Recombinant murine IL-12 promotes a protective Th1/cellular response in Mongolian gerbils infected with Sporothrix schenckii

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Antimicrobial Original Research Paper

Recombinant murine IL-12 promotes a protective Th1/cellular response in Mongolian gerbils infected with *Sporothrix schenckii*

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Sporotrichosis is a cutaneous fungal infection caused by *Sporothrix schenckii*. It is known to be mainly contained by Th1 responses. As IL-12 is crucial for Th1 response, we investigated if treatment with recombinant murine IL-12 (rmIL-12) promoted Th1 immunity and/or clinical improvement in an experimental sporotrichosis gerbil model. Gerbils were inoculated with *S. schenckii* in the footpad and treated with rmIL-12. Seven days post infection there was a significant increase in macrophage phagocytosis and oxidative burst, and in delayed-type hypersensitivity (DTH) reaction in rmIL-12 treated gerbils, as well as a $\sim$10-fold increase of serum IFN-gamma and a decrease of IL-4 and IL-10. Moreover, rmIL-12 substantially decreased ($\sim$70%) *S. schenckii* burden in liver and spleen and improved the clinical outcome preventing footpad ulcer and tail nodules observed in untreated gerbils. Our study demonstrates that rmIL-12 promotes Th1 immune response against *S. schenckii* favouring its clearance and preventing clinical symptoms.

Keywords: Sporotrichosis, *Sporothrix schenckii*, IL-12, *Meriones unguiculatus*, Th1 response

Introduction

Sporotrichosis is a subacute or chronic infection caused by the dimorphic fungus *Sporothrix schenckii*, which has worldwide distribution, especially in tropical and subtropical zones.[1] Typically, *S. schenckii* infection causes lesions usually restricted to the skin, subcutaneous cellular tissue, and adjacent lymphatic vessels due to inoculation, but inhalation resulting in pulmonary or disseminated disease may also rarely occur.[1] Disseminated sporotrichosis, cutaneous, or systemic develops mainly in immunocompromised individuals, particularly in those infected with HIV; where infection can be fatal.[2]

Virulence of *S. schenckii* is thought to play a role in the development and evolution of sporotrichosis, although discordant results have been reported in experimental sporotrichosis with clinical isolates from cutaneous and disseminated disease suggesting that host immune response has also an important participation in disease progression.[3]

Innate immune response appears to be the initial means by which *S. schenckii* infection is contained.[3,4] This response is largely mediated by macrophages (MΦ) apparently via Toll-like receptor 4, which is able to recognize molecules extracted from the yeast form of the fungus, leading to macrophage oxidative burst against it.[3,5]

On the other hand, cell-mediated response involving macrophages activated by CD4$^+$ T cells appear

to mainly exhibit the acquired immunity against *S. schenckii*.[6] This cell-mediated response has been previously described to be predominantly Th1 in human sporotrichosis.[7] Nevertheless, models of experimental murine sporotrichosis have shown to involve both Th1 and Th2 responses, but apparently the latter is presented only at advanced stages of the infection.[3,8]

Considering the above stated, Th1 cytokines production ought to be involved in the pathogenesis of *S. schenckii*. Of particular interest is IL-12, a heterodimeric cytokine mainly produced by activated phagocytes and dendritic cells.[9] This cytokine plays a critical role in the induction of Th1 response through IFN-gamma production, which activates macrophages enhancing their antimicrobial activities.[9,10]

Previous studies have shown that treatment with IL-12 can promote protective Th1 immune response *in vivo* against fungal pathogens such as *Histoplasma capsulatum*[11] and *Coccidiodes immitis*.[12] We hypothesized that a similar outcome might occur against *S. schenckii*. Therefore, we investigated the effect of IL-12 administration in an experimental sporotrichosis gerbil model.

Material and methods
Animals
Healthy 3-month old male Mongolian gerbils (*Meriones unguiculatus*), ranging in weight from 60 to 70 g, were supplied by the animal breeding facility of Centro de Investigación Biomédica de Occidente. Three groups of seven gerbils each were formed and housed in polycarbonate cages. They were kept under pathogen-free conditions in a room without windows with a set temperature of $22 \pm 2°C$, a 12-hour light schedule (lights on at 7:00 a.m. and off at 7:00 p.m.), and a relative humidity of $50 \pm 10\%$. Gerbils received pelleted food (Purina®, México) and water *ad libitum*.

