IFN-γ mediated signaling improves fungal clearance in experimental pulmonary mucormycosis.

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Abstract

We established three immunocompetent murine models of pulmonary mucormycosis to determine the involvement of the adaptive immune response in host resistance in pulmonary mucormycosis, a rapidly fatal disease caused mainly by *Rhizopus* spp. Immunocompetent BALB/c, C57BL/6, and Swiss mice were inoculated with *R. oryzae* via the intratracheal route. The inoculation resulted in an angioinvasive infection that spread to the brain, spleen, kidney, and liver. After 7 and 30 days of *R. oryzae* infection, C57BL/6 and BALB/c mice showed the lowest fungal load and highest production of IFN-γ and IL-2 by splenocytes, respectively. Swiss mice showed a higher fungal load 30 days p.i. and was associated with a weak development of the Th-1 profile. To confirm our findings, *R. oryzae*-infected IFN-γ$^{-/-}$ mice were evaluated after 60 days, where the mice still showed viable fungi in the lungs. This study showed, for the first time, that pulmonary mucormycosis in three widely used mouse strains resulted in an acute fungal dissemination without immunosuppression whose outcome varies according to the genetic background of the mice . We also identified the partial role of IFN-γ in the efficient elimination of *R. oryzae* during pulmonary infection.

Introduction

Mucormycosis is a severe fungal disease caused by various ubiquitous filamentous fungi of the order Mucorales, wherein *Rhizopus* spp. and *Mucor* spp. are the most prevalent causal agents [1]. Although aspergillosis remains the most frequent opportunistic mold infection, mucormycosis has emerged as an important systemic mycosis due to its increased incidence and high lethality [2]. Pulmonary mucormycosis is a rapidly fatal illness that typically develops in patients with profound neutropenia and graft-versus-host disease [2,3], with an overall mortality rate of 76%, which increases to 95% after extrathoracic dissemination [4–10]. Combined surgical/medical treatment may provide a better survival outcome in pulmonary mucormycosis compared to medical therapy alone [11].

Despite the importance of fungus–host interactions in pulmonary mucormycosis, few studies have been conducted on this infection. In an experimental model of pulmonary mucormycosis, it was demonstrated that the inhibition of *Rhizopus* growth inside alveolar macrophages is a central host defense mechanism that depends on nutritional immunity via iron starvation [12]. In addition, the same study showed that although bronchoalveolar macrophages can inhibit the germination of *R. oryzae* spores, they are not capable of killing them [12,13]. It has been demonstrated that the inability of macrophages to kill *Rhizopus* sp. is due to the inhibition of LC3-associated phagocytosis (LAP), a specialized pathway of phagosome biogenesis that plays a central role in the regulation of immune homeostasis and antifungal host defense [12,14,15]. These early inhibition effects result in phagosome maturation arrest and account for resistance to macrophage killing [12]. However, several questions on the signaling pathways, cytokine responses, and activation of macrophages by the adaptive immune response remain unanswered.

Few studies have addressed the association of acquired T cell deficiency with increased susceptibility to infection by Mucorales [16]. However, investigators recently observed the presence of Mucorales-specific

T cells in patients with invasive mucormycosis [17]. In addition, immunotherapy strategies have been a promising tool for controlling mucormycosis since anti-*Rhizopus* T cells from healthy volunteers demonstrated reactivity against Mucorales and enhanced the phagocyte killing effect of Mucorales [18,19]. It has also been shown that IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated human polymorphonuclear leukocytes to augment hyphal damage in *Rhizopus* species [20], and immunotherapy in combination with IFN-γ resulted in an effective treatment of a patient with intractable invasive mucormycosis [21].

In vitro and experimental studies suggest the protective role of the Th-17 type of adaptive immune response in the context of invasive mucormycosis, as well as the unprotective role of Th-2. It was shown that the hyphae of *Rhizopus oryzae* triggered a common innate sensing pathway in human dendritic cells, resulting in the robust production of IL-23 and Th-17 responses [22]. Indeed, the important role of the Th-17 response in disseminated mucormycosis was recently confirmed by our group. IL-$17^{-/-}$ mice are susceptible to intravenous *R. oryzae* infection. In addition, more efficient fungal clearance in immunocompetent models of disseminated mucormycois was associated with IL-17, while less efficient elimination of *R. oryzae* was associated with increased levels of IL-10 [23].

Taken together, the *in vitro* and experimental studies in mucormycosis seem to be consistent with that of human disease and collaborate with the improvement of current therapies for invasive mucormycosis. However, studies investigating the adaptive immune response, specifically in the context of pulmonary mucormycosis, have been rarely investigated. Considering this lack of knowledge, we established a murine model of pulmonary mucormycosis to study the adaptive immune response related to resistance during *R. oryzae* pulmonary infection.

Materials And Methods

Mice. Female BALB/c and Swiss mice were purchased from the Lauro de Souza Lima Institute (ILSL), Bauru, SP, Brazil. Wild type C57BL/6 (WT) and IFN-γ-deficient C57BL/6 mice (IFN-$\gamma^{-/-}$), 6–10 weeks old, were bred in a specific pathogen-free animal facility at the School of Medicine of Ribeirao Preto, University of São Paulo, Brazil. The genotypes of these knockout mice (IFN-$\gamma^{-/-}$ mice) were determined using a previously established polymerase chain reaction technique [24]. All mice were maintained in the animal facilities at the Experimental Immunopathology Laboratory (LIPE), UNESP - Univ. Estadual Paulista, Bauru, SP, Brazil. Food and sterile water were provided *ad libitum*. All protocols used were in accordance with the ethical principles for animal research adopted from the National Council for Animal Experimentation Control (CONCEA). The Ethical Committee of the School of Science, UNESP, Bauru, SP, Brazil approved this study (#1608/46/01/2013).

