Is the production of reactive oxygen and nitrogen species by macrophages associated with better infectious control in the experimental disseminated and pulmonary mucormycosis?

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Abstract

Different levels of resistance against *Rhizopus oryzae* infection have been observed between inbred (BALB/c) and outbred (Swiss) mice, with is associated with the genetic background of each mouse strain. Considering that macrophages play an important role in host resistance to *Rhizopus* species, we use the different infectious outcomes observed in experimental mucormycosis to identify the most efficient macrophages responses pattern against *R. oryzae in vitro* and *in vivo*. For this, we compared BALB/c and Swiss macrophage activity pre-and-post intravenous or intratracheal *R. oryzae* infections. Production of hydrogen peroxide (H_2O_2) and nitric oxide (NO) was determined in cultures of peritoneal (PMΦ) or alveolar macrophages (AMΦ) challenged, or not, with heat-killed spores of *R. oryzae*. Levels of TNF-α and IL-10 were also measured to enhance our findings. Naïve PMΦ from BALB/c increased the production of H_2O_2, TNF-α, and IL-10 in the presence of heat-killed spores of *R. oryzae,* while naïve PMΦ from Swiss mice was less responsive. Naïve AMΦ from two strains of mice were less reactive to heat-killed spores of *R. oryzae* than PMΦ. On 30 days of *R. oryzae* intravenous infection, lower fungal load in BALB/c strain of mice was accompanied by higher production of H_2O_2 by PMΦ when compared with Swiss mice. Differently, AMΦ from BALB/c mice showed higher production of NO, TNF-α, and IL-10 after 7 days of intratracheal infection and after 30 days, lower fungal load, when compared with Swiss mice. According to the set of experiments performed, our findings reveal that independently of mice strain, PMΦ is more reactive against *R. oryzae* in the first contact than AMΦ. In addition, increased PMΦ production of H_2O_2 at the end of disseminated infection is related to efficient fungal clearance observed in resistant (BALB/c). Our findings provide new evidence to understand the parasite-hosts relationship in mucormycosis.

48 **1. Introduction**

49 Mucormycosis is a devastating invasive fungal infection predominantly caused by
50 *Rhizopus* species (1). It has emerged globally as a public health threat during the COVID-
51 19 pandemic (2–4). The infection occurs via diverse routes such as inhalation,
52 percutaneous, or ingestion of spores. Mucormycosis may have a wide variety of disease
53 manifestations, with rhinocerebral and pulmonary diseases being the most common (5).
54 The mortality rate varies from 46%-70% and up to 90% upon dissemination (6–8).

55 Risk factors for mucormycosis include diabetes mellitus, neutropenia, sustained
56 immunosuppressive therapy, chronic prednisone use, and iron chelation therapy (7).
57 During the COVID-19 pandemic, the use of immunomodulatory drugs (e.g., systemic
58 corticosteroids and tocilizumab) and COVID-19-induced immune dysregulation
59 increased the risk of mucormycosis (9,10). Cases of COVID-19–associated
60 mucormycosis (CAM) have been reported worldwide (11–20), and have been mostly
61 associated with underlying uncontrolled diabetes mellitus diseases. Nevertheless,
62 cutaneous and soft-tissue mucormycosis has been observed in immunocompetent
63 individuals (21,22), such as those induced by traumatic implantation of contaminated soil
64 and water during tornadoes (23).

65 Considering the high rates of mortality and morbidity associated with this life-
66 threatening disease (14), it has a prognosis and outcome that have not significantly
67 improved over the last decades, mainly due to the difficulty in forming an early diagnosis
68 and the limited activity of current antifungal agents against Mucorales (24,25). In
69 addition, mucormycosis has an incompletely understood pathogenesis, particularly
70 related to the parasite-hosts relationship.

71 Experimental models of mucormycosis are widely used for the evaluation of
72 antifungal therapy and, in these studies, immunosuppression is used to induce fungal
73 dissemination (26–29). Nevertheless, the effect of immunosuppressive drugs restricts the
74 evaluation of the immunological mechanisms involved in fungal resistance. Recently, our
75 group has focused on studies exploring *Rhyzopus*-host interplay using immunocompetent
76 models, inducing both disseminated and pulmonary mucormycosis (30,31). Using
77 BALB/c and Swiss mice, we determined a resistant and less resistant model of
78 mucormycosis, respectively. While BALB/c mice has been shown better capacity in
79 decreasing fungal load until 30 days of intravenous and intratracheal infection, Swiss
80 mice has been shown be less responsive to *R. oryzae* infectious and consequently more
81 prolonged viable fungal presence in internal organs (30,31). We and others (32,33) have
82 highlighting the importance of evaluating immune response in immunocompetent mice
83 to understand the mechanisms involved in the resistance to Mucorales agents, as well as,
84 the mucormycosis pathogenesis in immunocompetent individuals (21,22).

85 Andrianaki and collaborators (2018) revealed the essential role of *Rhizopus*–
86 macrophage interplay for pulmonary mucormycosis. Using an immunocompetent model,
87 it was shown that the development of mucormycosis is related to the prolonged
88 intracellular survival of the fungus inside of the macrophages (33). Considering the poor
89 knowledge of macrophage activity during mucormycosis in the context of natural
90 resistance; in the present study, we employed resistant and less resistant models to
91 determine the reactive oxygen and nitrogen species production by *R. oryzae*-infected
92 macrophages

93

94 **2. Material and methods**

95 **2.1 Mice.** Two-month-old female inbred BALB/c and outbred Swiss mice from the
96 Animal House at the Laboratório de Imunopatologia Experimental (LIPE) of UNESP
97 (Univ Estadual Paulista, Bauru, SP, Brazil) were divided into groups randomly. We
98 provide food and water sterile *ad libitum*. This study was carried out in strict accordance
99 with the recommendations in the Guide for the Care and Use of Laboratory Animals of
100 the National Institutes of Health and Brazilian College of Animal Experimentation. The
101 study was approved by an Institutional Animal Care and Use Committee (IACUC)
102 (Protocol Number: 1608/46/01/2013-CEUA-FC) by Animal Experimentation Ethics
103 Committee of the School of Sciences of Bauru, UNESP. All surgery was performed under
104 ketamine/xylazine anesthesia, and all efforts were made to minimize suffering.

