasive pulmonary aspergillosis. Periodic acid-Schiff-stained sections were prepared from lungs of intact (A) or cyclophosphamide-treated (B) mice 1 day after last intranasal infection with $2 \times 10^7$ viable *Aspergillus fumigatus* conidia, given on days 0, 1, and 2. There were few infiltrates in intact mice (arrows), consisting of rare hyphae and inflammatory mononuclear cells (inset, A) in peribronchial region. Numerous septate-branching hyphae penetrating bronchial wall with signs of peribronchial necrosis and infiltrating neutrophils were observed in cyclophosphamide-treated mice (inset, B). Bar = 400 μm.
Figure 3. Cell recruitment to lung of intact or cyclophosphamide (Cy)-treated mice with invasive pulmonary aspergillosis. Mice were infected intranasally with $2 \times 10^7$ viable *Aspergillus fumigatus* conidia on days 0, 1, and 2. Cytospins of collagenase-treated lung cells were prepared 1 day after last fungal inoculation and stained with May-Grünwald–Giemsa. Each point is mean ± SE of 4–6 mice. $P < .05$, *infected vs. uninfected intact or ** intact vs. infected mice.

Figure 4. Conidiocidal activity and hyphal damage of lung cells from intact or cyclophosphamide (Cy)-treated mice with invasive pulmonary aspergillosis. Mice were infected intranasally with $2 \times 10^7$ viable *Aspergillus fumigatus* conidia on days 0, 1, and 2. Day after last fungal challenge, ability of total lung cells to kill conidia and damage hyphae was assessed (see Materials and Methods). $P < .05$, *infected vs. uninfected intact or ** Cy-treated infected vs. intact infected mice.
inoculation (figure 1). Infection was nearly cleared in 10 days (data not shown). In contrast, extensive fungal growth, particularly in the lungs, occurred in cyclophosphamide-treated mice (figure 1) and in mice treated with cortisone acetate or neutrophil-depleting MAb (data not shown).

Histopathologic examination of lung sections from mice with IPA revealed a pattern of lesions similar to those observed in invasive and noninvasive pulmonary aspergillosis [6, 42]. Intact resistant mice had few infiltrates; these consisted of rare, small hyphae with a moderate infiltration of inflammatory (predominantly mononuclear) cells in the peribronchial region (figure 2A). In contrast, lung sections from cyclophosphamide-treated mice had numerous septate branching hyphae that penetrated through tissue with signs of bronchial wall damage, peribronchial necrosis, and numerous infiltrating polymorphonuclear cells (figure 2B).

Cell recruitment in the lungs of mice with IPA. Because recruitment of leukocytes into the lung correlated with clearance in a model of fungal pneumonia [43], we characterized the cell influx in the lungs of mice resistant or susceptible to IPA. Cytospin preparations of collagenase-treated lung cells were prepared from intact or cyclophosphamide-treated mice 1 day after the last fungal inoculation. An early moderate influx of lymphocytes and phagocytic cells (e.g., neutrophils and monocytes) was observed in the lungs of intact mice upon infection. These mice also had a significant increase in eosinophils. Despite profound leukopenia, the number of neutrophils, eosinophils, and macrophages was significantly increased in the lungs of mice that succumbed to infection (figure 3). Flow cytometry of lung cells stained with relevant MAb showed the results obtained with cytospin preparations and also showed that upon infection in intact and cyclophosphamide-treated mice, respectively, interstitial lymphocytes consisted of αβ T cell receptor (TCR)-bearing CD3+ cells (31% and 21%), γδ TCR-bearing CD3+ cells (9% and 14%), B220+ cells (35% and 37%), and NK 5E6+ cells (19% and 27%).

Antifungal activity of phagocytic cells in mice with IPA. Monocytes and neutrophils are a first line of defense against Aspergillus conidia and hyphae, respectively [7]. Therefore, we assessed the conidoidal activity and hyphal damage of lung cells from intact or cyclophosphamide-treated mice 1 day after the last fungal inoculation. Because the numbers of recruited lung monocytes and neutrophils were different in the 2 groups of mice, graded numbers of lung cells were seeded for a better comparison (on a per cell basis) of the antifungal activity between groups. It was found that upon infection, both the co-
Production of inflammatory cytokines in mice with IPA. Cytokines and chemokines regulate the development of lung inflammatory responses [43–45]. We assessed the production of cytokines known to be involved in lung inflammatory responses: TNF-α and IL-6, -10, and -12. For this purpose, BAL fluid was collected from intact or cyclophosphamide-treated mice soon after infection and assessed for cytokine content by specific ELISAs. Increased production of TNF-α, IL-6, IL-12p70, and, to a lesser extent, IL-10 was observed in intact mice upon infection. In contrast, production of IL-12p70 and TNF-α was impaired but that of IL-6 and IL-10 was greatly increased in cyclophosphamide-treated (figure 5) and in neutropenic mice (data not shown).
Table 3. Course of pulmonary invasive aspergillosis in tumor necrosis factor (TNF)/LT-α−deficient mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>MST</th>
<th>D/T</th>
<th>Day 1</th>
<th>Day 7</th>
</tr>
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<tbody>
<tr>
<td>TNF/LT-α−/−</td>
<td>&gt;60</td>
<td>0/6</td>
<td>14.44</td>
<td>0.08</td>
</tr>
<tr>
<td>TNF/LT-α+/−</td>
<td>&gt;60</td>
<td>0/6</td>
<td>31.36</td>
<td>0.29</td>
</tr>
</tbody>
</table>