Fungal strains. *R. oryzae* (IAL 3796) was originally obtained from the fungal collection of ILSL, and species identification was confirmed by the Adolfo Lutz Institute (São Paulo, SP, Brazil). The fungi were maintained through monthly subculturing on Sabouraud dextrose agar (SDA) slants (Difco Laboratories Detroit, Michigan, USA).

Experimental design. Groups of 6–7 *R. oryzae*-infected BALB/c, Swiss, and C57BL/6 mice were evaluated 7 and 30 days post-inoculation (p.i.). The IFN-γ KO group was composed of *R. oryzae*-infected mice that were followed up for 60 days. The control groups were composed of mice subjected to the same procedures but inoculated with sterile saline solution (SSS).

Fungal infection. Fungi were washed carefully with SSS, and the suspensions were vortexed twice for 10 s and decanted off for 5 min. The supernatants were collected and washed twice with SSS. Fungal viability was determined using cotton blue staining and plating of the suspension on SDA plates. For the intratracheal inoculation, mice were anesthetized via intraperitoneal administration of ketamine and xylazine at doses of 80 and 10 mg/kg body weight, respectively. After tracheal exposition, each mouse received a volume of 40 µL (5×10^7 spores per mL) of the suspension. The incision was sutured with surgical thread, and the animals were kept in a warm place and observed for recovery.

Collection of biological materials. Mice were anesthetized with isoflurane and then euthanized via CO_2 asphyxiation. Fragments of the brain, liver, lung, spleen, kidneys, and thymus were collected and subjected to microbiological and histopathological analyses. Fragments of the spleen were also subjected to cell culture procedures.

Recovery of viable fungi. Ten fragments (2 × 2 mm) of the brain, liver, lung, spleen, and kidneys were cultured on SDA plates at 25 °C for 7 days [23,25]. Fungal growth on the fragments were counted, and the results were expressed as the frequency of *R. oryzae*-positive fragments per total number of cultivated fragments. Preliminary experiments confirmed that homogenization of tissues from *R. oryzae*-infected mice failed to recover viable fungi.

Spleen cell culture. The spleen fragments were collected and homogenized in ice-cold sterile PBS. Red blood cells were lysed with 0.15 M ammonium nitrate. After washing, the cellular suspension was adjusted to 2.0×10^6 cells per mL as determined by 0.1% trypan blue staining. A total of 2.0×10^5 spleen cells were placed into each well of 48-well flat-bottom microtiter plates and incubated at 37 °C in a 5% CO_2 humidified chamber. The cells were cultured with heat-killed spores of *Rhizopus* (1 spore : 1 cell) and after 48 h, the cell-free supernatants were harvested and stored at -80 °C for cytokine analysis.

Cytokine production. Levels of IFN-γ, IL-2, IL-17, and IL-4 were measured in the cell-free supernatants of the cell cultures using a cytokine Duo-Set Kit (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions.

Histopathological score analyses. Tissue fragments were fixed in 10% neutral-buffered formalin. Paraffin slides (4 µm) were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Microscopic examination was carried out using an optical light microscope coupled to a camera (Zeiss®, Jena, Germany), under 10× and 40× magnifications, throughout the length of the cut, and involved qualitative and semi-quantitative analyses of the organs. In the qualitative analyses, the following parameters were observed: presence/absence of a) hyphae, b) tissue damage, c) vascular alteration, and d) inflammatory

cell infiltrates. The semi-quantitative analyses were based on the scores for inflammatory infiltrates, assigning the following subjective scores according to the intensity of the inflammatory cells: (0) absence, (1) mild, (2) moderate, and (3) severe. Representative images of qualitative microscopic aspects were captured and recorded for comparison between the different lineages.

***Statistical analyses*.** Comparison of two independent samples was performed using the t-test, and multiple comparisons of three independent samples were performed using ANOVA with the Tukey post-test. All statistical tests were performed using GraphPad Prism (version 5.0) for Windows (GraphPad Software, San Diego, CA), and the statistical significance level was set at 5% ($p \leq 0.05$) for all analyses.

Results

Development of the intratracheal R. oryzae *infection in immunocompetent mice*

In pulmonary mucormycosis, the infection occurs through inhalation of sporangiospores, causing pulmonary infection [26], and can spread contiguously into other internal organs, such as the abdomen, increasing the mortality rate from 76% to 95% in immunocompromised patients [4–10]. In order to investigate whether *R. oryzae* pulmonary infection induces sustained dissemination in immunocompetent mice, we infected three strains of mice via the intratracheal route and evaluated the mice after 7 and 30 days, first evaluating disease progression via recuperation of viable fungi and histopathological analyses.

After 7 days of *R. oryzae* intratracheal inoculation, all mice showed viable fungi in the brain, kidney, liver, lung, and spleen (Table 1). After 30 days, BALB/c mice showed a reduction in fungal load in all evaluated tissues. C57BL/6 mice showed reduced recovery of viable fungi in the brain and Swiss mice showed reduced fungal loads in the liver and lungs after 30 days p.i. (Table 1). Comparison of the total fungal load among mouse strains showed that BALB/c mice showed the highest fungal load after 7 days of infection, and after 30 days, BALB/c mice showed the lowest fungal load among the three strains (Table 1).