Fungus
Sporothrix schenckii yeast-like form cells (SsY) were obtained by culturing a clinical isolate of *S. schenckii* in brain–heart infusion broth following previously described conditions.[4] Above 95% cells harvested from culture were differentiated into yeast-like form. *Sporothrix schenckii* yeast-like form cells were washed three times with Hank's balanced salt solution (HBSS), pH 7.4, and cell numbers were adjusted to a concentration of 1.2×10^7 SsY/mL. Viability of SsY was ascertained by determining the number of colony-forming units (CFU) after 7 days on incubation on brain–heart infusion plate agar at $37°C$ according to the protocol reported by Fernandes *et al.*[4]

Experimental infection
Gerbils from two of the three groups were infected by injecting subcutaneously 6×10^6 SsY suspended in 0.05 mL of sterile phosphate-buffered saline (PBS), pH 7.4, in the left hind footpad. Gerbils from the remaining group were injected with the vehicle (sterile PBS) alone, and were used as the healthy group.

IL-12 administration
Recombinant murine IL-12 (rmIL-12) was purchased as I-8523 (Sigma, St. Louis, MO, USA), and was diluted in 0.1 M PBS, pH 7.4. A group of infected gerbils were given rmIL-12 doses of 500 ng in a volume of 300 µL per day by intraperitoneal (i.p.) administration for 5 days, starting at the same time as the infection. This group was labelled as the infected + rmIL-12 group. The same dose/schedule/via were administered to both the remaining group of infected gerbils, hereafter called infected group, and the healthy group; but receiving PBS, pH 7.4, instead of rmIL-12.

Peritoneal macrophage isolation
On day 5 after the infection, gerbils were i.p. injected with 1 mL of sterile fluid thioglycollate broth (Merck, México 15867). Peritoneal macrophages (MΦ) were obtained 2 days later as previously described.[13] Final cell suspension contained more than 93% of peritoneal MΦ as identified by non-specific esterase colouration and phagocytosis. Viability, confirmed by the trypan blue (SIGMA, México 468) exclusion test, was above 95%. Peritoneal MΦ were resuspended at a density of 1×10^6 cells/mL in RPMI-1640 medium (R-6504, SIGMA) supplemented with 10% foetal calf serum (S-2442, SIGMA) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, Sector Salud, México 1921).

Phagocytosis assay
Phagocytosis assay was performed following the protocol of Fernandes *et al.*[14] with slight modifications. Briefly, peritoneal MΦ (5×10^5 cells) were plated on glass slides and incubated for 2 hours at $37°$ in an atmosphere of 5% CO_2. Non-adherent cells were removed by two washes with pre-warmed medium. Suspensions of SsY were placed on slides previously coated with a monolayer of adherent peritoneal MΦ at a ratio of 10 : 1 (SsY–MΦ). Slides were then incubated at $37°C$ in an atmosphere of 5% CO_2 for 2 hours Phagocytosis was stopped by washing the slides three times with cold RPMI-1640 to remove non-ingested fungi. Cells were then fixed with methanol–acetone (1 : 1) for 10 minutes stained with Wright's stain and observed with an optical microscope using $100 \times$ objective. The average number of ingested SsY per peritoneal MΦ was determined after counting the total of ingested SsY in 200 peritoneal MΦ.

Macrophage oxidative burst evaluation
Oxidative burst in peritoneal macrophages was measured by luminol-dependent chemoluminescence assay following a previously described protocol.[15] Briefly, 10^6 peritoneal MΦ suspended in 1 mL of phenol red-free HBSS (SIGMA) were used for each

test. Chemoluminescence elicited by 15 mg/ml zymosan (SIGMA Z-4250) was measured in the presence of 40 µL of luminol (SIGMA A-8511) (final concentration of 5 µM) using a luminometer 1250 (LKB Instruments, Inc) at 5-minute intervals for a total of 60 minutes, at 37°C.

Sensitization and elicitation of delayed-type hypersensitivity reaction

Gerbils were sensitized to 2,4,-dinitro-1-fluorobenzene (DNFB) by placing 20 µL of 0.5% DNFB in 4 : 1 acetone–olive oil on the shaved abdominal wall skin of gerbils on days 0 and 1 after infection. Delayed-type hypersensitivity (DTH) reaction was elicitated by the ear-swelling assay. Gerbil ear was painted with 10 µL of 0.2% DNFB on day 5 post infection. Ear thickness was measured 48 hours after DNFB challenge using a Mitatoya engineer micrometer and results expressed in units of 0.01 mm.