NOTE: MST = median survival time (days); D/T = no. of dead mice/no. infected.
a Mice were given multiple intranasal (in) injections of viable *A. fumigatus* conidia (2 × 10⁵) on days 0, 1, and 2.
b Chitin content in lungs expressed as μg of glucosamine/organ at different days after last fungal challenge.
c P < 0.05 (TNF/LT-α−/− vs. TNF/LT-α+/− mice).

d (treated vs. untreated mice).

d sIL-4R (10 g/injection) was given day of 1st fungal challenge and 1 day later. Anti–IL-10 MAb (100 μg/injection) was given day of 1st fungal challenge and 1 day later.

e Chitin content in lungs expressed as μg of glucosamine/organ.
f P < 0.05 (treated vs. untreated mice).

Th1 and Th2 cytokine production in mice with IPA. To correlate susceptibility and resistance to IPA with Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokine production by intraparenchymal lymphocytes, cytokine levels were measured in culture supernatants of mitogen-stimulated lymphocytes from infected intact or cyclophosphamide-treated mice. Total IgEs were also measured in BAL fluids. The results showed that the production of IFN-γ, IL-2, and IL-5 greatly increased in intact but not in cyclophosphamide-treated mice upon infection (figure 6) and remained high until clearance (data not shown). In contrast, IL-4 production was only minimally detected in intact mice but was greatly increased in cyclophosphamide-treated infected mice (figure 6). Production of IL-10 paralleled that of IL-4 and was maximally produced in leukopenic mice (data not shown). IgE levels were increased in both types of mice upon infection, although to a lesser extent in intact than in leukopenic mice (figure 6). A similar pattern of antibody and Th cytokine production was observed in neutropenic mice (data not shown).

Effect of cytokine administration or neutralization on the course of IPA. Because cytokine administration or neutralization could profoundly affect the course of fungal infections [46, 47], we evaluated the course of IPA in TNF/LT-α−/−deficient mice or in immunocompromised mice upon IL-4 or IL-10 neutralization or rIL-12 administration. Mice were monitored for mortality and fungal growth in the lungs. The numerous fungal cells recovered from the lungs 1 and 7 days after the last fungal challenge (table 3) showed that TNF/LT-α−/−deficient mice were more susceptible to infection than were wild type mice. Of interest, neutrophils and macrophages were similarly recruited to the lungs of both types of mice (data not shown). Eventually, all mice survived infection. Survival was associated with production of IFN-γ and IL-2, but not IL-4, by interstitial lymphocytes (data not shown). By assaying the effects of sIL-4R, anti-IL-10 MAb, or rIL-12 administration by different routes, we found that treatment with sIL-4R given subcutaneously greatly reduced the fungus load in the lungs and rendered mice resistant to infection (table 4). The same results, although to a lesser extent, were obtained with intranasal sIL-4R or anti-IL-10 MAb treatment. rIL-12 was effective when given subcutaneously. It significantly decreased the fungus burden and increased survival of neutropenic mice (table 4).

Discussion

The results of this study show that in a murine model of IPA, the production of inflammatory and Th cytokines differs in mice resistant or susceptible to infection. Resistance was associated with elevated production of TNF-α, IL-6, and IL-12 early in infection and with intraparenchymal Th1 lymphocytes producing IFN-γ and IL-2. Mice resisting primary infection also had increased resistance to subsequent local or systemic reinfection, which indicates that protective immunity was acquired after primary IPA. Conversely, in susceptible mice, early production of TNF-α and IL-12 was decreased but that of IL-10 was increased; this correlated with high-level production of IL-4 and IL-10 by intraparenchymal lymphocytes. Of interest, IL-5 production did not increase in immunocompromised mice upon infection, indicating that production of IL-5 and of IL-4 and -10 are differently regulated in the lungs of mice with IPA. Production of IL-4, although at a minimal level, was detected in resistant mice together with measurable levels of IgE in BAL fluid. This finding may reflect a complex immunoregulatory network occurring at the mucosal level in response to fungal colonization or infection, or both, as shown by results in mice with mucosal candidiasis in which both Th1 and Th2 cell responses are present, although resistance is mediated by Th1 cells [48, 49]. It is also possible that fungal allergens could be generated in vivo to induce Th2 cytokine production as suggested [50]. This likely occurs in persons with allergic bronchopulmonary aspergillosis whose antigen-specific T cell lines are characterized as CD4 Th2-like in their cytokine synthesis pattern [51] and in mice that produce high levels of...
IL-4 or IL-5, circulating IgE, and eosinophils in response to particulate *Aspergillus* antigens [52].