Next, we performed qualitative and semi-quantitative analyses of the histopathological patterns of infection in the brain, kidneys, liver, and lungs. In the semi-quantitative analyses, we observed that the infiltrate scores did not differ among the strains (Fig. 1A). In the qualitative analyses, we observed hemorrhage and inflammatory infiltrates in the three mouse strains from days 7 and 30 p.i. (Fig. 1B), especially in the foci, and were diffused near the meninges of the brain. In the kidney, the lesions were observed to have dilated blood vessels, foci of inflammatory cells in the cortex, and diffuse inflammatory cell infiltration in the medulla of the three strains of mice on days 7 and 30 p.i. (Fig. 1B). In the liver, congested and dilated blood vessels and perivascular infiltration of inflammatory cells were observed in all three strains after 7 and 30 days p.i. (Fig. 1B). In the lungs, congested and dilated blood vessels, hemorrhagic regions, and perivascular and peribronchial inflammatory cell infiltration was observed in all three strains after 7 and 30 days p.i. (Fig. 1B). No fungal structures were observed in sections of the brain, kidney, liver, or lungs.

Cytokine variation profile under R. oryzae*-Ag presence following* R. oryzae *infection*

As observed in the comparison of the total fungal load, BALB/c mice showed the lowest fungal load while Swiss mice showed the highest fungal load among the three strains after 30 days of infection. Considering the different capacities of the mice to eliminate their fungal load until 30 days p.i., we next characterized their specific immune responses by analyzing the *in vitro* production of cytokines by spleen cells from infected or non-infected mice co-cultivated with *R. oryzae*.

After 7 days of infection, increased production of IL-2 (Fig. 2A) and IFN-γ (Fig. 2B) in heat-killed *R. oryzae*-stimulated spleen cells from BALB/c and C57BL/6 mice were observed, whereas IL-2 and IFN-γ levels in Swiss mice did not change (Fig. 2A). After 30 days of infection, the production of IL-2 remained elevated in the heat-killed *R. oryzae*-stimulated spleen cells from the more resistant BALB/c and C57BL/6 strains than in both the non-infected mice and Swiss mice. In addition, the production of IFN-γ (Fig 2B) remained elevated in heat-killed *R. oryzae*-stimulated spleen cells from C57BL/6 mice, and the production of IFN-γ (Fig 2B) in heat-killed *R. oryzae*-stimulated spleen cells from BALB/c mice decreased. After 30 days p.i., we observed that C57BL/6 mice showed a higher production of IFN-γ (Fig 2B) by stimulated spleen cells than BALB/c and Swiss mice. No detectable levels of IL-4 and IL-17 were observed in any group (data not shown).

R. oryzae *infection in IFN-γ KO mice.*

Considering the association between fungal clearance and increased production of IFN-γ, we next investigated the role of IFN-γ in IFN-γ KO mice infected with *R. oryzae*. As shown in Figure 3, the absence of IFN-γ did not influence the survival of infected mice (Fig. 3A), but IFN-γ KO mice showed a higher fungal load in the lungs than WT mice after 60 days of infection (Fig. 3B).

Discussion

This study showed, for the first time, that experimental pulmonary mucormycosis in three widely used mouse strains resulted in fungal dissemination in the absence of immunosuppression. We also identified the partial role of IFN-γ in the efficient elimination of *R. oryzae* during pulmonary infection.

In mucormycosis, experimental models are widely used for the evaluation of antifungal therapy and, in these studies, immunosuppression was used to induce dissemination in murine models [27–30]. Nevertheless, the effect of immunosuppressive drugs restricts the evaluation of the immunological mechanisms involved in fungal resistance. Recent studies exploring host-parasite relationships in mucormycosis have used immunocompetent models, such as disseminated mucormycosis models, using BALB/c, C57BL/6, and Swiss mice [23]; mucormycosis in a skin model using BALB/c mice [31]; and a pulmonary mucormycosis model using C57BL/6 mice [12]. Although pulmonary mucormycosis in immunocompetent C57BL/6 mice has been reported once [12], dissemination was not investigated, and the study focused on the innate immune response against *R. oryzae,* while the adaptive immune response in pulmonary infection is still unknown. Considering this, our study was the first to characterize

a model of a pulmonary infection of *R. oryzae* that induces angioinvasion and dissemination in immunocompetent mice. Angioinvasion has been associated with the ability of the pathogen to disseminate hematogenously from the primary site of infection to other organs [32]. In humans, pulmonary mucormycosis occurs due to the inhalation of fungal spores into the bronchioles and alveoli, which typically results in the rapid progression of pneumonia or endobronchial disease, hemoptysis with vascular invasion causing necrosis, and invading tissue to spread locally or disseminate systemically [4].

Our *R. oryzae*-infected mice were characterized via the recovery of viable fungal growth, hemorrhage, vessel alteration, and inflammatory cell infiltration in the lungs as well as in the brain, kidney, liver, and spleen after seven days of infection, which showed an angioinvasive infection, indicating that our models are similar to the human pattern of infection [33].

