Interferon- γ and Tumor Necrosis Factor- α Determine Resistance to *Paracoccidioides brasiliensis* Infection in Mice

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To investigate the role of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) in the resistance to *Paracoccidioides brasiliensis* (Pb) infection, mice with homologous disruption of the IFN- γ (GKO) or TNF- α receptor p55 (p55KO) were infected with the parasite. GKO and p55KO, but not wild-type (WT) mice, were unable to control the growth of yeast cells and the mice succumbed to infection by days 16 and 90 after infection, respectively. Typical inflammatory granulomas were found only in WT mice. In contrast, knockout mice presented an inflammatory infiltrate composed of a few neutrophils, mononuclear, epithelioid, and multinuclear giant cells forming incipient granulomas in GKO mice and without granuloma formation in p55KO mice. Besides, both groups of knockout mice exhibited elevated numbers of yeast forms in agreement with colony-forming unit counts in organs. Compared with WT, splenocytes from infected GKO mice cultured with the Pb F1 fraction produced lower TNF- α levels, whereas leukocytes from infected p55KO mice produced similar amounts of TNF- α but higher levels of IFN- γ . Moreover, splenocytes from infected WT mice produced higher levels of nitric oxide (NO) resulting in a lower T-cell proliferative response to Con A than uninfected WT, or infected p55KO and GKO mice. On the contrary, the addition of IFN- γ to splenocytes from infected GKO mice resulted in higher NO production and lower T cell proliferation. Taken together, these findings suggests that endogenous TNF- α , acting through the p55 receptor, and IFN- γ mediate resistance to Pb infection and induce NO production that determines marked T cell unresponsiveness. (*Am J Pathol* 2000, 156:1811–1820)

Paracoccidioidomycosis (PCM), the major systemic mycosis in Latin America, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis* (Pb). The infection is acquired by inhalation of airborne propagules produced by the fungal mycelium form, which then change into the pathogenic yeast form when at core body temperature.¹ The yeast forms in the mammalian host can be eliminated by immune competent cells or disseminate to tissues by the lymphatic and hematogenous route. After infection there are no immediate signs or symptoms of the disease, characterizing silent PCM but the host develops a specific immune response against fungal antigens which can be detected by a skin test. In endemic areas, up to 60% of asymptomatic individuals may be skin test positive.²

In the acute form of clinically manifest infection, there is an involvement of the reticuloendothelial system, a non-specific hypergammaglobulinemia, a depressed cellular immune response, a diffuse inflammatory granulomatous response, and concomitant fungus dissemination. The chronic form presents a broad spectrum of clinical manifestations with frequent damage of the lung and oropharyngeal mucosa and a profound impairment of the immune response.³ In this phase of infection, an epithelioid inflammatory granulomatous reaction is known to prevent the dissemination of the fungus.⁴

Athymic mice do not control dissemination of the fungus, suggesting that cell-mediated immunity is an important host defense mechanism against Pb infection.^{5,6} However, there is a T cell dysfunction in patients and infected mice that may be related to alterations in the ratio of T cell subpopulations, suppressive effects of fungal components, and an imbalance in the levels of cytokine production.^{7–10} The cellular immune response against fungal antigens that occurs in resistant mice results in macrophage activation and granuloma formation.¹¹ Tumor necrosis factor- α (TNF- α) produced by macrophages in response to the Pb cell wall component¹¹ is required for macrophage accumulation and differentiation into epithelioid cells, and for persistence of

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well-formed granulomas.¹² Interferon- γ (IFN- γ) can activate infected macrophages to secrete TNF- α and to inhibit the replication of Pb.¹³ Recently, experiments performed on Pb-infected mice treated with anti-IFN- γ revealed an exacerbation of pulmonary infection and earlier fungal dissemination.¹⁴

In the present study we evaluated the role of IFN- γ and TNF- α in the resistance to Pb infection in mice. Using mice genetically deficient in IFN- γ (GKO) or TNF- α receptor p55 (p55KO), we could demonstrate that both cytokines are involved in the resistance to Pb infection, granuloma formation, and control of fungus dissemination. In addition, our data suggest that IFN- γ and TNF- α modulate the production of cytokines and nitric oxide (NO) and the T-cell proliferative response in Pb-infected mice.