105 **2.2 Fungal strains.** *R. oryzae* (IAL 3796) was previously obtained from the fungal
106 collection of Instituto Lauro de Souza Lima (ILSL), and submitted to species
107 identification by Adolfo Lutz Institute (São Paulo, SP, Brazil). The fungi were maintained
108 by monthly subculturing on Sabouraud dextrose agar (SDA) slants (Difco Laboratories,
109 Detroit, Michigan, USA).

110

111 **2.3 Experimental design.** Mice were randomly separated into two main groups
112 according to the route of inoculation: intravenous groups (*Rhi*-IV*)* and intratracheal
113 groups (*Rhi*- IT). For the *Rhi*-IV groups, Swiss and BALB/c mice were inoculated within
114 3.0×10^4 viable spores of *R. oryzae* in the caudal vein. For the *Rhi*- IT groups, Swiss and
115 BALB/c mice were inoculated within 2.0×10^6 viable spores of *R. oryzae* in the trachea.
116 Next, groups of six *R. oryzae*-infected mice were evaluated on days 7 and 30 post-
117 infection (p.i.). Non-infected groups were composed of BALB/c and Swiss mice
118 inoculated with sterile saline solution (SSS) by intravenous or intratracheal route.

119

120 **2.4 Fungal infection.** Fungi were washed carefully with SSS, and the suspension was
121 mixed twice for 10 s on a vortex-mixer and decanted off for 5 min. The supernatants were
122 collected and washed twice. We determined fungal viability by cotton blue staining. In
123 *Rhi*-IVgroups, a volume of 100 µL of fungal suspension (3×10^4 spores of *R. oryzae*) was
124 inoculated into the lateral tail vein. In *Rhi*-IT groups, mice were previously anesthetized
125 ketamine and xylazine (80 and 10 mg/kg body weight, respectively) by intraperitoneal
126 route. After the tracheal exposition, each mouse received a volume of 40 µL (2×10^6
127 spores of *R. oryzae*) of the suspension. The incision was sutured with surgical thread, and
128 the mice were kept in a warm place and observed for their recovery process.
129
130 **2.5 Collection of the biological material.** Mice were anesthetized with isoflurane then
131 euthanized via CO_2 asphyxiation. Peritoneal lavage (PL) and bronchoalveolar lavage
132 (BAL) was performed in *Rhi*- IV and *Rhi*-IT groups, respectively, using cold and sterile
133 phosphate-buffered saline (PBS). Fragments of the brain, liver, lung, spleen, and kidneys
134 were collected and submitted to microbiological evaluation.
135
136 **2.6 Recovery of viable fungi.** Quantitative colony culture is a wide method for organ
137 fungal burden determination in per gram tissue in which whole tissue is grinding into
138 suspension before inoculating onto culture plates. However this methods causes non-
139 viability in Mucorales (21), and to avoid damage to fragile hyphal and consequent false-
140 negative results in our quantitative analysis (34), the fragment method was employed in
141 our set of experiments, as previously reported (30,31,35). For this, ten fragments (2 x 2
142 mm) of the brain, liver, lung, spleen, and kidneys were cultured on Sabouraud agar plates
143 at 25ºC for a maximum of seven days. We counted fragments of fungal growths and

144 expressed the results as the frequency of *R. oryzae*-positive fragments per total of
145 cultivated.
146
147 **2.7 Macrophages cell culture.** BAL and PL suspensions were centrifuged for 10 minutes
148 at 410 *g*, and cells were resuspended in 1.0 mL of supplemented RPMI-1640 (Nutricell,
149 Campinas, SP, Brazil). The supplemented RPMI-1640 contained: 10% heat-inactivated
150 fetal calf serum (Nutricell), penicillin (100 UI mL^{-1}), streptomycin (100 mg mL^{-1})
151 (Sigma-Aldrich, St. Louis, MO, USA), and amphotericin B (0.25 µg mL^{-1}) (Sigma-
152 Aldrich). Cell concentration was adjusted to 1.0×10^5 mononuclear phagocytes mL^{-1} as
153 judged by the uptake of 0.02% neutral red (Sigma) and confirmed by expression of F4/80
154 by Fluorescence-Activated Cell Sorting (FACS). Cells were plated into 96-well flat-
155 bottomed microtiter plates (Greiner BioOne, Frickenhausen, Germany), and incubated for
156 two hours at 37°C and 5% CO_2 in a humidified chamber to allow cells to adhere and
157 spread. Non-adherent cells were removed by washing the wells three times with RPMI,
158 and we used the remaining adherent cells (>95% mononuclear phagocytes as assessed by
159 morphological examination) for experiments. The adherent cells were cultured at 37°C
160 and 5% CO_2 in supplemented RPMI-1640 with or without heat-killed spores of *R. oryzae*
161 (*R. oryzae*-Ag) (1 spore: 1 cell). As an internal control for macrophage activity, cells were
162 cultured with 10 µg ml-1 lipopolysaccharide (Sigma-Aldrich). After 24 hours, cell-free
163 supernatants were harvested and stored at -80°C for cytokine analysis.
164
165 **2.8 Production of hydrogen peroxide (H_2O_2).** The production of H_2O_2 was estimated
166 according Russo et al. (1989). Briefly, adhered mononuclear phagocytes obtained as
167 described before were maintained in RPMI-1640 culture medium at 37°C and 5% CO_2
168 for 24 h. At the end of the cell culture period, we removed the supernatants from the wells,