Serum cytokine measurement

Cytokine levels in serum were assessed by enzyme-linked immunosorbent assay (ELISA) on day 7 after infection. Commercially available kits using specific monoclonal antibodies (mAbs) for IFN-gamma, IL-4, or IL-10 (Amersham Pharmacy Biotech, NJ, USA) were used. The assays were performed according to the manufacturer instructions. Cytokine concentration in picograms per millilitre was calculated from a standard curve for each assay.

Determination of fungal burden in tissue

Fungal burden of liver and spleen tissues of gerbils infected with *S. schenckii* and treated with rmIL-12 was assessed 7 days post infection. Gerbils were euthanized by asphyxiation and their livers and spleens were taken aseptically and homogenized in 3 mL of sterile PBS. Serial dilutions of the homogenates were plated on Saboraud dextrose agar and colonies were estimated by quantitative counts of CFU as elsewhere described.[4]

Clinical evolution

Two gerbils from each group were kept alive under the conditions afore mentioned for 60 days after infection in order to observe its clinical evolution of sporotrichosis with and without rmIL-12 treatment.

Statistical analysis

Statistical analyses and graphics were performed using Prism v.6 (Graphpad software, Inc; San Diego CA, USA). Student *t*-test was used to assess mean differences between groups. *P*-values of <0.05 were considered statistically significant. Values were corrected with the Sidak–Bonferroni method.

Results
Effect of rmIL-12 over MΦ activities

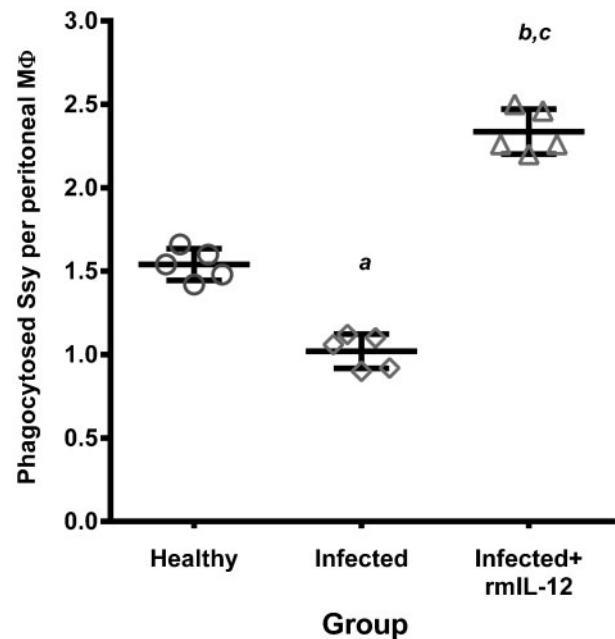Phagocytic activity from gerbil peritoneal MΦ was evaluated by challenging them with Ssy 7 days after

Figure 1 Effect of rmIL-12 on Sporothrix schenckii infected gerbil peritoneal MΦ phagocytosis. Peritoneal MΦ were isolated from treated gerbils at day 7 and were cocultured with Ssy for 2 hours at 37°C, 5% CO_2. The average of ingested Ssy per MΦ was calculated after counting 200 MΦ. Values are expressed as mean±SD for five individual gerbils per group. Ssy: S. schenckii yeast-like form cells; (a) infected vs healthy $P<0.0001$; (b) infected+rmIL-12 vs healthy $P<0.0001$; (c) infected+rmIL-12 vs infected $P<0.0001$

receiving the respective treatments. An over two-fold increase of phagocytic activity was observed in peritoneal MΦ of *S. schenckii* infected, rmIL-12 treated gerbil group (infected+rmIL-12) compared to the infected group ($P<0.0001$) (Fig. 1). When comparing against the healthy group we can see how *S. schenckii* infection caused a $\sim$50% decrease in MΦ phagocytic activity against Ssy ($P<0.0001$), while the infection plus rmIL-12 increased it around 70% ($P<0.0001$) (Fig. 1).

A similar trait was observed while evaluating the oxidative burst of peritoneal MΦ by luminol-dependent chemiluminescence assay (Fig. 2). The results show how oxidative burst is above two-fold increased in the infected+rmIL-12 group over the infected group at all evaluated times ($P<0.0001$), while comparisons against the healthy group show that oxidative burst is $\sim$50% decreased in the infected group at all times ($P<0.0001$) but the 5 minutes measure, in which the decrease was 20% ($P=0.002$); and $\sim$70% increased in the infected+rmIL-12 group at all times ($P<0.01$) but the 50 and 60 minutes measures, in which the observed increase was not statistically significant (Fig. 2). Time 0 was not included in afore mentioned group comparisons.