In a murine model of mucormycosis, angioinvasion was frequently observed following the establishment of pulmonary mucormycosis after intranasal challenge with Mucorales of corticosteroid-treated mice [34]. In the study above, it was observed that mice infected with up to 10^6 spores of *Lichtheimia corymbifera* were susceptible to infection until 10 days p.i. [34]. In addition to the angioinvasion observed in our immunocompetent models of pulmonary mucormycosis, no fatality was observed in the mice after administration with an inoculum of 2×10^6 spores of *R. oryzae.* The lack of immunosuppression may explain the absence of susceptibility in our model; in addition, an intranasal inoculum of 2×10^6 spores of *R. oryzae* in BALB/c, C57BL/6, and Swiss immunocompetent mice did not lead to the recovery of viable fungal or histological tissue alterations (data not shown). These data suggest that for the establishment of *R. oryzae* pulmonary infection in immunocompetent mice, intratracheal inoculation is more efficient than intranasal inoculation. Similar to our study, in C57BL/6 mice intratracheally infected with *R. oryzae* established as an immunocompetent model of pulmonary mucormycosis, considerable tissue edema, neutrophil infiltration, and viable *R. oryzae* in the lungs were observed five days p.i. [12]. In addition to the study above, we compared three different strains of mice and observed that differences in the capacity for fungal clearance was related to the genetic background.

It is well-established that inbred mouse strains have varying dynamics of infection. The differences in the type of immune response developed and the resulting severity of disease allows the applicability of mice with different genetic bases as effective tools for studying the dynamics of infections [35–38]. In this study, we observed that under pulmonary *R. oryzae* infection, C57BL/6 and BALB/c mice were more resistant, while Swiss mice were less resistant . Inbred lines of highly susceptible (A/J, A/Sn, DBA / 2) and highly resistant (BALB/c, C57BL/6J) mice have been described to be infected with *Aspergillus fumigatus* [35], *Candida albicans* [36], *Cryptococcus neoformans* [37], *Paracoccidoides brasiliensis* [38], and *Rhizopus oryzae* [23]. Some genetic targets such as C5-deficiency (Hc^0 allele, hemolytic complement) and C5-independent pathways (such as the loci *Carg3* and *Carg4*) have been identified to modulate the host's initial response and cause an ineffective inflammatory response against *Candida albicans* in some inbred mouse strains [36]. In Swiss mice, C5 deficiency is the cause of the high susceptibility to fungal infections [39–41]. In addition, in experimental models of disseminated

mucormycosis, Swiss mice were the less resistant strain, compared with the other two inbred strains, showing an anti-inflammatory response during intravenous *R. oryzae* infection.

Considering the lack of knowledge in the adaptive immune response in the context of pulmonary mucormycosis, we used the differences in response of three strains of mice against *R. oryzae* to identify relevant resistance mechanisms, and we observed that greater resistance against pulmonary infection was associated with the Th-1 response mediated by higher IFN-γ and IL-2 levels, as observed in the more resistant BALB/c and C57BL/6 strains, while less resistance was associated with an absolute adaptive immune response observed in the Swiss strain. It is known that IFN-γ-producing Th-1 cells confer protective immunity against fungi, whereas Th-2 responses increase susceptibility to fungal infections [42]. In addition, Th-17 cells have also been implicated in mucosal immunity against fungi [42]. IL-2 induces T cell clonal expansion and is a marker of adaptive immune response development [43].

In patients with invasive mucormycosis, Mucorales-specific T-cells showed high levels of IL-4, IFN-*γ*, IL-10, and IL-17 [17]. In the same study, it was observed that all patients showed a high expression of IL-10 at the time of diagnosis and close to the time of their death. Interestingly, the absence of Mucorales-specific T cells producing IL-10 was found in one patient at the time of complete resolution [17]. In contrast to our results, in an immunocompetent BALB/c and C57BL/6 model of disseminated mucormycosis, resistance against *R. oryzae* was associated with higher levels of IL-17.

It has been noticed that different routes of inoculation may, at times, give rise to different host responses, which could be another explanation for the observed differences [44]. In this context, during intravenous inoculation of *C. albicans*, it was observed that Th-1 and Th-17 responses mediated protective adaptive immunity [45], while in experimental *C. neoformans* intratracheal infection, a type of Th-1 response mediated by IFN-γ was mainly developed [46]. In aspergillosis, major T-helper cell lineages, Th-1, Th-2, and Th-17, have been demonstrated to play important roles in patients and animal models depending on the type of infection [47–51]. For invasive aspergillosis, the Th-17 type of response was essential for fungal control [52,53], but in pulmonary infections caused by *A. fumigatus*, the Th-1 response profile is more effective [54]. In pulmonary infections, the Th-17 type of response seems to be pathogenic to the lung due to an uncontrolled or prolonged inflammatory response that caused persistent inflammation (50–52). For mucormycosis, the pattern of adaptive immune response seems to be similar to that observed in aspergillosis. In disseminated mucormycosis, IL-17 signaling is crucial to control the infection and, as observed here in *R. oryzae* pulmonary infection, the Th-1 response was developed as evidenced by higher levels of IFN-γ (Fig. 4).

It has been shown that the Th-1 response is crucial for fungal clearance since it potentiaties the fungicidal activity of innate immune cells through IFN-γ release [54–57]. To elucidate the possible involvement of IFN-γ signaling in the protection against *R. oryzae* pulmonary infection, we induced pulmonary mucormycosis in IFN- $\gamma^{-/-}$ mice and found that they were incapable of eliminating *R. oryzae* in the lungs until 60 days of infection. These results indicate that, as observed in other fungal infections

[45,46,54], the Th-1 response through IFN-γ signaling is partially important for fungal clearance in mucormycosis [17,27,58,59].