169 added the phenol red solution containing dextrose (Sigma), phenol red (Sigma), and
170 horseradish peroxidase type II (Sigma), and incubated the plate at 37°C in 5% CO_2 for
171 one hour. Reaction was stopped by adding 1 N NaOH. The H_2O_2 concentration was
172 determined using a colorimetric microreader (ELx 800; BioTek Instruments Inc.,
173 Winooski, VE, USA)(36).
174
175 **2.9 Detection of levels of nitric oxide (NO).** The production of NO was estimated
176 following the Griess' method (Green et al., 1982). Production of nitrite, a stable end
177 product of NO, was measured in the cell-free supernatants of adhered mononuclear
178 phagocytes cultured. Briefly, a volume of 0.1 mL of cell-free supernatant with an equal
179 volume of Griess reagent was incubated for 10 minutes at room temperature. Griess
180 reagent was prepared using: 1% sulfanilamide (Synth, Diadema, SP, Brazil), 0.1%
181 naphthalene diamine dihydrochloride (Sigma), and 2.5% H3PO4. The nitrite
182 accumulation was quantified using a colorimetric microreader (ELx 800; BioTek
183 Instruments Inc., Winooski, VE, USA). Nitrite concentration was determined using
184 sodium nitrite (Sigma) diluted in RPMI-1640 medium as a standard (36).
185
186 **2.10 Dosage of TNF-α and IL-10**. IL-10 and TNF-α levels were measured in the cell-
187 free supernatants of cell cultures using a cytokine Duo-Set Kit (R&D Systems,
188 Minneapolis, MI, USA), according the manufacturer's instructions.
189
190 **2.11 Statistical analyses.** Normality tests of data was determined by Shapiro–Wilk's test.
191 For comparing two independent samples *t*-test was performed. For multiple comparisons,
192 ANOVA with the Tukey post-test was used. Pearson's correlation coefficient was
193 determined to measure the statistical association between two continuous variables. All

194 statistical tests were performed using GraphPad Prism version 5.0 for Windows
195 (GraphPad Software, San Diego, CA). *P*-value of 5% or lower was considered
196 statistically significant (Zar, 2010).
197

198 **3. Results**

199 **3.1 Production of reactive oxygen reactive species and IL-10 by heat-killed *R. oryzae*-**
200 **infected peritoneal macrophages is dependent of mouse genetic background.**

201 First, we evaluated the *in vitro* response of peritoneal (PMΦ) and alveolar (AMΦ)
202 macrophages obtained from non-infected BALB/c and Swiss mice challenged with heat-
203 killed *R. oryzae* (*R.oryzae*-Ag). To evaluate the PMΦ and AMΦ response we measured
204 reactive oxygen and nitrogen species (H_2O_2 and NO) in the cultures. We also measured
205 the levels of TNF-α and IL-10 to validate our findings.

206 In the presence of heat-killed spores of *R. oryzae*, PMΦ from the two mouse
207 strains showed decreased production of the NO (Fig 1A) and increased the TNF-α
208 production (Fig 1C). Interestedly, *R. oryzae*-infected PMΦ from BALB/c showed
209 increased production of H_2O_2 and IL-10. (Fig 1B, Fig 1D).

210 In general, AMΦ from both Swiss and BALB/c mice showed similar production
211 of NO and H_2O_2 (Fig 1E, Fig 1F). A slight increased production of TNF-α by *R. oryzae*-
212 infected AMΦ obtained from BALB/c was observed. In this set of experiments, no
213 detectable production of IL-10 was observed.
214

215 **Fig 1. Peritoneal and alveolar macrophages from naive Swiss and BALB/c mice**
216 **react differently in front of heat-killed *R. oryzae*.** (A) NO, (B) H2O2, (C) TNF-α, (D)
217 IL-10 levels in cell-free supernatant of peritoneal macrophages, and (E) NO (F) H2O2,
218 (G) TNF-α levels in cell-free supernatant of alveolar macrophages of non-infected Swiss,
219 and BALB/c mice co-cultured or not with heat-killed spores of R. oryzae. Student's t-test;
220 n=5-7; *p < 0.05, ** p< 0.01, ***p< 0.001). NO: nitric oxide; H2O2: hydrogen peroxide;
221 TNF-α: tumor necrosis factor-alpha; IL-10: interleukin 10; PMΦ: peritoneal
222 macrophages; AlvMΦ: alveolar macrophages.

223 **3.2 Enhanced production of H_2O_2 by peritoneal macrophages was associated with**
224 **better clearance of *R. oryzae* in a model of disseminated mucormycosis.**

225 In the present study, BALB/c and Swiss mice strains showed more and less
226 resistance, respectively, against *in vivo R. oryzae* infection, as previously reported
227 (30,31). The course of infection was characterized by similar fungal load on day 7 for
228 both mouse strain, followed by a decreased fungal load on day 30, that was more
229 expressive in BALB/c mice (Fig 2A).

230 In order to explore the results of *in vitro R. oryzae*-infected macrophages
231 experiments, we hypothesized that the production of reactive species of oxygen and
232 nitrogen by macrophages could be involved in the differential response observed between
233 these two mice strains.

234 To assess our hypothesis, we first evaluated the specific activity of PMΦ derived
235 from BALB/c and Swiss mice that were intravenously infected with *R. oryzae*. For this,
236 on days 7 and 30 p.i., PMΦ were recovered from the peritoneal cavity and challenged
237 with heat-killed spore of *R. oryzae*.