Materials and Methods

Animals

Breeding pairs of mice with targeted disruption of the IFN- γ (GKO mice)¹⁵ and TNF- α receptor p55 (p55KO) genes¹⁶ were obtained from Jackson Laboratories (Bar Harbor, ME) and Amgen Institute (Toronto, Canada), respectively. Breeding stock backcrossed on C57Bl/6 were obtained and the genotype of p55KO and GKO mice determined by polymerase chain reaction of DNA as previously described.^{16,17} Male C57Bl/6 wild-type (WT), GKO, and p55KO mice, 6 to 8 weeks old, were bred and maintained in microisolator cages in the animal housing facility of the Department of Immunology, University of São Paulo, Ribeirão Preto, Brazil.

Parasite and Mice Infection

Yeast cells of virulent Pb 18 strains were cultured at 37°C in Fava-Netto's medium¹⁸ for 7 to 14 days. The yeast cells were harvested and washed three times in phosphate-buffered saline (PBS), pH 7.2. Viability of yeast cells was determined as previously described.¹⁹ The animals were infected intravenously with 1×10^6 viable yeast cells in 100 μ l of PBS.

Assay for Organ Colony-Forming Units

The dissemination of fungus in lungs, livers, and spleens was assayed as previously described.²⁰ The organs were removed, weighed, homogenized in sterile PBS, pH 7.2, and serially diluted. Aliquots of 100 μ l were dispensed into Petri dishes, in duplicates, containing brain-heart infusion agar (Difco Laboratories, Detroit, MI) supplemented with 4% (v/v) of normal horse serum and 5% (v/v) of Pb 192 broth yeast culture filtrate from 2-week-old cultures as source of growth-promoting factor.²¹ Plates were incubated at 37°C, and colonies were counted 7 to 14 days later. Results were expressed as numbers of colony-forming units (CFU) $\pm$ SE per gram of tissue.

Preparation and Fractionation of Pb18 Cell Walls

Fungus cell walls were obtained as previously described.¹¹ Briefly, yeast forms were sonicated and lipids from the cell walls were removed by treatment with chloroform/methanol (2:1, v/v) with stirring at room temperature for 2 hours. Extracts were separated by centrifugation at $500 \times g$ for 5 minutes. The resulting insoluble cell residue was named the cell wall fraction. The cell wall was further treated with 1 N NaOH and gently stirred at room temperature for 1 hour. The alkali-insoluble sediment was washed with water until it reached pH 7.0 and then washed with ethanol, followed by acetone and diethyl ether. The resulting white powder was named F1 fraction and contained <0.05 ng/ml of bacterial endotoxin, as determined by the *Limulus ameobocyte* assay (Sigma Chemical Co., St. Louis, MO).

Proliferation Assay and Nitrite Quantification

Spleen cells from uninfected (day zero) or Pb-infected mice were washed in Hanks' medium and incubated for 4 minutes with lysis buffer (one part of 0.17 mol/L Tris and nine parts of 0.16 mol/L ammonium chloride). The cells were washed and suspended to a concentration of 5×10^6 cells per ml in RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 5% fetal calf serum (Life Technologies Inc., Bethesda, MD), 5×10^{-2} mol/L 2- β -mercaptoethanol, 2 mmol/L L-glutamine, and antibiotics (all from Sigma). The cells were cultured in flat-bottom 96-well plates (Corning Glass Works, Corning, NY) at 1×10^6 /well with or without Con A (2 μ g/ml) for 72 hours at 37°C in a humidified 5% CO₂ incubator. To assess proliferation, 0.5 μ Ci/well of [³H]TdR (Amersham Corp., Arlington Heights, IL) was added during the final 18 hours of culture, the cells were harvested and radioactivity was measured in a scintillation counter. In some experiments, to inhibit the inducible nitric oxide synthase (iNOS), we added 200 μ mol/L of N^G-methyl-L-arginine (LNMMMA, Sigma). Data were expressed as means ($\pm$ SE) of counts per minute of triplicate cultures.

For nitrite quantification, splenocytes (2×10^6 cells/ml) from WT, GKO, and p55KO animals were cultured in triplicate for 48 hours in medium alone or medium containing Con A (2 μ g/ml) in the presence or absence of 100 U/ml of IFN- γ . Duplicates of nitrite concentrations in each culture supernatant were assayed in a microplate by mixing 0.1 ml of culture supernatant with 0.1 ml of Griess reagent.²² The A₅₄₀ was read 10 minutes later, and the nitrite concentration was determined by reference to a standard curve of 1 to 100 μ mol/L NaNO₂.

