Medical Mycology, 2018, 56, 703–710
doi: 10.1093/mmy/myx109
Advance Access Publication Date: 8 December 2017
Original Article

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Azole-resistant and -susceptible *Aspergillus fumigatus* isolates show comparable fitness and azole treatment outcome in immunocompetent mice

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Received 4 July 2017; Revised 6 September 2017; Accepted 5 October 2017; Editorial Decision 13 September 2017

Abstract

No data are available on the *in vivo* impact of infections with *in vitro* azole-resistant *Aspergillus fumigatus* in immunocompetent hosts. Here, the aim was to investigate fungal fitness and treatment response in immunocompetent mice infected with *A. fumigatus* (parental strain [ps]) and isogenic mutants carrying either the mutation M220K or G54W (*cyp51A*). The efficacy of itraconazole (ITC) and posaconazole (PSC) was investigated in mice, intravenously challenged either with a single or a combination of ps and mutants (6×10^5 conidia/mouse). Organ fungal burden and clinical parameters were measured. In coinfection models, no fitness advantage was observed for the ps strain when compared to the mutants (M220K and G54W) independent of the presence or absence of azole-treatment. For G54W, M220K, and the ps, no statistically significant difference in ITC and PSC treatment was observed in respect to fungal kidney burden. However, clinical parameters suggest that in particular the azole-resistant strain carrying the mutation G54W caused a more severe disease than the ps strain. Mice infected with G54W showed a significant decline in body weight and lymphocyte counts, while spleen/body weight ratio and granulocyte counts were increased. In immunocompetent mice, *in vitro*

azole-resistance did not translate into therapeutic failure by either ITC or PSC; the immune system appears to play the key role in clearing the infection.

Key words: fitness, immune response, itraconazole, posaconazole, fungal infection.

Introduction

Antifungal treatment of invasive aspergillosis (IA) consists of systemically applied azoles, such as voriconazole (VRC), posaconazole (PSC), and itraconazole (ITC), or amphothericin B.[1,2,3,4] One major advantage of azoles compared to other antifungals is the availability of oral formulations,[2,5] facilitating home care of chronically ill patients. However, complications in the management of IA, representing as breakthrough infections, are rising due to increased incidence of acquired azole resistance in *A. fumigatus*.[6–13] Particularly, pan-azole resistant *A. fumigatus* isolates are associated with a worse outcome. The most frequent underlying genetic mechanism for azole resistance is single nucleotide polymorphisms (SNPs) in the *cyp51A* gene (lanosterol 14α-demethylase), the primary cellular target of azoles. Depending on the position of the SNP, the mutation causes a single-, a cross-, or pan-azole resistance. Substitutions in amino acid (aa) methionine 220 (M220) were linked to ITC,[14–16] while mutations in glycine 54 (G54) were linked to ITC and PSC resistance.[15,17–20] These two amino acid changes are often associated with extensive environmental or clinical azole exposure.[8,15,21,22] In clinical practice both immunocompetent[23–26] and immunocompromised patients[27,28] are suffering from aspergillosis. Aspergillosis in immunocompetent patients, such as chronic pulmonary aspergillosis, is classified as emerging infectious diseases. The diseases burden estimates a prevalence of more than 3 million patients worldwide. Oral azole treatment represents the standard of care.[29] As most studies refer to immunodeficiency, the current study aimed to evaluate the fitness and treatment response of azole-resistance isolates in a mouse model of a systemic *A. fumigatus* infection in the immunocompetent host.

Previous studies in immunocompetent invertebrate models found that drug resistance in *A. fumigatus* comes along with fitness cost, resulting in decreased virulence/pathogenicity.[30] For azole-resistance such differences in fitness were so far not observed if the host has an impaired immune system.[31,32] We aimed to evaluate whether differences in fitness are observed when the host is immunocompetent. To the best of our knowledge, the impact of azole-resistance on fitness and azole treatment response is investigated for the first time in an immunocompetent vertebrate model.