In contrast to our study, in an experimental model of invasive aspergillosis, the absence of IFN-γ resulted in an increase in susceptibility to infection [60], while our IFN-$\gamma^{-/-}$ mice did not succumb to *R. oryzae* infection. Two hypotheses can explain these results. First, there was an absence of an immunosuppressive drug in our model to induce mucormycosis, while in the study above, a neutropenic model of mice induced to have invasive aspergillosis was used. Second, other immune signaling may be involved in the prevention of death in IFN-$\gamma^{-}/^{-}$ mice infected with *R. oryzae* [23]. For exemplo, IL-2 has already been demonstrated to be capable of activating human NK cells to damage *R. oryzae* hyphae, but it did not affect resting conidia in a previous *in vitro* study (58).

Although responses other than Th-1 are capable of preventing death in infected IFN-$\gamma^{-}/^{-}$ mice, they were not as efficient as the response mediated by IFN-γ, since the IFN-$\gamma^{-}/^{-}$ mice were unable to eliminate live *R. oryzae* conidia in the lungs. The absence of additional experiments to explore the mechanisms of survival of infected IFN-$\gamma^{-}/^{-}$ mice was the main limitation of the present study, and we suggest that more studies should be done to clarify the survival mechanisms observed here.

In summary, we introduced three immunocompetent models of pulmonary *R. oryzae* infection resulting in angioinvasive mucormycosis, which permits new studies to explore infection dynamics in this context. Using the differences in capacity of murine models in eliminationg *R. oryzae*, we gathered new insights into the immune response during pulmonary mucormycosis, and, in addition, we recognized that IFN-γ contributes to a more effiective mechanism of eliminating *R. oryzae*.

Declarations

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Conflicts of interest/Competing interests. The authors do not have a commercial or other association that might pose a conflict of interest.

***Availability of data and material*.** The authors confirm that the data supporting the findings of this study are available within the article.

***Code availability*.** Not applicable.

Authors' contributions. Conceived of or designed study: OSA, MSPA, JV, VSL. Performed research: ARS, DFAD, RFS, ACF, CTS, VSL, NLMA, MIA. Analyzed data: ARS, TFFS, DFAD, NLMA, VSL, JV. Contributed new methods or model:VSL, NLMA. Wrote the paper:ARS, JV.

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Table

Table 1. *R. oryzae* dissemination after intratracheal infection in three mouse strains.

	Stage of infection	BALB/c	C57BL/6	Swiss	*P* value
Brain	7 days	72.00	48.00	36.00	0.4019
	30 days	0.020	0.0020	4.000	0.3984
	P value	0.0007*	0.0322*	0.1950	
Kidney	7 days	64.00	24.00	24.00	0.2684
	30 days	0.020	5.00	15.00	0.5345
	P value	0.0082*	0.4248	0.7231	
Liver	7 days	68.00	32.00	52.00	0.2330
	30 days	4.000	20.00	12.00	0.6789
	P value	0.0019*	0.6329	0.0462*	
Lung	7 days	100.0^{A}	53.00^{B}	96.00^{AB}	$0.0295^{\#}$
	30 days	28.33	15.00	44.00	0.4118
	P value	0.0002*	0.1637	0.0303*	
Spleen	7 days	80.00^{A}	8.00^{B}	60.40^{A}	$0.0015^{\#}$
	30 days	0.020	25.00	32.00	0.2703
	P value	0.0002*	0.3645	0.2277	
Total fungal load	7 days	76.80^{A}	33.00^{B}	53.68^{AB}	$0.0043^{\#}$
	30 days	6.333^{B}	14.00^{AB}	24.60^{A}	$0.0207^{\#}$
	P value	<0.0001*	0.1106	0.0152*	

Data were expressed as the percentage of *R. oryzae*-positive fragments in a total fragments per group. *Indicate the difference between 7 and 30 days p.i., $p<0.05$, Unpaired T-test (vertical analysis). $^{\#}$Letters indicate the difference among three mouse strains, $p<0.05$, ANOVA with Tukey's Multiple Comparison Test (horizontal analysis).