Morphology

Five to seven animals selected at random from each group were sacrificed at 7 and 15 days (GKO) and at 15, 30, and 60 days (p55KO) after infection. The lungs obtained were fixed in 10% formalin for 24 hours and embedded in paraffin. Tissue sections (5 μ m) were stained

with hematoxylin and eosin (H&E) or impregnated with silver for demonstration of reticulum fibers using standard protocols. WT mice were used as a control.

Cytokine Detection in Culture Supernatants

Spleen cells (2×10^6 cells/ml) from normal or infected mice were cultured in 24-well tissue culture plates (Corning) with 2 μ g/ml of Con A, 40 μ g/ml of Pb18 F1 fraction, or medium alone for 48 hours at 37°C in a humidified 5% CO₂ incubator. The supernatants were harvested and stored at -20°C until assayed for IFN- γ , TNF- α , interleukin (IL)-10, and IL-12, using a two-sandwich enzyme-linked immunosorbent assay. XM1.2 (anti-IFN- γ), XT22.11 (anti-TNF- α), JES5-2A5 (anti-IL-10), and C17.15.10 (anti-IL-12) were used as capture monoclonal antibody (mAb). IFN- γ and TNF- α bound to the mAb were visualized with polyclonal rabbit anti-IFN- γ or anti-TNF- α (both from Santa Cruz Biotechnology, Santa Cruz, CA), followed by goat anti-rabbit IgG conjugated with peroxidase (Life Technologies Inc., Gaithersburg, MD), whereas IL-10 and IL-12 were detected using appropriated biotinylated mAbs SXC1 (anti-IL-10) and C15.6 (anti-IL-12), respectively. A standard curve was prepared with specific cytokines (all from Sigma) and enzyme-linked immunosorbent assay sensitivities were 0.625 U/ml for IFN- γ , 312 pg/ml for TNF- α , and 390 pg/ml for IL-10 and IL-12.

Statistical Analysis

The results are expressed as the mean $\pm$ SE of the indicated number of animals or experiments. Statistical analysis was performed using analysis of variance followed by the parametric Tukey-Kramer test (INSTAT software, GraphPad, San Diego, CA). A *P* value < 0.05 was considered to indicate statistical significance.

Results

Absence of IFN- γ or the TNF- α Receptor p55 Causes Increased Susceptibility to *Pb* Infection

To determine the role of IFN- γ and TNF- α in the resistance to *Pb* infection, IFN- γ and TNF- α receptor p55 knockout mice were infected with yeast cells and their survival was compared with that of littermate controls. We found that whereas 100% of the control littermate mice survived during the 90-day period of the experiment, the infected GKO and p55KO mice succumbed by days 16 and 90 after parasite inoculation, respectively (Figure 1). The infected p55KO mice started to die after day 60 of infection and the mortality rate at day 90 of infection was 100% when compared with 100% of survival in infected WT animals. Because infected GKO mice did not survive beyond 16 days, the fungal loads in these animals were determined on days 4, 7, and 15 postinfection. The results showed a significantly higher amount of fungus in GKO mice than that verified in infected WT mice (*P* < 0.001; Figure 2, A–C). It is possible that the CFU numbers

Figure 1. Absence of IFN- γ leads to high susceptibility to *P. brasiliensis* infection. Survival of WT (triangles), p55KO (circles), and GKO (squares) mice after intravenous infection with 1×10^6 *P. brasiliensis* yeast cells. Eight mice were used in each group. Similar results were obtained in a second experiment.

at days 7 and 15 are underestimated, because the data represent the CFU load of mice that have survived. The fungal loads obtained in the organs from infected p55KO mice (Figure 2, D–F) also were higher than those in control-infected WT mice (*P* < 0.001), except for the spleen at day 15 after infection, for which a statistically significant difference could not be detected (Figure 2F).

Figure 2. Lack of resistance of infected GKO and p55KO mice to control *P. brasiliensis* yeast cell proliferation and dissemination. Colony-forming units (CFU) of lungs (A and D), liver (B and E), and spleen (C and F) of WT (open bars), GKO (black bars), and p55KO mice (hatched bars) were determined on different days, as described in Material and Methods. The scale bars represent the mean $\pm$ SE of CFU obtained of duplicates cultures performed in groups of three animals and are representative of two independent experiments. *, *P* < 0.001 compared with infected WT mice.