Methods

Fungal isolates and media

The strain set consisted of three *A. fumigatus* strains, namely a parental strain (ps) *A. fumigatus* CEA1_ΔakuB^{KU80} for the *cyp51A* gene and two isogenic mutant strains built on its basis, carrying either the mutation G54W or M220K.[10,14] Strains were labeled with barcode sequences that allow their discrimination with real-time polymerase chain reaction (PCR), as previously described by Valsecchi et al.[32] Strains carrying the mutation M220K display ITC resistance, those carrying G54W show ITC and PSC cross-resistance.[33] Susceptibility patterns were established using broth microdilution method for filamentous fungi according to Clinical and Laboratory Standards Institute (CLSI) document M38-A2;[34] minimal inhibitory concentration (MIC) results were classified according to recently proposed interpretative breakpoints[35,36] (see Supplementary Table 5). For inoculum preparation, all isolates were cultured on Sabouraud agar supplemented with gentamycin (160 mg/l) for 7 days at 37° C.

In vivo fitness studies and treatment response

Inoculum

Conidia were harvested from Sabouraud agar plates with physiological saline containing 0.01% Tween 20 and filtered through a 40 μm nylon cell strainer (BD Falcon), followed by a 5.0 μm filter to remove hyphae. The conidia were counted by means of a hemocytometer, and a final inoculum concentration of 1.0×10^6 colony forming units (cfu)/ml was adjusted. For coinfection experiments inocula were prepared by mixing the respective strains in equal proportions; final concentration from coinfection experiments did not differ from the mono-infection experiments.

In vitro fitness experiments

Strain fitness was compared with radial growth experiments. Inoculum was adjusted to 5×10^3 cfu/ml, and 5 μl were doted on Saboraud 2% medium (Sigma) without or with drugs (ITC; Sporanox, Janssen and PSC; Noxafil, Merck) at the following final concentrations 0.25 μg/ml and 1.0 μg/ml ITC and PSC 0.25 μg/ml. Experiments were performed in triplicates, and diameter was measured at three positions of each colony.

Animal model

In vivo significance of azole-resistance was evaluated using *A. fumigatus* CEA1_ΔakuB^{KU80} (parental strain) and two azole-resistant strains (G54W and M220K). Response to ITC (Sporanox, Janssen) and PSC (Noxafil, Merck) was evaluated using 7-week-old, female BALB/c mice ($n = 224$; Charles River Laboratory, Wilmington, MA, USA) with a body weight between 17.0 g and 18.0 g. The suitability of BALB/c mice for studying systemic IA in immunocompetent mice was previously demonstrated.[37] Mice were treated in accordance with the guidelines of the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' and the Austrian law. Animal experiments were approved by the ethics committee of the Austrian federal ministry of science and research (BMWF-66.011/0115-II/3b/2013). Mice were housed in standard boxes and fed with normal mouse chow and sterile water *ad libitum*. Mice were randomly divided in groups of seven individuals and either infected with a dose of 6×10^5 conidia/mouse[37] of a single strain or coinfected with two or three strains via injection into the lateral tail vein (day 0). Control groups (uninfected) received sterile physiological saline via lateral tail vein (day 0). ITC or PSC treatment (12.5 mg/kg p.o. BID) or administration of physiological saline (orally by gavage twice per day) started 24 h after infection (day +1). Animals were daily checked for clinical symptoms (reduced food/water uptake, apathy, rigidness, weight loss, and heavy breathing). All mice were killed by cervical dislocation (day +3 to simulate an early stage of infection in an immunocompetent host; in concordance with Mirkov et al.[37] and Valsecchi et al.[32] and dissected with preparation of spleen, kidneys, liver, and brain. The organs were weighted and correlated with the body weight at death.