Figures

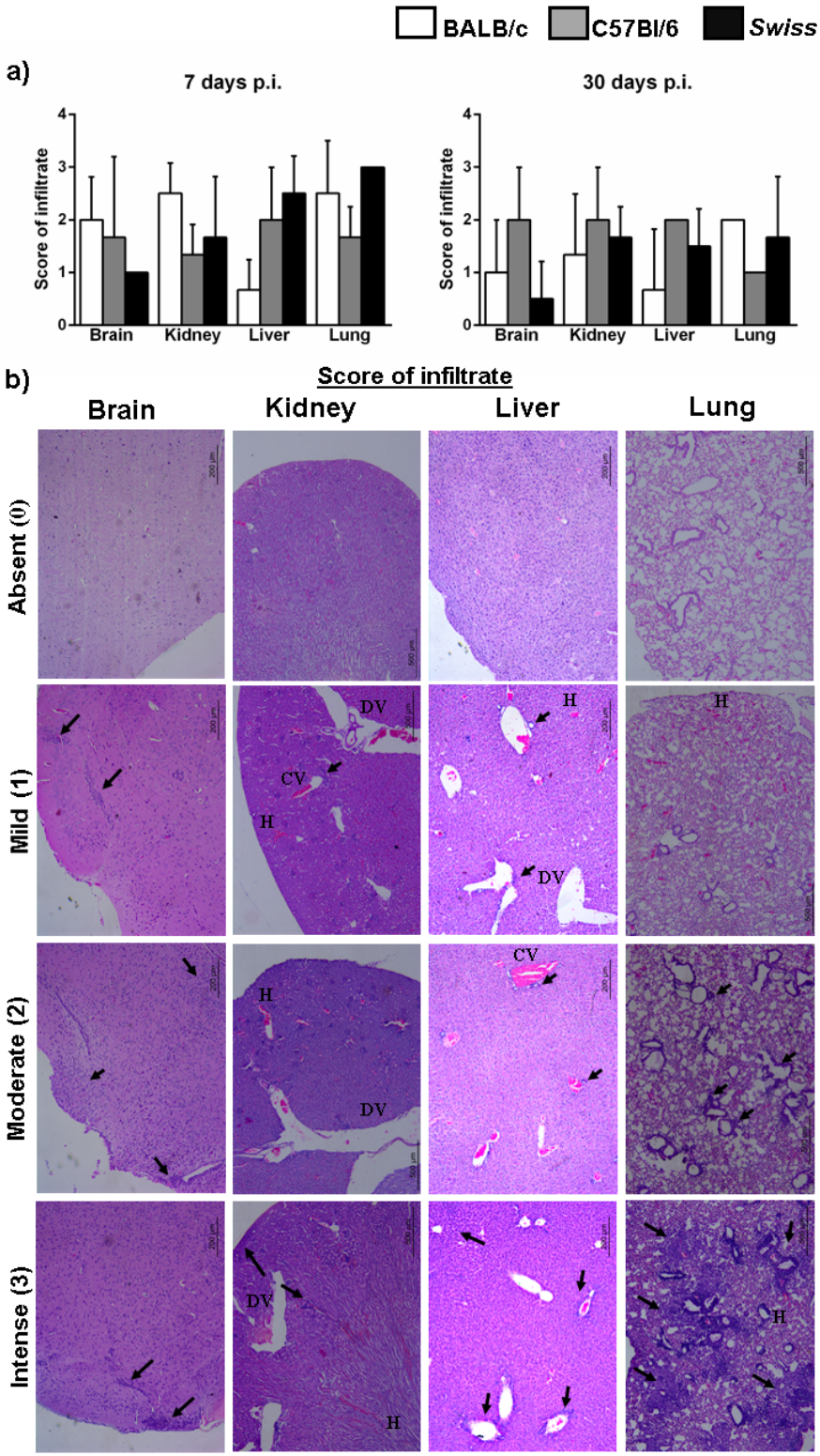

Figure 1

Characterization of pulmonary mucormycosis in three immunocompetent strains of mice. BALB/c, C57BL/6, and Swiss mice were infected intratracheally with 40 µL of a solution containing 5 × 107 spores per mL of R. oryzae and were evaluated after 7 and 30 days. (A)Fragments of organs such as the brain, kidneys, liver, lungs, and spleen were collected and evaluated for their inflammatory cells infiltrate score. Graphs show means with standard deviations. (ANOVA with post-hoc Tukey test; n=5-7; *p < 0.05, ** p<

0.01, ***p< 0.001). (B) Infiltrate scores in H&E stained sections. Absent infiltrate column: brain, kidneys, liver, lung, and spleen of non-infected mice (Control). Arrows indicate inflammatory infiltrate foci or places with diffuse infiltrates; CV: congested vessels; DV: dilated vessels; H: hemorrhage;