Effect of rmIL-12 over DTH reaction

Gerbils from the three groups were sensitized to DNFB at day 0 and 1 and challenged with it at day 5
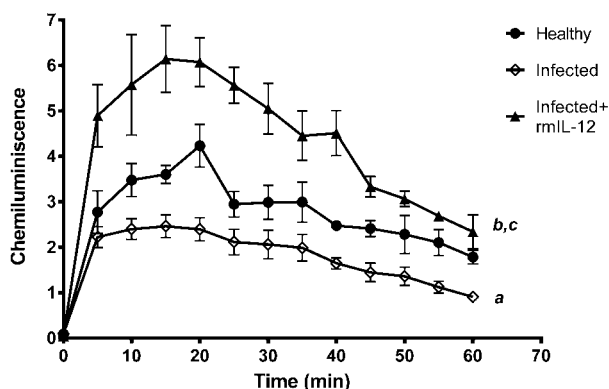
Figure 2 Effect of rmIL-12 on Sporothrix schenckii infected gerbil peritoneal MΦ oxidative burst. Peritoneal MΦ were isolated from treated gerbils at day 7. 10^6 peritoneal MΦ were cultured at 37°C in the presence of 15 mg/ml zymosan and 5 μM luminol. Chemiluminescence was measured at 5 minutes intervals in an LKB 1250 luminometer. Values are expressed as mean±SD for five individual gerbils per group. All comparisons lacked significance at time 0. (a) infected vs healthy $P<0.01$ at all times; (b) infected+rmIL-12 vs healthy $P<0.01$ at all times but 50 and 60 minutes; (c) infected+rmIL-12 vs infected $P<0.0001$ at all times

Figure 3 Effect of rmIL-12 on Sporothrix schenckii infected gerbil DTH reaction. Treated gerbils were sensitized with 0.5% DNFB on days 0 and 1. On day 5, gerbils were challenged with 0.2% DNFB in the ear. Ear thickness was measured before challenge (0 hour) and 48 hours after challenge. Values are expressed as mean±SD for five individual gerbils per group. DTH: Delayed-type hypersensitivity; DNFB: 2,4,-dinitro-1-fluorobenzene; (a) infected vs healthy $P<0.0001$; (b) infected+rmIL-12 vs healthy $P<0.0001$; (c) infected+rmIL-12 vs infected $P<0.0001$.

post infection to elicitate DTH reaction using the ear-swelling assay. Figure 3 shows the result of the assay, expressed in mm, for the measures before the challenge (0 hour) and 48 hours after. As expected, there was no difference in ear thickness between groups at time 0, as no DTH reaction had been elicited (Fig. 3). On the other hand, 48 hours after DNFB challenge, ear thickness, and hence DTH, was significantly higher in gerbils from the infected+rmIL-12 group than in gerbils from the infected and healthy groups ($P<0.0001$ in both comparisons) (Fig. 3). Moreover, DTH was decreased in the infected group compared to the healthy group ($P<0.0001$).

Effect of rmIL-12 over serum cytokine concentration

We sought to evaluate the type of immune response elicited by *S. schenckii* infection with and without rmIL-12 treatment by measuring serum levels of Th1 and Th2 cytokines. For that purpose serum was obtained from all gerbil groups at day 7 after infection and put through ELISA for the Th1 cytokine IFN-gamma, and the Th2 cytokines IL-4 and IL-10. Serum concentration of all cytokines evaluated increased significantly in both infected and infected+rmIL-12 groups compared to the healthy group ($P<0.001$), meaning *S. schenckii* infection promotes these cytokines production (Fig. 4). Furthermore, we found that *S. schenckii* infection promotes a Th2 response by augmenting serum concentrations of IL-4 and IL-10 over IFN-gamma, whereas treatment with rmIL-12 polarized the response towards Th1 as shown by the almost 10-fold increase of IFN-gamma ($P<0.0001$) and the 30
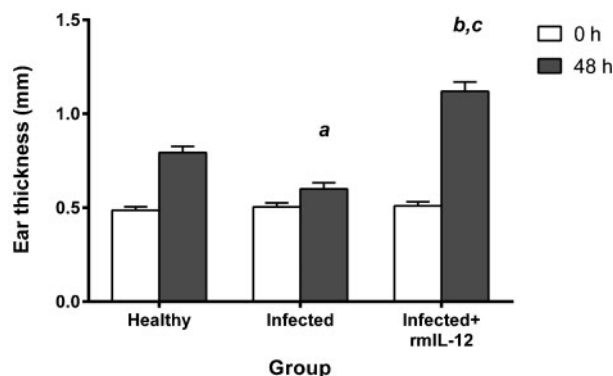
and 50% decrease of IL-4 and IL-10, respectively ($P<0.0001$) compared to the infected group (Fig. 4).