Drug levels

ITC and PSC serum levels were measured at the local ISO15189 accredited ZIMCL (= Zentralinstitut für med. u chem. Labordiagnostik) with a commercial IVD-CE certified HPLC-FLD method (Chromsystems, Munich, Germany). Blood was obtained after a minimum of three given dosages. Blood was sampled 3, 6, 9, and 12 h after last administered drug dose, from seven mice per drug concentration and time point. Drug concentrations were measured for each individual sample. Values are given as mean (+SD) of the seven individual measurements per time point. Briefly, 20 μl samples were mixed with internal standard and precipitation solutions. Precipitates were removed by centrifugation; the supernatant was subjected to HPLC-FLD analysis. External calibration was performed on each measurement day; quality control samples were measured in each batch. The lower limit of quantification was 0.1 mg/l; the upper limit of quantification was 10 mg/l. The inter-day assay precision was better than 4.0%.

Fitness of ps and the two mutant strains (G54W and M220K) was evaluated in coinfection models (detection of the individual strains by real-time PCR) with and without ITC or PSC treatment. To investigate if azole resistance has an impact on the fitness of *A. fumigatus* strains, coinfection experiments were performed. Therefore, mice were either challenged simultaneously with a combination of the parental strain and either of the azole-resistant strains (ps + M220K or ps + G54W), or with a combination of all three strains with same proportions of each strain (ps + M220K + G54W) (total amount of 6×10^5 conidia/mouse did not differ between single- and coinfection). Kidney burden was relatively quantified by real-time PCR, as in intravenous infection model with *A. fumigatus* represents the most affected organ.

To evaluate the fitness of all three strains, the relative proportion of fungal strains in co-infected mice was determined using a real-time PCR assay, which was previously described by Valsecchi et al.[32] In short, PCR conditions were as follows: 95°C for 5 s; 45 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 2 min; 72°C for 10 min and a melt curve analyses (from 60°C to 95°C, 0.5°C steps for 10 s). Sso Fast SybrGreen (Bio-Rad, Munich, Germany) master contained: 5.0 μl Sso Fast SybrGreen, 10 mM of each primer, 3.0 μl PCR ultra-pure water, and a DNA concentration of 50 ng/reaction. Either of the following primer sets were used in singleplex PCR reactions: (1) ps ^{KU80}fwd *5′-CACATSCAAGTGAGACTGTTGTAACC-3′* and ps^{KU80}rev *5′-CTCAGTATAGGCAACAACACTTCAGG-3′*, (2) M220Kfwd *5′-CCTCCAAAACCACCAAGACCAC-3′* and M220Krev *5′-ACCTATTCCGATCACACCAAATCC-3′*, (3) G54Wfwd *5′-GTGGTGTGTGGGAGGTTTAGAGGTTTA-3′* and G54Wrev *5′-TCAGTATAGGCAACAACACTTCAGGGC-3′*.

All reactions were run in duplicates per marker and sample. A five-point standard curve was used to calculate relative percentages of WT and respective mutant strains. CFX96 Touch™ was used as PCR cycler together with CFX Manager™ Software v3.1. and Precision Melt AnalysisTM v.1.2. Software (Bio-Rad). Relative quantification was automatically calculated with CFX Manager™ Software v3.1.

Kidney homogenates were plated on Sabouraud agar supplemented with gentamycin (160 mg/l). After 48 h of incubation, colonies were harvested from Sabouraud agar plates with physiological saline containing 0.01% Tween 20 and transferred to an Eppendorf tube for DNA extraction, which was performed as already described by Moller et al.[38] DNA concentrations were measured by NanoVue spectrophotometer (Ge Healthcare, Vienna,

Austria). DNA was adjusted and used in the PCR described above.

Treatment response was studied by determining organ fungal burden. Organs (spleen, kidneys liver, and brains) were mechanically homogenized in 1.0 ml of 0.9% NaCl and serially 10-fold diluted. Dilutions were plated on Sabouraud agar supplemented with gentamycin (160 mg/l), and cfu were counted after 24 h and 48 h at 37°C. Limit of detection was 10 cfu/ml organ homogenate.