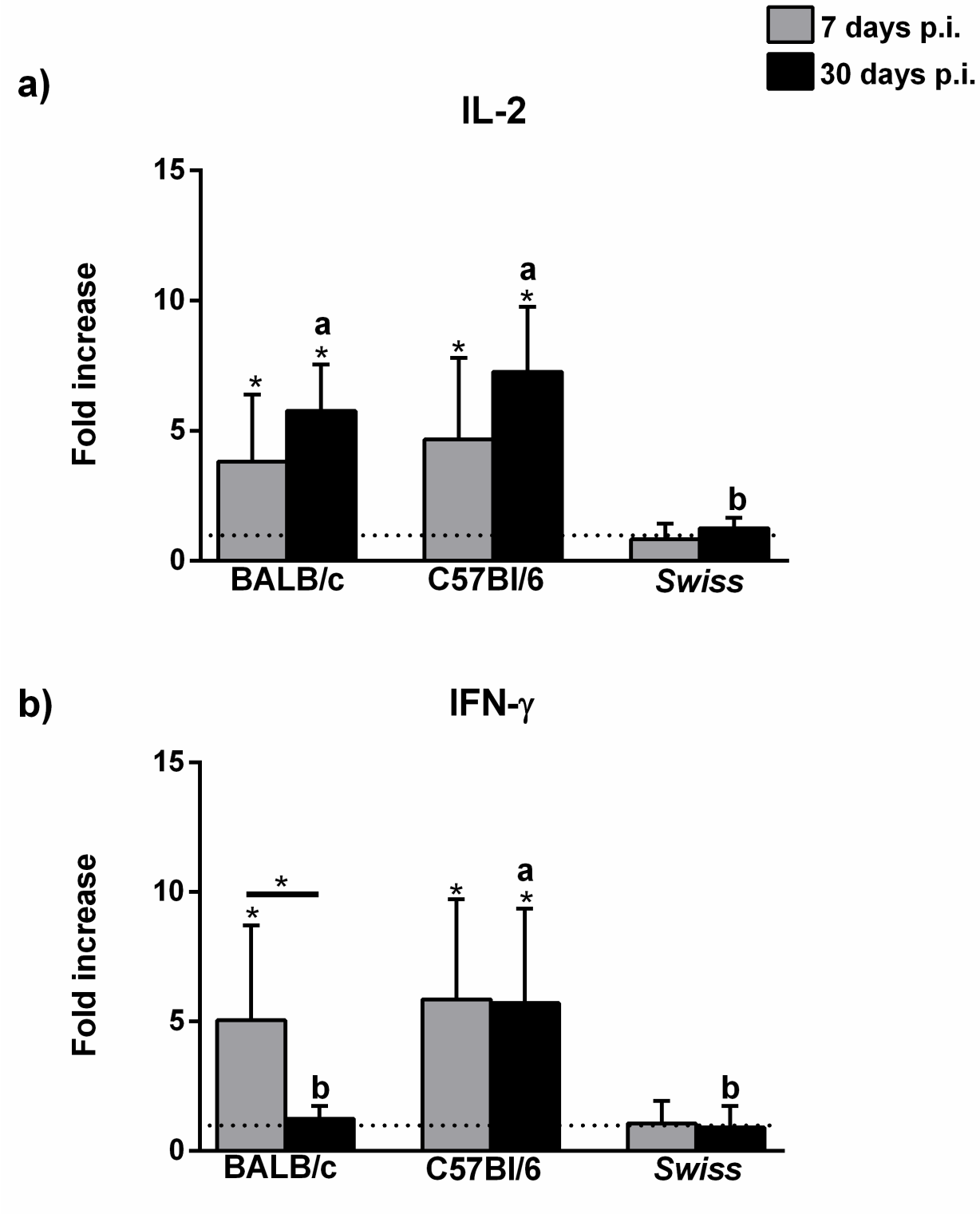

Figure 2

Specific response of spleen cells challenged with R. oryzae-Ag. BALB/c, C57BL/6, and Swiss mice were infected intratracheally with 40 µL of a solution containing 5 × 107 spores per mL of R. oryzae and were

evaluated after 7 and 30 days. Fragments of the spleen were collected and cell-free supernatants of spleen cells co-cultured with heat-killed spores of R. oryzae were evaluated for levels of cytokines. The fold increase in IL-2 (A) and IFN-γ (B) levels were determined after normalization to naïve uninfected control mice (n=5). Any significant differences relative to naïve samples (without bars), to infected samples (with bars) or to different strains (letters) are indicated. (ANOVA with post-hoc Tukey test; n=5-7; *p < 0.05, ** p< 0.01, ***p< 0.001).