238 On day 7, PMΦ from Swiss mice showed higher production of NO (Fig 2B) and
239 TNF-α (Fig 2D) than PMΦ from BALB/c mice. On the other hand, on day 30, more
240 resistant BALB/c mice showed higher levels of H_2O_2 (Fig 2C). No differences in the
241 production of NO, TNF-α, and IL-10 were observed between both mouse strains (Fig 2B,
242 Fig 2D, Fig 2E).

243 Next, we performed a correlation test between production of H_2O_2 and fungal
244 load. An inverse correlation was observed between the production of H_2O_2 and total
245 fungal load in the BALB/c mice (Fig 2G), but not in the Swiss mice (Fig 2F).

246

247 **Fig 2. Increased PMΦ production of H_2O_2 at the end of disseminated infection is**
248 **related to efficient fungal clearance in BALB/c mice.** (A) Total fungal load (B) NO,

(C) H_2O_2, (D) TNF-α, and (E) IL-10 levels in cell-free supernatant of peritoneal macrophages from Swiss or BALB/c mice co-cultured with heat-killed spores of *R. oryzae*. Linear regression analysis between H_2O_2 levels and total fungal load in BALB/c (F) and Swiss (G) mice. The infected group was composed of mice inoculated intravenously with 3 x 10^4 spores of *R. oryzae* and evaluated after 7 and 30 days. Any significant differences relative to infected samples compared to different times post infection (letters) and to different strains (*) are indicated. (Student's t-test; n=5-7; *$p < 0.05$, ** $p< 0.01$, ***$p< 0.001$). NO: nitric oxide; H_2O_2: hydrogen peroxide; TNF-α: tumor necrosis factor alpha; IL-10: interleukin 10.

3.3 Enhanced initial pro-inflammatory response and NO production by alveolar macrophages of BALB/c mice seems to better control the pulmonary mucormycosis.

BALB/c and Swiss mice were intratracheally infected with *R. oryzae* to evaluate the tissue-specific response. As observed in intravenous infection, both BALB/c and Swiss mice showed a decrease in viable fungal load after 30 days p.i., and BALB/c mice showed better fungal clearance than Swiss mice (Fig 3A).

On day 7, AMΦ from BALB/c mice showed higher production of NO (Fig. 3B), TNF-α (Fig 3D), and IL-10 (Fig 3E) than AMΦ from Swiss mice. Lower production of H_2O_2 by AMΦ from BALB/c mice was observed (Fig 3C).

On day 30, no differences in the production of NO, H_2O_2, TNF-α, and IL-10 by AlvMΦ were observed (Fig 3B, Fig 3C, Fig 3D, Fig 3E).

Fig 3. AMΦ from BALB/c mice showed higher production of NO, TNF-α, and IL-10 than AMΦ from Swiss mice after 7 days of *R. oryzae* pulmonary infection. (A) Total fungal load, (B) NO, (C) H_2O_2, (D) TNF-α, and (E) IL-10, levels in cell-free supernatant of alveolar macrophages from Swiss or BALB/c mice co-cultured with heat-killed spores of *R. oryzae*. The infected group was composed of mice inoculated intratracheally with 2 ×10^6 spores of *R. oryzae* and evaluated after 7 and 30 days. Any significant differences relative to infected samples compared to different times post infection (letters) and to different strains (*) are indicated. (Student´s t-test; n=5-7; *$p < 0.05$, ** $p< 0.01$, ***$p< 0.001$). NO: nitric oxide; H_2O_2: hydrogen peroxide; TNF-α: tumor necrosis factor alpha; IL-10: interleukin 10.

4. Discussion

283 Here we use the different outcomes observed in experimental mucormycosis
284 between the immunocompetent BALB/c and Swiss mice to highlight the protective
285 pattern of macrophages responses against *R. oryzae*. We found that macrophages from
286 different microenvironments showed different responses against *R. oryzae* not only *in*
287 *vitro* but also during *in vivo* infection. Also, the better outcome was associated with higher
288 H_2O_2 produced by PM, and higher initial NO, TNF-α, and IL-10 produced by AMΦ
289 during experimental disseminated and pulmonary mucormycosis, respectively. In
290 addition, the genetic background interferes in the macrophage response, so, regardless of
291 the microenvironment, the BALB/c's macrophage is more effective in eliminating the
292 fungus.

293 Inbred mouse strains vary widely in their degree of innate susceptibility to
294 systemic fungal infections (37–41). BALB/c and Swiss mice are resistant and susceptible
295 strains, respectively, in experimental infections caused by *Cryptococcus neoformans*
296 (Rhodes et al., 1980), *Candida albicans* (38,42), and *Paracoccidioides brasiliensis* (41).
297 It was identified that the genetic target C5-deficiency (Hc0 allele, hemolytic complement)
298 modulates the host's initial response and causes susceptibility and ineffective
299 inflammatory response against fungal infections (38,39). Active as an anaphylatoxin, the
300 C5 molecule is a power chemotactic factor for polymorphonuclear leukocytes. Lack of
301 C5 would block complement, thereby decreasing the opsonization and recruitment of
302 phagocytic cells (39). Although the higher susceptibility of Swiss mice to fungal
303 infections may be explained by a deficiency in the Hc0 allele (41–43), our results suggest
304 that other genetic targets may be involved in the less responsiveness of macrophages from
305 non-infected Swiss mice to heat-killed *R. oryzae*.