Clinical parameters such as body weight, spleen/body weight ratio, C-reactive protein (CRP), and blood values with and without ITC or PSC treatment were measured. Blood was taken at day +3 by puncture of the submandibular vein with EDTA as anticoagulant. After centrifugation at 1377 *g* for 10 min, the supernatant (= platelet-poor plasma; PPP) was used immediately or stored at −80°C for further use. The whole blood cell count was analyzed using scil Vet abc analyzer (Scil Animal Care Company, Gurnee, IL, USA). For quantification of CRP levels, the mouse CRP Duoset ELISA (R&D, Minneapolis, MN, USA) was performed according to the manufacturer's instructions. Each sample was processed in duplicates, and concentrations were calculated by creating a standard curve with a four-parameter logistic cure-fit (4-PL).

Statistical analyses

Data were graphically summarized in cross tables where mean and standard deviation were calculated as measures of central tendency and dispersion. Since data were in general not normally distributed, Kruskal–Wallis H tests were applied to test for differences between mice groups (uninfected, ps, M220K, G54W, ps+M220K, ps+G54W, ps+M220K+G54W), as well as therapy groups (none, ITC, PSC). In case of a significant omnibus *P*-value (i.e., <.05) from the Kruskal–Wallis H test, pairwise comparisons were performed according to the Dunn-Bonferroni method accounting for type 1 error rate inflation in multiple testing. *P* values below .05 (two-tailed) were regarded as statistically significant. Statistical analyses were performed using Graph Pad Prism, version 6 and SPSS, version 22 (SPSS, Inc., Chicago, IL, USA) software.

Results

Fitness of azole-resistant isolates and ps was compared in both: single infection and coinfection experiments.

In vitro experiments found that under drug-free conditions, no differences in radial growth speed was observed. Growth speed was in concordance with MIC results. WT failed to growth at all media containing ITC and PSC. (Supplementary Fig. 1).

In coinfection models, no statistically significant difference in the fitness (without antifungal treatment) was found between the parental strain and the two azole-resistant mutants, as similar proportions of all strains were found in the kidneys using PCR analyses (Supplementary Table 1).

Based on body weight loss, azole-resistant mutants were found to cause even more severe clinical symptoms than the ps strain, indicating no reduced virulence in infected animals. Mice infected with azole-resistant strains showed a more pronounced body weight loss than animals infected with the ps (Fig. 1), when they were untreated or treated with ITC. At the end of experiment, mice infected with G54W had only 88.80% of their body weight at experimental start, compared to 95.8% for ps-infected animals and 102% of mock-treated mice.

In addition, the outcome of azole therapy was compared between *in vitro* azole-resistant strains (G54W, M220K) and azole-susceptible parental strain.

Moreover, ITC and PSC treatment did not statistically significant impact on the distribution of strains found in the kidneys (Supplementary Table 1), even though sufficient serum levels of PSC and ITC were achieved in mice (Supplementary Fig. 2).

No significant difference in fungal burden of kidney, spleen, brain, and liver was observed between untreated, ITC- or PSC-treated mice and *A. fumigatus* ps and mutant strains (M220K and G54W; Supplementary Fig. 3). ITC treatment resulted in a by trend (not statistically significant) less pronounced body weight loss; particularly in G54W-infected mice. However, PSC-treatment had a highly significant impact on the sustain of body weight in all infected mice [ps ($P = .016$), M220K ($P = .003$), and G54W ($P = .001$)] (Fig. 1, Supplementary Table 2, data on coinfections: Supplementary Table 3).

Infection of the mice with ps did result in a reduction of total leucocyte number; this effect was much pronounced and even reached significance when infection was performed with G54W alone (Table 1; Supplementary Fig. 4) or in coinfection with ps (Supplementary Table 4). The phenomenon of leukocyte loss was independent of treatment. When monitoring in more detail the respective leucocyte subtractions, it can be shown that leukocyte reduction is mainly due to reduced lymphocyte numbers. Again, lymphocyte loss was more pronounced for G54W than for ps, independent of antimycotic treatment (Table 1). Neither monocyte nor granulocyte numbers did change significantly in the observed time period.