Effect of rmIL-12 on tissue fungal burden and clinical evolution

The measurement of tissue *S. schenckii* burden of infected and treated and infected alone gerbils showed that rmIL-12 treatment improved pathogen elimination 7 days post infection reducing ~70% of liver and spleen fungal burden compared to the untreated group (Fig. 5). Likewise, treatment with rmIL-12 improved the clinical response to *S. schenckii* showing inflammation at the infected footpad and no further clinical events at the period of time of observation (60 days), contrasting with the ulcer in infected footpad and tail nodules developed in the untreated group (data not shown).
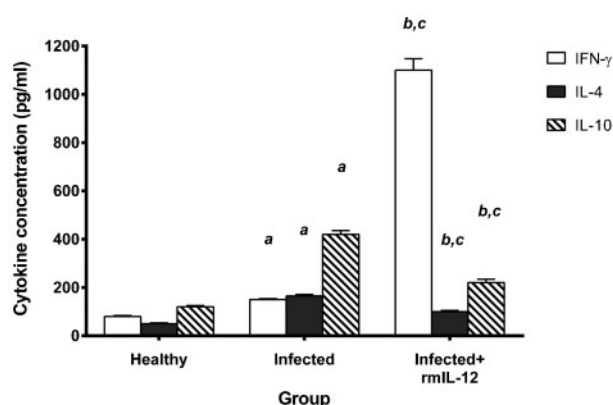

Figure 4 Effect of rmIL-12 on Sporothrix schenckii infected gerbil serum cytokine levels. Serum from treated gerbils was obtained on day 7. Levels of IFN-gamma, IL-4, and IL-10 were assessed by ELISA. Values are expressed as mean±SD for five individual gerbils per group. (a) infected vs healthy $P<0.0001$; (b) infected+rmIL-12 vs healthy $P<0.001$; (c) infected+rmIL-12 vs infected $P<0.0001$.
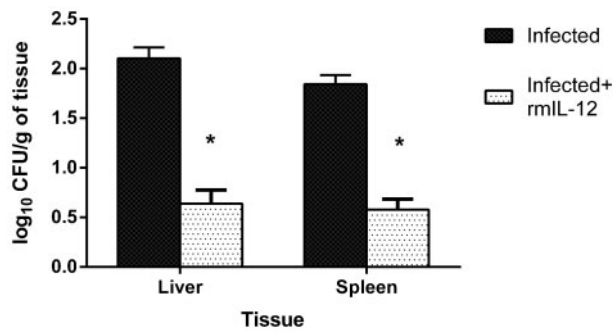
Figure 5 Tissue fungal burden of Sporothrix schenckii infected and rmIL-12 treated gerbils. Liver and spleen were collected from euthanized gerbils at day 7, homogenized in PBS and plated on Saboraud dextrose agar. Resulting CFU were count. CFU: colony-forming units; * $P < 0.0001$

Discussion

Sporothrix schenckii is a dimorphic fungus responsible for sporotrichosis. Although virulent *per se*, *S. schenckii* infection severity has been observed to relate to the immune status of the host as it is mainly contained, as some other fungi pathogens, by Th1 responses.[3] Considering that IL-12 is pivotal for this kind of response,[9,10] we evaluated whether this cytokine promoted Th1 response and/or clinical improvement in a gerbil experimental model of sporotrichosis.

Macrophages are important effectors of the Th1/cellular immune response killing pathogens via phagocytosis through oxidative burst.[10] A previous investigation reported the role of IL-12 in MΦ phagocytosis of *S. schenckii*, showing that antibody blockade of IL-12 lead to a decrease in Ssy phagocytosis by gerbil peritoneal MΦ.[16] Here we corroborate the importance of IL-12 in MΦ phagocytosis showing that i.p. treatment with rmIL-12 enhanced peritoneal MΦ phagocytic activity against Ssy (Fig. 1) in a similar experimental model. Also, we found rmIL-12 increased oxidative burst of peritoneal MΦ (Fig. 2) showing a direct relation with the results of Ssy phagocytosis. These findings are in accordance with the reported biological function of IL-12 of promoting IFN-gamma production from T and NK cells, which ultimately contributes to enhance MΦ killing abilities.[9] In contrast, we observed a decrease of MΦ phagocytosis and oxidative burst in the infected group. The former might be attributed to the inhibitory action of *S. schenckii* lipid membrane on MΦ phagocytosis previously reported;[17] the latter is of interest as it gives clue of the role of *S. schenckii* infection in production of reactive oxygen species in MΦ other than nitric oxide as zymosan, used as activator in our study, does not elicit its production;[17,18] concordant with the reported for polymorphonuclear leucocytes.[19]