306 According to clinical and experimental data, individuals who lack phagocytes or
307 present impaired phagocytic functions show a higher risk of developing mucormycosis

308 (6,44,45). We observed in the *in vitro* part of this study that peritoneal macrophages from
309 the most resistant strain (BALB/c) showed a potent inflammatory response mediated by
310 H_2O_2 and TNF-α. (46) in the first contact with *R. oryzae* antigen. A previous *in vitro* study
311 showed that the inactivated Mucorales cells can stimulate high levels of TNF-α, IL-1β,
312 IL-6, I-L8, GM-CSF, and MCP-1 production by humans cells of different immune
313 subsets, with paralleled upregulation of transcriptional activity of IL-1β and TNF-α
314 (47,48). Since the oxidative microbicidal production by neutrophils and monocytes is
315 related to *R. oryzae* hyphae damage and killing (49,50), our results indicate an initial
316 proinflammatory response against *R. oryzae* can lead to a better outcome in experimental
317 mucormycosis.

318 Unexpectedly, in the first contact with *R. oryzae* antigen peritoneal macrophages,
319 in general, decreased the production of NO. Similar to our results, it was demonstrated
320 that *Aspergillus nidulans* melanin inhibited the NO production by lipopolysaccharide
321 (LPS)-stimulated peritoneal macrophages, accompanied by a slight stimulatory effect on
322 TNF-α production (51). Melanin is a type of pigment in the fungal cell wall of black
323 molds and has been shown to block the effects of hydrolytic enzymes on the cell wall
324 (52). Considering the importance of this finding, we suggest that the immunomodulatory
325 effects induced by the *R. oryzae* cell wall on peritoneal macrophages need to be better
326 investigated.

327 Differently from peritoneal macrophages, alveolar macrophages from the BALB/c
328 strain produced higher NO and TNF-α levels than less resistant strain, during the *in vivo*
329 model of pulmonary mucormycosis. It is known that pulmonary activated macrophages
330 are the major defense against fungal invasion (53). In contact with macrophages
331 receptors, TNF-α provides signals that lead to an induction of antimicrobial activity. This
332 activity depends on NO synthase activation and NO production (54). Some studies are

333 demonstrating that this process is essential to fungal death. In paracoccidioidomycosis
334 (PCM), TNF-a induces *P. brasiliensis* killing by H_2O_2 and NO release (55). In
335 cryptococcosis, TNF-α significantly promoted macrophage NO production and
336 anticryptococcal activity(56). In addition, TNF-α enhances pulmonary alveolar
337 macrophage phagocytosis and oxygen production during initial contact with *A. fumigatus*
338 (57). It is known that TNF-α contributes to the influx and activation of neutrophils and
339 mononuclear cells in the lungs during the filamentous fungal challenge (58). In
340 mucormycosis, the TNFα signaling may have a protective response since patients treated
341 with a tumor necrosis factor inhibitor have a higher risk to developed disseminated
342 mucormycosis(59,60).

343 Although the association of NO with the killing of yeast cells supports the
344 importance of this metabolite in host protection (61), other studies have been
345 demonstrating that the overproduction of this metabolite is associated with susceptibility
346 in experimental PCM (62,63). Nascimento, et. al (2002) observed that high levels of NO
347 induce T cell immunosuppression during *P. brasiliensis* infection (Nascimento et al.,
348 2002). These reports suggest that the protective or deleterious NO role *in vivo* depends
349 on the balance between its fungicidal and immunosuppressive properties. Our data
350 demonstrate that after intravenous *R. oryzae* infection, stronger and early production of
351 NO and TNF-a by peritoneal macrophages from Swiss mice does not result in late better
352 fungal clearance. On the other hand, after a pulmonary *R. oryzae* infection, higher and
353 early production of NO and TNF-a by alveolar macrophages from BALB/c mice was
354 accompanied by higher levels of IL-10, resulting in later better control of pulmonary
355 mucormycosis. Suggesting that in BALB/c mice, an initial balanced immune response is
356 essential for better infection control.

357 IL-10 is an anti-inflammatory cytokine that can impede pathogen clearance. But
358 it also can ameliorate immunopathology (64). The balance between pro-and anti-
359 inflammatory cytokines is crucial for the host´s defense against *A. fumigatus*. An *in vitro*
360 study with mononuclear cells (MNC) stimulated with *A. fumigatus* showed that hyphae
361 of this fungi induced the release of IL-10 by MNC, and this process was dependent on
362 endogenous IL-1 (65). Whereas inflammatory cytokines such as IFN-γ, TNF-α, and IL-
363 18 activate monocytes and neutrophils to ingest and kill *A. fumigatus* conidia and hyphae,
364 the subsequent release of the anti-inflammatory cytokine IL-10, are responsible for down-
365 regulating the potential deleterious overstimulation induced by the inflammatory
366 mediators (66).

367 In general, one of the biological effects of IL-1 is an increased synthesis of IL-10
368 (67). Netea et al., (2004) showed that *Candida* stimulated IL-1 release by cells, and
369 secondarily IL-10 release (68). In Mucormycosis, although high production of IL-10 by
370 Mucorales-specific T cells from patients was related to susceptibility in the late phase of
371 the disease (69), our results showed a correlation between higher IL-10 production by
372 alveolar macrophages in the initial moments of *R. oryzae* infection and better outcome in
373 experimental pulmonary mucormycosis. We suggest that high levels of IL-10 were a
374 biological effect to balance the pro-inflammatory response mediated by high levels of
375 TNF-α and NO. A fundamental step for an efficient immune response.