Moreover, the relative spleen weight increased by infection. Whereas uninfected controls had a spleen/body weight ratio of 6.02, the ratio did increase by infection with ps, M220K and G54W to 7.65, 7.75, and 7.12, respectively. Similar spleen enlargement was visible, when the parallel

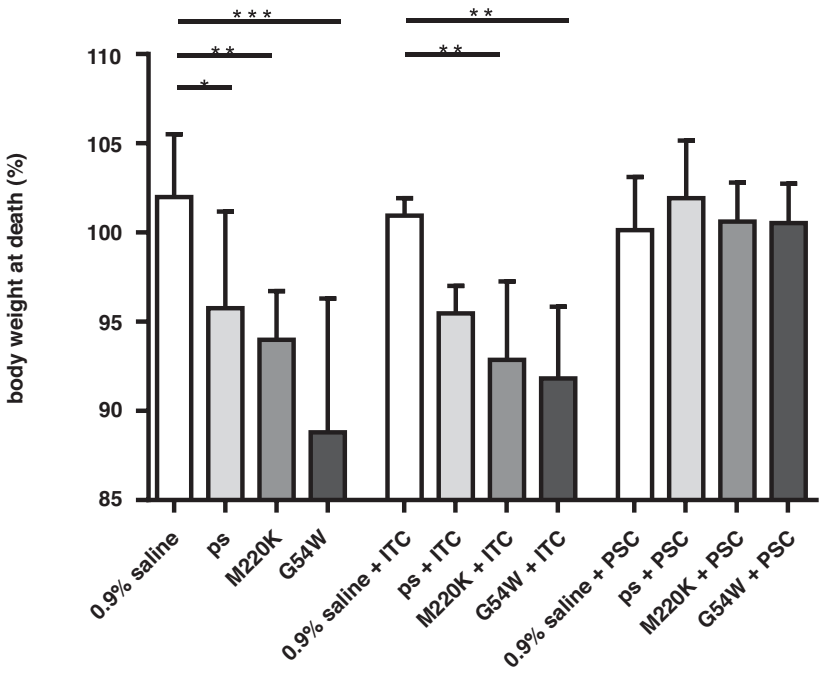

Figure 1. Body weight at time point of death (day +3) of BALB/c mice ($n = 7$) infected with 6×10^5 conidia/mouse of *A. fumigatus* ps, M220K, and G54W via lateral tail vein. Mice were treated with 12.5 mg/kg itraconazole (ITC) or posaconazole (PSC) orally by gavage twice a day. Control groups received sterile physiological saline. Bars denote the mean, whiskers the standard deviation. ps, parental strain *Aspergillus fumigatus* CEA1_ΔakuB^{KU80}; M220K, isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation M220K; G54W, isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation G54W. ** represents $P < 0.01$ and *** $P < 0.001$.

groups were treated with ITC or PSC (Fig. 2; Supplementary Table 2). For the CRP, no statistically significant differences were observed (see Supplementary Table 4, Supplementary Fig. 4c).

Discussion

In *Aspergillus* spp. azole-resistance is increasing, and clinical failure linked to azole-resistance is being reported in immunocompromised patients.[21,39,40] Azole-resistant *A. fumigatus* were not associated with a decreased fitness in immunodeficient hosts.[31,32] However, lack of knowledge exists about the impact of azole-resistance on strains' fitness, and the consequences on treatment response in intravenously infected immunocompetent vertebrate models. In the present study, these open questions were addressed.