IL-4 or IL-10 neutralization increased resistance to IPA in immunocompromised mice, suggesting a pathogenetic role for Th2 cytokines in infection. We also observed the efficacy of sIL-4R treatment upon local administration. These results are reminiscent of those seen in a murine model of mucosal candidiasis in which administration of IL-4 or -10 exacerbated the infection [53] but IL-4 [33] or IL-10 (unpublished data) neutralization increased resistance to it.

In addition to their role in regulating the generation and effector functions of antifungal Th cells [27], cytokines also affect the expression of antifungal effector and immunomodulatory functions of phagocytic cells [22]. Against *Aspergillus* species, different cytokines have opposite effects on phagocytic host defenses [21, 54–59]. The oxidative responses and hyphal damage of phagocytic cells are potentiated by IFN-γ and colony-stimulating factors [54–59] and suppressed by IL-10 [21].

In the present study, we found that the ability of pulmonary phagocytic cells to kill hyphal elements and conidia is up-regulated in mice resistant to infection and severely depressed, particularly the killing of hyphae in mice with progressive invasive infection. Quantitative changes in phagocytic antifungal activity were evident soon after infection when cytokines with positive and negative effects on phagocytic antifungal effector functions were also detected. Thus, the regional cytokine milieu may dictate the effectiveness of phagocytic host defenses against *Aspergillus* infection in the lungs. In addition, cytokines, such as TNF-α [60] and chemokines [43], may also actively participate in initial host defense against pulmonary fungal infection via cell recruitment. We found that TNF-α production was increased in resistant mice and decreased in susceptible mice. The early control of infection was greatly impaired in TNF/IL-10-α/deficient mice. However, lung recruitment of phagocytic cells in TNF/IL-10-α-deficient mice was not modified compared with wild type mice, which may indicate multiple mechanisms through which TNF-α contributes to host defense against *A. fumigatus*. A murine model of pulmonary cryptococcosis showed that endogenous TNF-α may mediate its protective effect through induction of IL-12 [61].

IL-12 is required and prognostic for development of protective Th1 responses against fungal infections [62], and exogenous administration of IL-12 has a protective effect on mice with pulmonary cryptococcosis [63]. We found that production of IL-12 was impaired early in infection in highly susceptible mice and that exogenously administered IL-12 increased resistance in these mice. Also, resistance of intact mice to infection was impaired upon IL-12 neutralization (unpublished data). These findings indicate that IL-12 may be required for optimal development of antifungal immunity in mice with IPA. Whether IL-12 exerts its protective effect through inhibition of Th2 cytokine production, as found in a murine model of allergic asthma [64], or through its ability to sustain production of cytokines activating the fungicidal activity of phagocytic cells remains to be determined. The finding that exogenous IL-12 could only partially restore resistance in cyclophosphamide-treated mice (data not shown) is in line with previous findings in mice with candidiasis. In those studies, exogenous IL-12 failed to increase resistance of susceptible mice to systemic or gastrointestinal *Candida albicans* infection. This appeared to be due to the ability of IL-12 to induce production of counteracting IL-10 by neutrophils [65–67]. Thus, the efficacy of IL-12 may be blunted by the presence of high numbers of neutrophils, as found in the lungs of cyclophosphamide-treated but not neutrophil-depleted mice. Whether induced or not by IL-12, IL-10 production appeared to exacerbate the course of IPA as evidenced by the beneficial effect observed upon its neutralization.

The mechanism underlying the physiologic regulation of Th phenotype development in mice with IPA remains to be elucidated. The type of Th cell responses to antigens encountered at the mucosal surfaces of the body may be influenced by regional environmental factors, such as the nature of regional innate immunity [68] and factors present in the local microenvironment [69]. We recently showed that expression of co-stimulatory molecules on phagocytic cells influences Th cell development in mice with candidiasis [29] and that IL-4, and thus Th2 development, is produced mainly through the CD28/B7-2 costimulatory pathway [70]. When the expression of co-stimulatory molecules, such as B7-1 (CD80) and B7-2 (CD86), was examined in lung cells from mice with IPA, preliminary results showed an increased expression of B7-2 molecules in mice highly susceptible to infection (unpublished data). This finding suggests that induction of lung mucosal antifungal Th2 cell responses requires costimulation through the B7-2 molecule as demonstrated in response to allergens [71, 72] and helminths [73].

In conclusion, the results of our study demonstrate that, similar to observations in mice with disseminated aspergillosis [23], innate host defense and the generation of Th1 cytokines play an essential role in resistance of mice to IPA. Inhibition of Th2 cytokines or administration of Th1-promoting IL-12 resulted in a beneficial effect, suggesting that strategies aimed at redirecting the lung mucosal Th phenotype may be helpful in IPA.

References

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