376 While alveolar macrophages from a more resistant strain showed an initial higher
377 TNF-α, NO, and IL-10 response to a pulmonary infection, the peritoneal macrophages
378 from the same strain showed a late and strong response mediated by H_2O_2 after the
379 intravenous *R. oryzae* infection. It is important to note that after infection by the
380 intravenous route, the decrease in viable *R. oryzae* was inversely proportional to the
381 release of H_2O_2 by peritoneal macrophages from a more resistant strain of mice. It

382 indicates the relation of this mediator with protection in Mucormycosis. This result agrees
383 with the study done by Andrianaki et al. (33), where they prove the susceptibility of *A.*
384 *fumigatus* and *R. oryzae* conidia to oxidative damage induced by H_2O_2. In this context,
385 an important detail was that the BALB/c strain only showed a more efficient fungal
386 clearance after 30 days of infection. We suggest that the adaptative immune response
387 potentiated the activity of the macrophages evaluated in this study.

388 Macrophages are the first immune cells to interact with invasive pathogens.
389 Depending on macrophage's activity, they can prime the adaptive immune response,
390 which is far more aggressive and specific to pathogens (70). Generally, high levels of
391 ROS production by macrophages are linked to intracellular events, including the
392 activation of transcription factors such as NFκB (71), and induction of mitogenesis (72).
393 In addition, *in vitro*, and *in vivo* studies already showed that *R. oryzae* could trigger a Th-
394 17 response mediated by high amounts of IL-23 by dendritic cells (73), and also the
395 association of high levels of IL-17 and IFN-γ with better *R. oryzae* elimination by
396 immunocompetent BALB/c and C57BL/6 mice (30,31). Considering the studies above
397 and the evidence found here, we hypothesized that adaptive immune responses developed
398 by BALB/c mice were essential to enhance the fungicidal activity of peritoneal
399 macrophages and efficiently kill *R. oryzae*.

400 The absence of additional experiments to explore the intracellular mechanisms
401 mediating the differences in ROS production and to confirm the adaptative immune
402 response related to a more efficient macrophages response front of *R. oryzae*-Ag was the
403 main limitation of the present study, and we suggest that more studies should be done to
404 clarify the results observed here.

405 In summary, our findings reveal that independently of mice strain, PMΦ is more
406 reactive against *R. oryzae* in the first contact than AMΦ. In addition, increased PMΦ

407 production of H_2O_2 at the end of disseminated infection is related to efficient fungal
408 clearance observed in resistant (BALB/c). Our findings provide new evidence to
409 understand the parasite-hosts relationship in mucormycosis.

410

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640

641 **Striking image. Overview of *in vitro* and *in vivo* macrophages response in BALB/c**
642 **and Swiss mice infected with *R. oryzae* via different routes of infection**. Macrophage
643 activity of BALB/c and Swiss mice were evaluated pre-and-post intravenous or
644 intratracheal *R. oryzae* infection. NO, H_2O_2, TNF-α, and IL-10 concentrations were
645 evaluated in macrophages co-cultured or not with heat-killed spores of *R. oryzae*. Infected
646 mice were intravenously or intratracheally inoculated with 3×10^4 and 2×10^6 spores of
647 *R. oryzae* respectively, and evaluated after 7 and 30 days. Summarized results of the
648 fungal load on day 30 are also shown. NO: nitric oxide; H_2O_2: hydrogen peroxide; TNF-
649 α: tumor necrosis factor alpha; IL-10: interleukin 10; PMΦ: peritoneal macrophages;
650 AlvMΦ: alveolar macrophages. Image by Débora Almeida-Donanzam via Inkscape

651

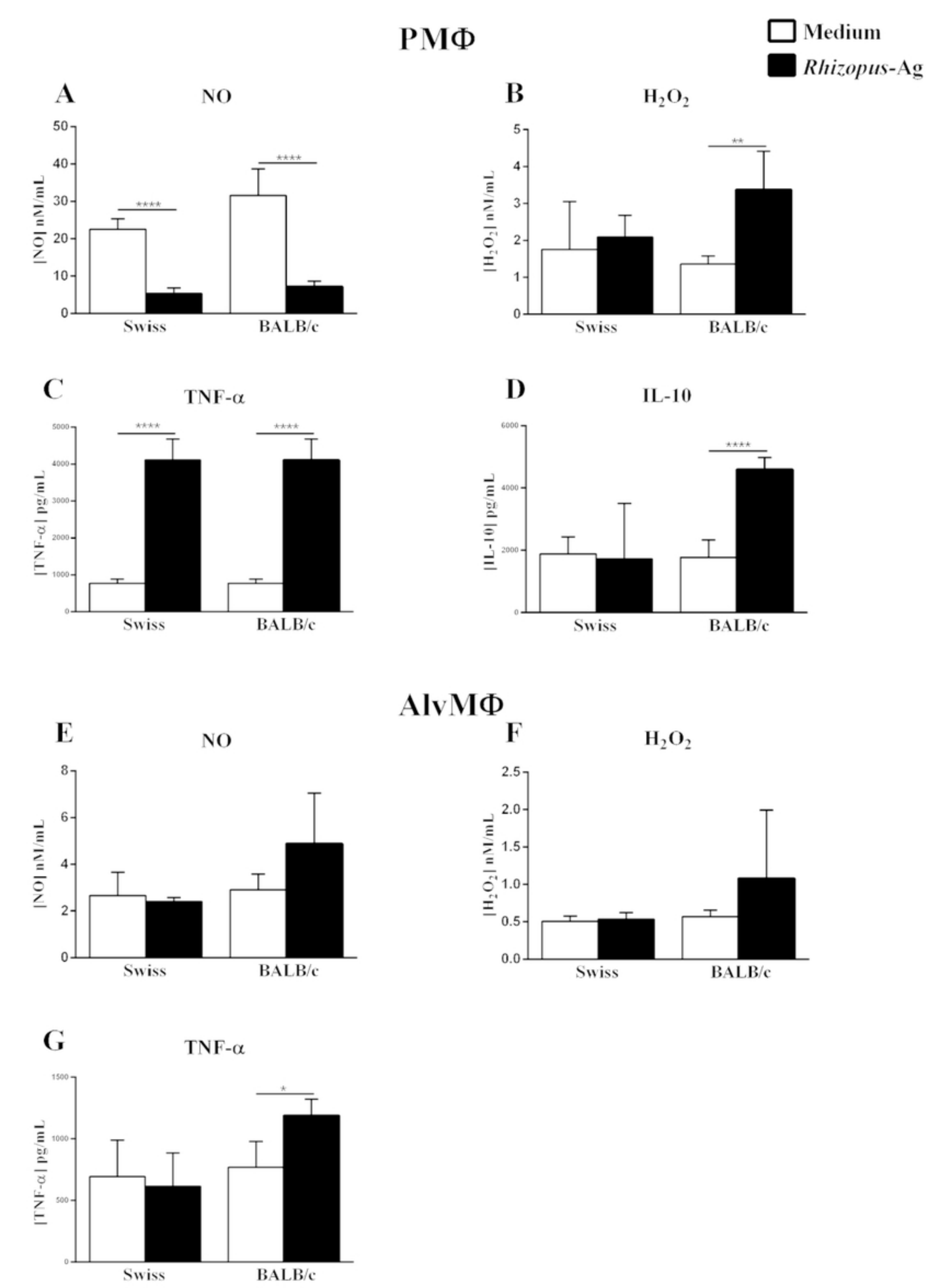
PMΦ
Medium
Rhizopus-Ag
A
NO
[NO] nM/mL
50
40
30
20
10
0
****
****
Swiss
BALB/c
B
H2O2
[H2O2] nM/mL
5
4
3
2
1
0
**
Swiss
BALB/c
C
TNF-α
[TNF-α] pg/mL
5000
4000
3000
2000
1000
0
****
****
Swiss
BALB/c
D
IL-10
[IL-10] pg/mL
6000
4000
2000
0
****
Swiss
BALB/c
AlvMΦ
E
NO
[NO] nM/mL
8
6
4
2
0
Swiss
BALB/c
F
H2O2
[H2O2] nM/mL
2.5
2.0
1.5
1.0
0.5
0.0
Swiss
BALB/c
G
TNF-α
[TNF-α] pg/mL
1500
1000
500
0
*
Swiss
BALB/c