Our *in vitro* data demonstrate that the mutations G54W and M220K in *cyp51A* are linked with cross-azole resistance for ITC and PSC (Supplementary Table 5). Other authors also found increased ITC and PSC MICs for isolates carrying these mutations.[14–16,20,22,41]

Fitness data showed that the ps was unable to outcompete the mutant strains in untreated mice; therefore, we conclude that these mutations do not lead to a reduced fitness. Also, Valsecchi et al.[32] recently showed that strains which carry the mutations M220K or G54W have no fitness loss *in vitro* and in immunocompromised mice. However, the fitness of strains carrying these mutation in an azole-exposed environment and the *in vivo* treatment response were not investigated by Valsecchi et al.[32] In our study, the mutations G54W and M220K are not of importance for the persistence of *A. fumigatus* in mice receiving ITC or PSC (Supplementary Table 1), as mutant strains were unable to outcompete the ps. However, intra-group variability was high, introducing some uncertainty to the results. In addition, in immunocompetent hosts, no fungal survival benefit was observed for azole-resistant *A. fumigatus* strains when these strains were challenged with azoles.

In our study, a statistically significant therapy-related result was that mice challenged with any of the three *Aspergillus* strains had higher body weights at point of death when they received PSC compared with mice receiving ITC or 0.9% saline. However, a therapeutical response in terms of reduction of fungal organ burden was not found, even though PSC serum levels were sufficient for both the ps and azole-resistant mutants (Supplementary Fig. 2). Hence, we hypothesize that PSC exerts some immune modulatory activity rather than having an enhanced antifungal activity. The beneficial effect of PSC on immune modulation was already described by others.[42] Fungal burden data for spleen and kidneys (Supplementary Fig. 2a and 2b) showed no statistically significant differences between mice challenged

Table 1. Number of different blood cells of the animals at day +3.

Therapy[a]	Mice groups[b]	Leucocytes ($\times 10^3/\mu$l)	Lymphocytes ($\times 10^3/\mu$l)	Monocytes ($\times 10^2/\mu$l)	Granulocytes ($\times 10^3/\mu$l)	Platelets ($\times 10^5/\mu$l)	Erythrocytes ($\times 10^6/\mu$l)
None	**0.9% saline**	9.88 (±1.44)	7.85 (±1.21)	3.44 (±0.86)	1.64 (±0.43)	10.29 (±2.36)	10.40 (±0.63)
	ps[c]	7.43 (±2.49)	**5.16 (±1.76)**	2.79 (±0.70)	1.44 (±0.32)	9.39 (±1.97)	10.27 (±0.60)
	M220K[d]	7.19 (±1.41)	**5.14 (±1.40)**	3.64 (±0.84)	1.72 (±0.35)	8.84 (±3.74)	10.40 (±0.33)
	G54W[e]	**5.00 (±1.35)**	**3.20 (±0.73)**	2.86 (±1.23)	1.49 (±0.43)	9.68 (±1.24)	10.58 (±0.77)
ITC	**0.9% saline**	10.13 (±1.76)	8.12 (±1.47)	3.57 (±0.79)	1.77 (±0.15)	12.38 (±1.64)	11.49 (±0.38)
	ps[c]	8.03 (±1.75)	6.11 (±1.36)	2.86 (±0.69)	1.17 (±0.27)	11.12 (±3.6)	10.52 (±0.47)
	M220K[d]	7.55 (±2.51)	**5.13 (±2.37)**	2.86 (±0.90)	1.34 (0.24)	11.51 (±1.66)	10.54 (±0.44)
	G54W[e]	**5.72 (±1.86)**	**3.91 (±1.40)**	3.43 (±0.97)	1.49 (±0.27)	10.64 (±1.29)	10.48 (±0.67)
PSC	**0.9% saline**	7.94 (±0.66)	6.36 (±0.69)	2.00 (±0.81)	1.39 (±0.24)	10.97 (±0.69)	10.79 (±0.43)
	ps[c]	8.65 (±1.27)	6.88 (±0.93)	3.00 (±0.89)	1.48 (±0.38)	8.66 (±2.11)	9.87 ± 0.63)
	M220K[d]	7.33 (±1.21)	**5.53 (±1.15)**	3.00 (±0.63)	1.50 (±0.25)	10.32 (±2.18)	9.88 ± 0.36)
	G54W[e]	**5.02 (±1.08)**	**3.65 (±0.83)**	2.33 (±0.37)	1.13 (±0.26)	9.12 (±1.19)	9.70 (±0.50)

Results are presented as mean ± standard deviation.