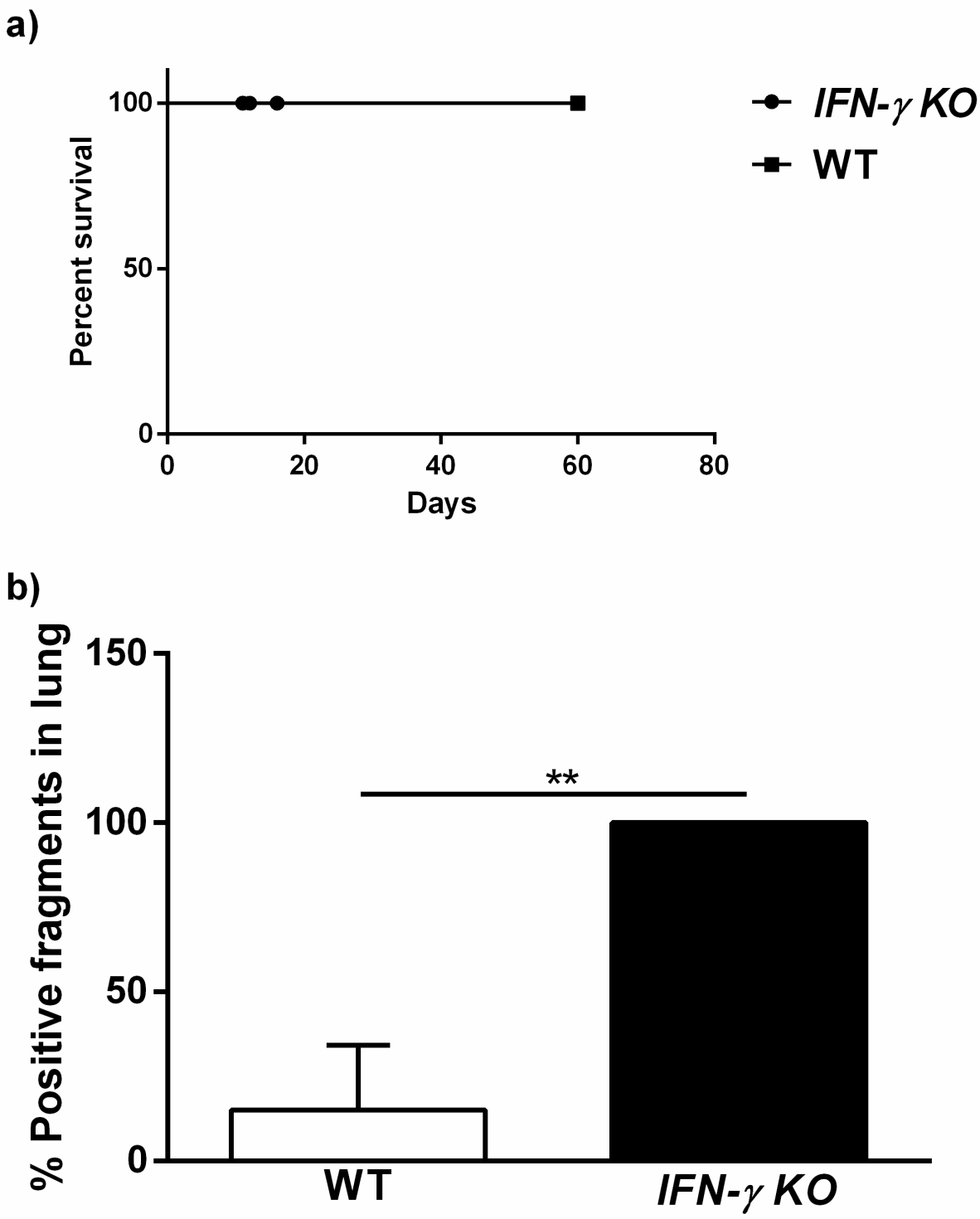

Figure 3

Rhizopus oryzae infection in IFN-γ-/- mice. For IFN-γ evaluation, IFN-γ-/- mice (mice of a C57BL/6 background with genetic-deficient IFN-γ production) and WT (C57BL/6 mice) were infected intratracheally with 40 µL of a solution containing 5 × 107 spores per mL of R. oryzae and evaluated as follows: A) mortality rate (Kaplan-Meier survival curves, n=5), B) viable fungal recovery (ANOVA with post-hoc Tukey test; n=5-7; *p < 0.05, ** p< 0.01, ***p< 0.001; data were expressed as the percentage of R. oryzae-positive fragments out of the total fragments per group).

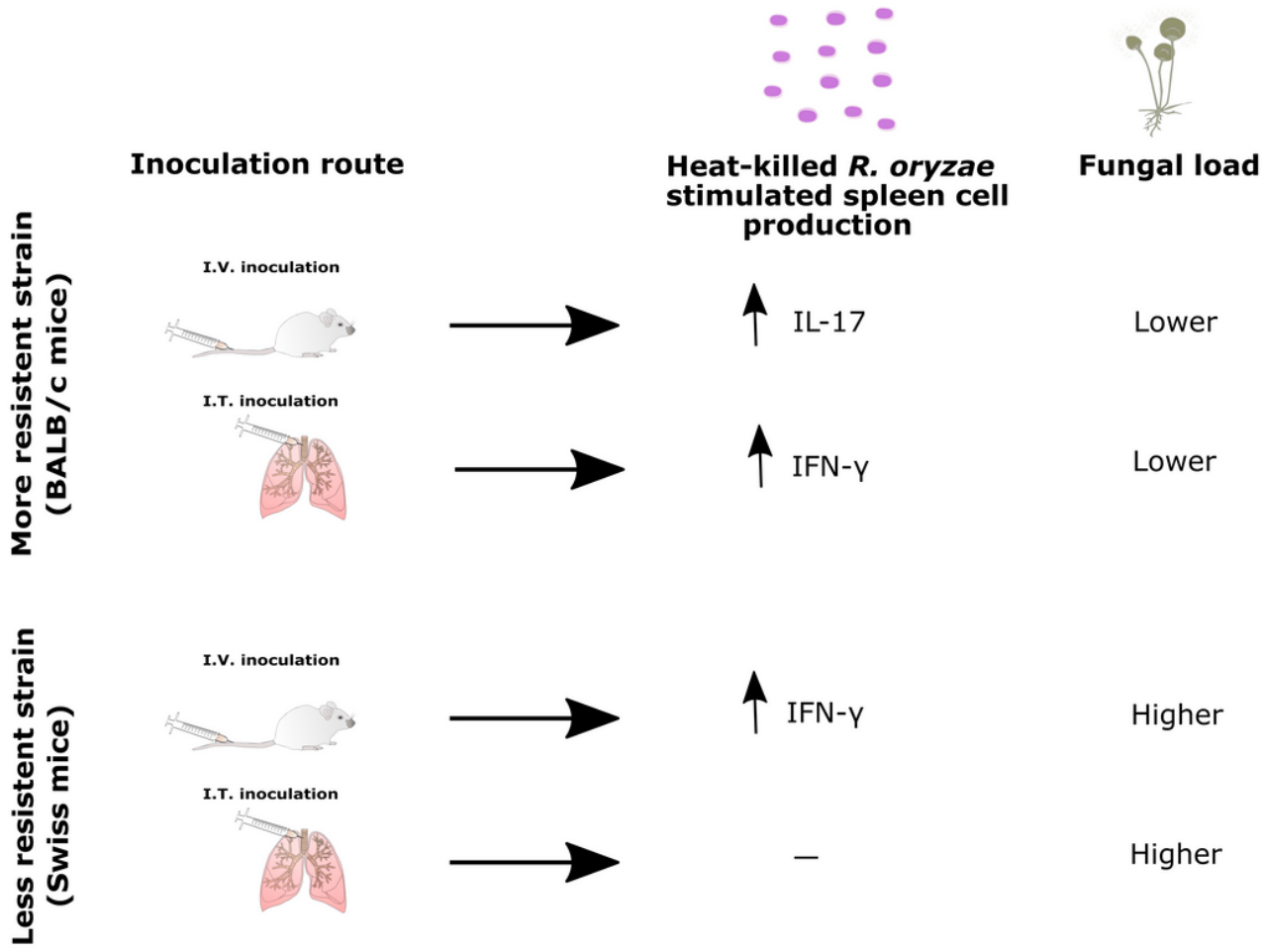

Figure 4

Overview of immune response and fungal burden in BALB/c and Swiss mice infected with R. oryzae via different routes of infection. BALB/c and Swiss mice were infected intravenously with 3 × 104 spores [23] or intratracheally with 2 ×106 spores of R. oryzae and evaluated after 30 days. Cytokine concentrations were evaluated in cell-free supernatants from spleen cells co-cultured with heat-killed spores of R. oryzae. Summarized results of the fungal burden on day 30 are also shown.