Figure 1

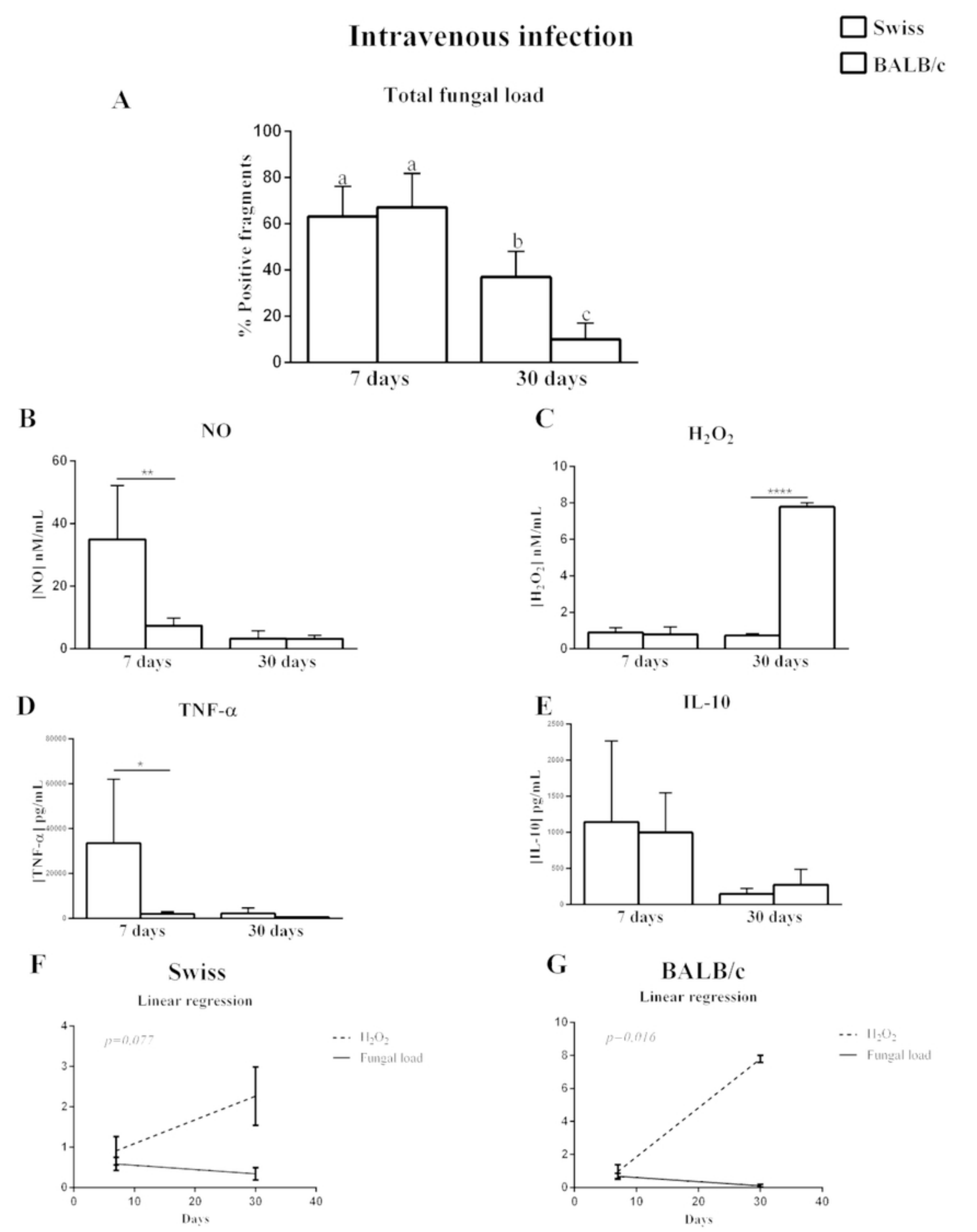
Intravenous infection
Swiss
BALB/c
A
Total fungal load
% Positive fragments
100
80
60
40
20
0
a
a
b
c
7 days
30 days
B
NO
[NO] nM/mL
60
40
20
0
**
7 days
30 days
C
H_2O_2
$[H_2O_2]$ nM/mL
10
8
6
4
2
0
****
7 days
30 days
D
TNF-α
[TNF-α] pg/mL
80000
60000
40000
20000
0
*
7 days
30 days
E
IL-10
[IL-10] pg/mL
2500
2000
1500
1000
500
0
7 days
30 days
F
Swiss
Linear regression
p=0,077
H_2O_2
Fungal load
4
3
2
1
0
0
10
20
30
40
Days
G
BALB/c
Linear regression
p=0,016
H_2O_2
Fungal load
10
8
6
4
2
0
0
10
20
30
40
Days