[a]ITRA (12.5 mg itraconazole /kg orally by gavage twice a day), PSC (12.5 mg posaconazole /kg orally by gavage twice a day), none (animals received sterile physiological saline).

[b]Groups of BALB/c mice ($n = 7$) were challenged with 6×10^5 conidia/mouse via lateral tail vein

[c]Parental strain *Aspergillus fumigatus* CEA1_ΔakuB^{KU80}.

[d]Isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation M220K.

[e]Isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation G54W.

Bold numbers indicate statistical significance based on *P*-values gained for Kruskal–Wallis test and Dunn Bonferroni's multiple comparison test, the later was only performed when a statistically significant omnibus *P*-value was found (overview on statistical results is given in Supplementary Table 2 and 6). *P*-values smaller than 0.05 were regarded as statistically significant.

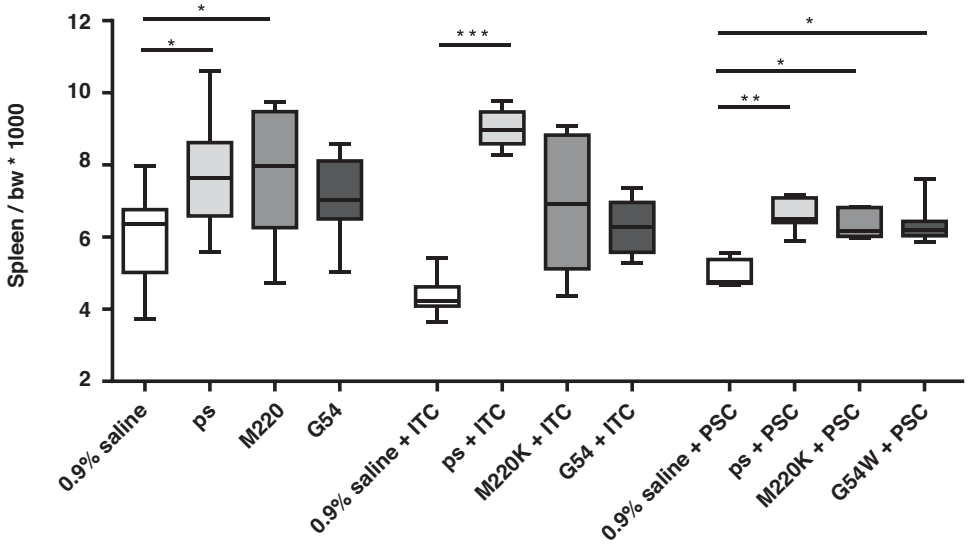

Figure 2. Box plot of spleen/body weight ratio at time point of death (day+3) of BALB/c mice (7 mice/group) challenged with 6×10^5 conidia/mouse via lateral tail vein (spleen weight/body weight*1000). Mice were treated with PSC (12.5 mg posaconazole/kg orally by gavage twice a day), ITC (12.5 mg itraconazole/kg orally by gavage twice a day) or received 0.9% saline. ps, parental strain *Aspergillus fumigatus* CEA1_ΔakuB^{KU80}; M220K, isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation M220K; G54W, isogenic *A. fumigatus* mutant strain built on the basis of CEA1_ΔakuB^{KU80} carrying the mutation G54W. * represents $P < 0.05$ and *** $P < 0.001$.

with the ps strain or G54W (Supplementary Table 6). The lack of any statistically significant reduction of fungal organ burden in mice challenged with an *in vitro* ITC- and PSC-susceptible strain suggests that antifungal therapy is of limited value in the acute phase of infections in immunocompetent mice. Hence, *in vitro* observed resistance does not appear to directly translate into *in vivo* resistance, as infection is mainly cleared by the immune system.