Delayed-type hypersensitivity measurement results followed a pattern similar to that observed for MΦ phagocytosis and oxidative burst: a decrease in the infected group and an increase in the infected + rmIL-12 group (Fig. 3). Being DTH a cell-mediated immune mechanism modulated by Th1 lymphocytes,[20] the down-regulation of DTH by *S. schenckii* is most probably due to T-cell suppression mediated by reactive nitrogen species, particularly nitric oxide; previously reported by Fernandes *et al.*[4] Treatment with rmIL-12 evidently led the phagocytes to overcome this suppression, as shown by the increased phagocytosis and oxidative burst of peritoneal MΦ (Figs. 1 and 2, respectively); enhancing DTH *in vivo*.

Since phagocytosis, oxidative burst and DTH are Th1 mediated,[9,20] any change in Th1/Th2 cytokine balance will have directly repercussion over their performance. This was corroborated by our experiments, showing that the decrease in phagocytosis, oxidative burst and DTH observed in *S. schenckii* infected gerbils came along with an increase in serum levels of the Th2 cytokines IL-4 and IL-10 and low levels of the Th1 cytokine IFN-gamma (Fig. 4), results that are in agreement with previous reports regarding IL-10 production in sporotrichosis.[4,5] However, the moderate increase of IL-4 within 7 days after infection differs from the increase starting at the 5th week reported by Maia *et al.*,[8] suggesting there might be presence, in a minimal amount at least, of humoral response during early stages of sporotrichosis, although this also might be due to the use of a different experimental model.

On the other hand, the enhancement of phagocytosis, oxidative burst and DTH observed in rmIL-12 treated gerbils was accompanied with high serum levels of IFN-gamma (Fig. 4). This finding is novel, as the use of rmIL-12 has not been reported before in sporotrichosis experimental models. Nevertheless, our results are in accordance to those reported for the use of rmIL-12 with other fungal pathogens like *H. capsulatum*,[11] *C. immitis*,[12] *Pneumocystis carinii*,[21] *Aspergillus fumigatus*,[22] and *Candida albicans*[23] and for the bacterium *Streptococcus pneumoniae*;[24] in terms of IFN-gamma secretion.

Interestingly, the increase in serum IFN-gamma was consistent with a reduction of viable *S. schenckii* CFU in liver and spleen (Fig. 5) and thus with an improvement of the clinical course of the infection, confirming that Th1 immunity is pivotal for sporotrichosis clearing.[1,3] In fact, rmIL-12 promoting beneficial effects by IFN-gamma-dependent pathway has been previously reported in murine experimental models of mycoses like histoplasmosis,[11] coccidiodomycosis,[12] *Pneumocystis* pneumonia,[21] and aspergillosis,[22] and in respiratory infection by extracellular bacteria.[24] However, IFN-gamma increase by rmIL-12 treatment showed to be detrimental in *C. albicans* infection, leading to enhanced susceptibility.[23] Th1 importance in sporotrichosis was further evidenced by the findings in untreated infected gerbils, which showed high tissue

fungal load and developing of clinical symptoms, corresponding to the increase of IL-10 and IL-4 and very low levels of IFN-gamma in serum.

Considering our results and the fact that visceral *S. schenckii* isolates have been reported to induce a weaker Th1 response, hence being more virulent, than cutaneous isolates;[7] the use of IL-12 might be helpful in patients bearing visceral sporotrichosis, as it might boost the Th1 response contributing to the proper clearance of the infection. However, attention must be put in the proper delivery to infected tissue as previous reports showed the efficacy and safety of locally administrated rIL-12 in infection[25] and cancer;[26] unlike the high incidence of toxic side effects of systemic administration.[27]

Due to their capability of promoting innate anti-fungal immunity, it would have been interesting to assess Th17 serum levels, such as IL-17 and IL-22. Nevertheless, considering the strong Th1 response elicited by rmIL-12 treatment here shown and the fact that Th17 protection against fungus seems to be dispensable in the presence of functional Th1 response,[28,29] particularly for *S. schenckii* infection;[29] the lack of Th17 cytokine measures does not reduce relevance to our findings.