Figure 2

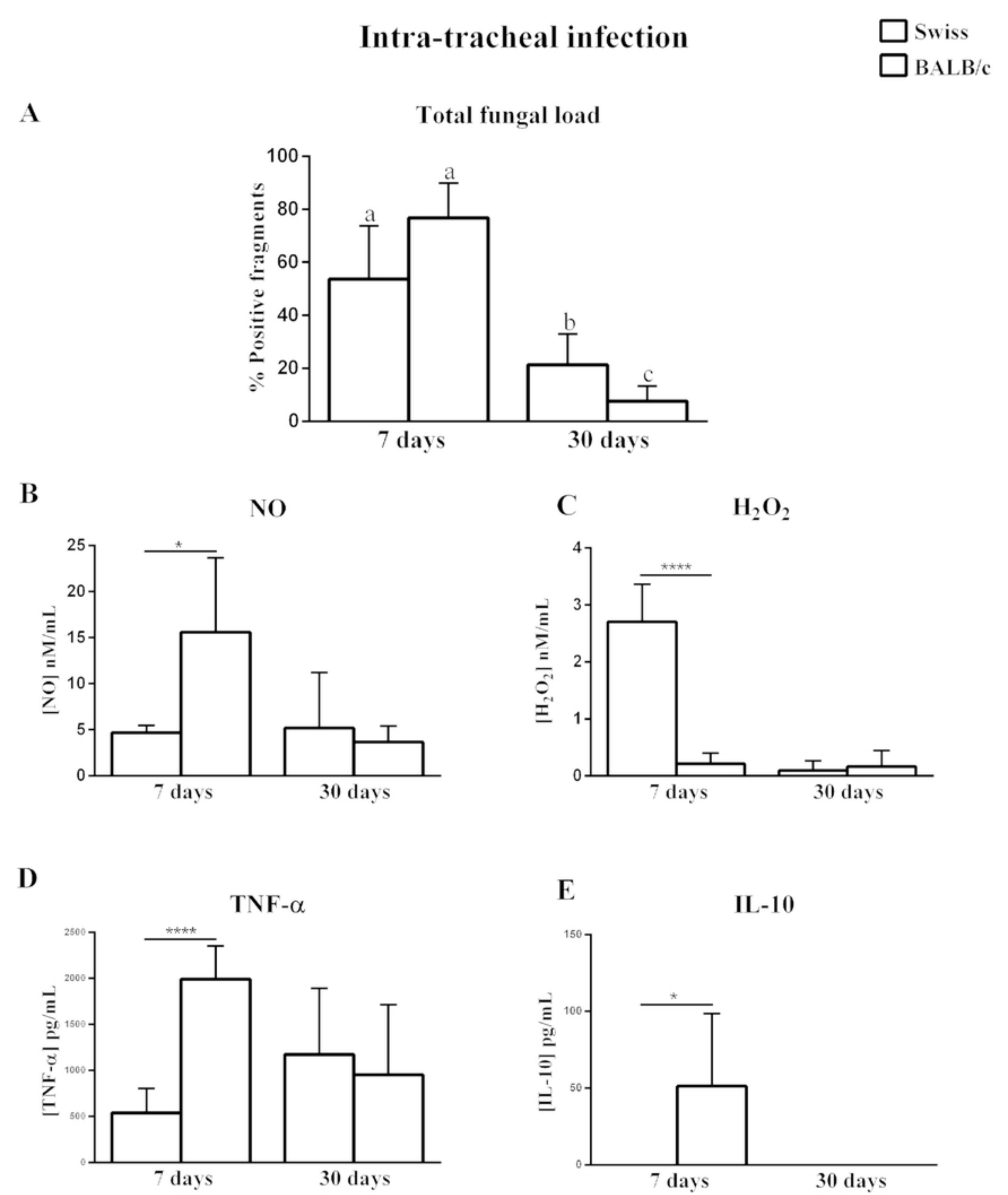
Intra-tracheal infection
Swiss
BALB/c
A
Total fungal load
% Positive fragments
100
80
60
40
20
0
a
a
b
c
7 days
30 days
B
NO
[NO] nM/mL
25
20
15
10
5
0
*
7 days
30 days
C
H_2O_2
$[H_2O_2]$ nM/mL
4
3
2
1
0
****
7 days
30 days
D
TNF-α
[TNF-α] pg/mL
2500
2000
1500
1000
500
0
****
7 days
30 days
E
IL-10
[IL-10] pg/mL
150
100
50
0
*
7 days
30 days

Figure 3