A potential limitation of our study is the relatively short time period to investigate treatment outcome. However, this experimental set up was chosen as Mirkov et al.[37] found the greatest difference in immunological response in

immunocompetent mice 3 days after their infection with *A. fumigatus*. To verify antifungal serum levels, ITC and PSC concentrations were measured at 3 h, 6 h, 9 h, and 12 h (immediately prior to the next dosage) after administration (Supplementary Fig. 1). Another limitation is the relatively low inoculum size (6×10^5 conidia/mouse), which was chosen based on (a) the rationale of Mirkov et al.[37] and (b) our inoculum doses experiments (data not shown), which found that a higher inoculum leads to a sudden inflammation syndrome and subsequent death in immunocompetent mice.

An enlargement of the spleen is known to reflect the intensity of immunological response to fungal infection. Increased spleen mass and cellularity in response to *Aspergillus* infection was also found by other groups and was diagnosed by immunohistochemistry to be mainly due to red pulp upgrowth, whereas germinal centers and marginal zone appearance were unaltered.[37] Since the mice spleen is a hematopoietic organ, the enhanced spleen/body weight ratio reflects enhanced hematopoietic activity and can be hypothesized to encounter the increased need of leucocytes during the acute phase of fungal infection.[37] Moreover, leucocyte influx from periphery to the spleens might have contributed to enlargement of this lymphatic organ. This hypothesis is supported by the finding in our study that peripheral leucocyte counts were reduced by infection and also by other studies in the literature (Mebius and Kraal 2005).[43]

In our study, reduction in leucocyte numbers in the acute immune response against *A. fumigatus* was observed to be strain-dependent, with highest manifestation in mice challenged with G54W (Table 1, Fig. 1). The effect was less pronounced when mice were challenged with ps (Supplementary Table 4). This loss in leucocytes was exclusively due to reduced lymphocyte numbers, while granulocytes and monocytes remained unchanged. Since lymphocytes are the main leucocyte subtraction to traffic to the spleen and orchestrate the immune response in this organ, these findings correlate perfectly with the above mentioned spleen enlargement. The relevance of lymphocytes, particularly of B-cells, for the antifungal immune defense is highlighted by the fact that individuals with B1 lymphocyte deficiencies were at increased risk for acquiring fungal infections such as paracoccidioidomycosis.[44]

The parameters of infection-induced body weight loss and lymphocyte number reduction suggest strain-dependent differences in immune response; the most prominent reaction was associated with G54W and the combination ps+G54W, followed by M220K, and the ps strain. Depletion of ergosterol and altered membrane permeability in the mutants[45] might result in altered cell wall synthesis and subsequently also in a modified immune response compared to the parental strain. Consequences might be a less efficient clearance and thus an enhanced virulence of the mutant strains.

So far for immunocompetent vertebrate host, our findings are in agreement with studies performed in immucompromised host where no decline of fitness was associated with azole resistance.[31,32] Studies from Mavridou et al.[31] support our data using non-neutropenic mice and a clinical azole-resistant *A. fumigatus* strain. Furthermore, in contrast to immunocompromised hosts,[21,40,41] *in vitro* resistance patterns of fungal isolates do not correlate with infection outcome under *in vivo* therapy in immunocompetent mice, underlining the central role of the immune system to overcome azole-resistant strains.

In immunocompetent mice, azole-resistant *A. fumigatus* strains carrying the point mutations M220K or G54W had no fitness disadvantage compared with wt strain. The resistant strains were unable to outcompete the ps in coinfected immunocompetent mice, either untreated or receiving ITC or PSC. No significant differences in outcome were observed independently of the antimycotic drug (ITRA, PSC) and strain applied, highlighting the prominent role of the immune system in clearing pathogens in the early phase of infection.

Acknowledgments

The authors thank the technical staff from the Division of Hygiene and Medical Microbiology for assistance.

This work was funded by the ERAnet grant AspBiomics Project Nr. FWF I656-B09 (Austrian Science Fund).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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