Despite the use of Mongolian gerbils instead of mice, evidence is here presented that rmIL-12 treatment has a beneficial effect on sporotrichosis. Furthermore, our results show similarities with both studies with sporotrichosis and with infections treated with rmIL-12.[4–6,11,12,19,21] In this respect, high homology gerbil-mice has been reported in cytokine genes particularly in IL-12, IFN-gamma, and IL-10,[30,31] supporting the results of the majority of cross-species assays here performed. Moreover, endogenous gerbil IL-12 related activity was reported to be abrogated by treatment with anti mouse IL-12 monoclonal antibody,[16] further supporting gerbil-mouse cross reactivity hypotheses.

In summary, our data suggest *S. schenckii* inhibits Th1 effector function *in vivo* by promoting anti-inflammatory cytokine production, such as IL-10 and IL-4, and exogenous administration of IL-12 overcomes that inhibition by enhancing IFN-gamma production, hence protecting gerbils against systemic infection. This is the first report about the use of rmIL-12 in experimental sporotrichosis. Further studies are required to elucidate the underlying molecular mechanisms involved in the process as well as the pharmacological implications of its use.

Disclaimer statements

Contributors Aurelio Flores-García: Concept and experimental design, technical analysis, contribution to article writing and revision. Jesús Salvador Velarde-Félix: Contribution to data interpretation, article writing and revision. Vicente Garibaldi-Becerra: Contribution to experimental design and technical analysis. Héctor Rangel-Villalobos: Contribution to data interpretation, article writing and revision. Olivia Torres-Bugarín: Contribution to data interpretation, article writing and revision. Eloy Alfonso Zepeda-Carrillo: Contribution to data interpretation, article writing and revision. Salvador Ruíz-Bernés: Contribution to statistical analyses and data interpretation. Luis Antonio Ochoa-Ramírez: Statistical analyses, data interpretation, main article writer, article draft.

References

1 Barros MB, de Almeida Paes R, Schubach AO. *Sporothrix schenckii* and sporotrichosis. Clin Microbiol Rev. 2011;24:633–54.
2 Galhardo MC, Silva MT, Lima MA, Nunes EP, Schettini LE, de Freitas RF, *et al. Sporothrix schenckii* in AIDS during immune reconstitution syndrome. J Neurol Neurosurg Psychiatry. 2010;81:696–9.
3 Carlos IZ, Sassá MF, da Graça Sgarbi DB, Placeres MC, Maia DC. Current research on the immune response to experimental sporotrichosis. Immunology. 2009;168:1–10.
4 Fernandes KS, Neto EH, Brito MM, Silva JS, Cunha FQ, Barja-Fidalgo C. Detrimental role of endogenous nitric oxide in host defence against *Sporothrix schenckii*. Immunology. 2008;123:469–79.
5 Sassá MF, Saturi AE, Souza LF, Ribeiro LC, Sgarbi DB, Carlos IZ. Response of macrophage Toll-like receptor 4 to a *Sporothrix schenckii* lipid extract during experimental sporotrichosis. Immunology. 2009;128:301–9.
6 Tachibana T, Matsuyama T, Mitsuyama M. Involvement of CD4+ T cells and macrophages in acquired protection against infection with *Sporothrix schenckii* in mice. Med Mycol. 1999;37:397–404.
7 Uenotsuchi T, Takeuchi S, Matsuda T, Urabe K, Koga T, Uchi H, *et al.* Differential induction of Th1-prone immunity by human dendritic cells activated with *Sporothrix schenckii* of cutaneous and visceral origins to determine their different virulence. Int Immunol. 2006;18:1637–46.
8 Maia DC, Sassá MF, Placeres MC, Carlos IZ. Influence of Th1/Th2 cytokines and nitric oxide in murine systemic infection induced by *Sporothrix schenckii*. Mycopathologia. 2006;161:11–9.
9 Hamza T, Barnett JB, Li B. Interleukin-12 a key immunoregulatory cytokine in infection applications. Int J Mol Sci. 2010;11:789–806.
10 O'Garra A, Murphy KM. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. Nat Immunol. 2009;10:929–32.

11 Zhou P, Sieve MC, Tewari RP, Seder RA. Interleukin-12 modulates the protective immune responses in SCID mice infected with *Histoplasma capsulatum*. Infect Immun. 1997;65:936–42.

12 Magee DM, Cox RA. Interleukin-12 regulation of host defenses against *Coccidioides immitis*. Infect Immun. 1996;64:3609–13.

13 Hernández-Flores G, Gómez-Contreras PC, Domínguez-Rodríguez JR, Lerma-Díaz JM, Ortíz-Lazareno PC, Cervantes-Munguía R, *et al.* γ-Irradiation induced apoptosis in peritoneal macrophages by oxidative stress. Implications of antioxidants in caspase mitochondrial pathway. Anticancer Res. 2005;25:4091–100.

14 Fernandes KS, Coelho AL, Lopes Bezerra LM, Barja-Fidalgo C. Virulence of *Sporothrix schenckii* conidia and yeast cells, and their susceptibility to nitric oxide. Immunology. 2000;101:563–9.

15 Bravo-Cuellar A, Balercia G, Levesque JP, Liu XH, Osculati F, Orbach-Arbouys S. Enhanced activity of peritoneal cells after aclacinomycin injection: effect of pretreatment with superoxide dismutase on aclacinomycin-induced cytological alterations and antitumoral activity. Cancer Res. 1989;49:1578–86.

16 Flores-García A, Garibaldi-Becerra V, Barba-Barajas M, Velarde-Félix S, Fernandez-Argüelles R. Endogenous interleukin-12 regulates macrophage phagocytosis of *Sporothrix schenckii*. Rev Fac Cien Med (Quito). 2010;35:56–7.

17 Carlos IZ, Sgarbi DB, Santos GC, Placeres MC. *Sporothrix schenckii* lipid inhibits macrophage phagocytosis: involvement of nitric oxide and tumor necrosis factor-alpha. Scand J Immunol. 2003;57:214–20.

18 Kim JS, Kwon HY, Choi WH, Jeon CY, Kim JI, Kim J, *et al.* Phagocytosis of serum- and IgG-opsonized zymosan particles induces apoptosis through superoxide but not nitric oxide in macrophage J774A.1. Exp Mol Med. 2003;35:211–21.

19 Ramos-Zepeda R, González-Mendoza A. Metabolic activity of phagocytes in experimental sporotrichosis. Mycopathologia. 1986;93:109–12.

20 Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech. 2001;53:241–5.

21 Ruan S, McKinley L, Zheng M, Rudner X, D'Souza A, Kolls JK, *et al.* Interleukin-12 and host defense against murine *Pneumocystis* pneumonia. Infect Immun. 2008;76:2130–7.

22 Zhang CR, Lin JC, Xu WM, Li M, Ye HS, Cui WL, *et al.* Interleukin-12 and interleukin-2 alone or in combination against the infection in invasive pulmonary aspergillosis mouse model. Mycoses. 2013;56:117–22.

23 Lavigne LM, Schopf LR, Chung CL, Maylor R, Sypek JP. The role of recombinant murine IL-12 and IFN-γ in the pathogenesis of a murine systemic *Candida albicans* infection. J Immunol. 1998;160:284–92.

24 Sun K, Salmon SL, Lotz SA, Metzger DW. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. Infect Immun. 2007;75:1196–202.

25 Liu Y, Egilmez NK, Russell MW. Enhancement of adaptive immunity to *Neisseria gonorrhoeae* by local intravaginal administration of microencapsulated interleukin 12. J Infect Dis. 2013;208:1821–9.

26 Lenzi R, Edwards R, June C, Seiden MV, Garcia ME, Rosenblum M, *et al.* Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease<1 cm) associated with ovarian cancer or primary peritoneal carcinoma. J Transl Med. 2007;5:66.

27 Hedlund J, Langer B, Konradsen HB, Ortqvist A. Negligible adjuvant effect for antibody responses and frequent adverse events associated with IL-12 treatment in humans vaccinated with pneumococcal polysaccharide. Vaccine. 2001;20:164–9.

28 De Luca, Zelante T, D'Angelo C, Zagarella S, Fallarino F, Spreca A, *et al.* IL-22 defines a novel immune pathway of antifungal resistance. Mucosal Immunol. 2010;3:361–73.

29 Zhang X, Zhang J, Huang H, Xue R, Hu X, Li M, *et al. Taenia taeniaeformis* in rat favors protracted skin lesions caused by *Sporothrix schenckii* infection: Dectin-1 and IL-17 are dispensable for clearance of this fungus. PLoS ONE. 2012;7:e52514.

30 Gaucher D, Chadee K. Molecular cloning of gerbil interleukin 12 and its expression as bioactive single-chain protein. Cytokine. 2001;14:177–83.

31 Crabtree JE, Court M, Aboshkiwa MA, Jeremy AHT, Dixon MF, Robinson PA. Gastric mucosal cytokine and epithelial cell responses to *Helicobacter pylori* infection in Mongolian gerbils. J Pathol. 2004;202:197–207.