Similar fungal loads were also obtained at days 4 and 7 postinfection in p55KO and WT mice (data not shown).

IFN- γ and TNF- α Control Dissemination of Pb18 Yeast Cells in the Lung

Histopathological examination of lung tissue sections revealed marked differences between granulomatous lesions developed in WT mice compared with the lesions observed in both GKO and p55KO after infection with Pb18.

At day 7 postinfection, the WT mice lungs showed a diffuse focal infiltrate of polymorphs and mononuclear cells in association with few yeast forms of the fungus. At day 15, diffuse foci of histiocytes, epithelioid cells, lymphocytes, and a few polymorphs aggregated around yeast cells, identifiable as epithelioid granulomas, could be seen (Figure 3A). The reticulum fiber staining clearly demonstrated the early structures of granuloma (Figure 3B). At day 30, well-organized granulomas composed of histiocytes, epithelioid cells, and multinuclear giant cells aggregated around yeast forms and surrounded by a rim of lymphocytes and fibroblasts, were formed (Figure 3C). The normal pattern of reticulum fibers present in granulomas could be clearly demonstrated (Figure 3D). At day 60 after parasite inoculation, multiple and confluent typical epithelioid granulomas were observed (data not shown).

The lungs of GKO mice at day 7 after infection showed pulmonary damage characterized by diffuse microfoci of lung parenchyma consolidation depicted as intra-alveolar edema and hemorrhage and exudation of polymorphs and lymphomononuclear cells. At day 15, in agreement with CFU counts, the number of yeast forms of Pb was remarkably increased in association with an inflammatory infiltrate composed of polymorph, lymphomononuclear, epithelioid, and multinuclear giant cells forming incipient granulomas (Figure 4A). The reticulum fiber pattern clearly showed the structure of these incipient granulomas and loss of pulmonary parenchyma architecture (Figure 4B).

At day 15 after infection, the p55KO mice showed diffuse foci of lung tissue consolidation characterized by intra-alveolar exudation of lymphomononuclear cells and a few polymorphs correlated with a great number of yeast forms of the fungus (Figure 5A). The reticulum fiber pattern of the lung parenchyma was preserved (Figure 5B). At day 30, the pulmonary lesions were focal and diffuse, characterized by lymphomononuclear, a few epithelioid, and multinuclear giant cells in association with abundant yeast forms, without granuloma formation (Figure 5C). The reticulum fiber pattern of the lung was fairly well preserved (Figure 5D). In agreement with CFU counts, the number of yeast forms of the fungus was markedly increased at both intervals after infection. Progressively (60 days), the lung parenchyma was severely damaged by a diffuse mononuclear cell infiltration associated with an increased number of yeast forms (data not shown).

Cytokine Production of Splenocytes from Infected WT, GKO, and p55KO Mice

Splenocytes from infected WT mice cultured with Con A produced less IFN- γ when compared with noninfected mice (day zero corresponds to baseline, before inoculation; Figure 6A) and with infected p55KO mice (Figure 6C). The TNF- α levels in supernatants of cells cultured with Pb F1 fraction increased progressively in infected WT mice on days 7 and 15, whereas infected GKO mice produced less TNF- α during the same period (Figure 6B). On the other hand, splenocytes from infected p55KO and WT mice cultured with Pb F1 fraction produced similar levels of TNF- α during the course of infection (Figure 6D). Cells from infected or uninfected mice cultured with medium only did not produce detectable levels of TNF- α or IFN- γ . Although the level of lipopolysaccharide in the F1 fraction was <0.05 ng/ml, we assayed for TNF- α levels in splenocytes from C3H/HeJ mice that are resistant to lipopolysaccharide. We found that the levels of TNF- α produced by the cells from C3H/HeJ in the presence of F1 fraction was similar to that found in C57Bl/6 mice (data not shown). Because IL-12 potentiates and IL-10 inhibits the IFN- γ production, we assayed for IL-12 and IL-10 in supernatants of splenocytes cultured with or without F1 fraction and Con A, respectively. We found that IL-12 levels in the supernatants of leukocytes from infected WT or knockout mice were similar. For example, on day 30 after infection, while the IFN- γ levels were significantly higher in p55KO than in WT mice (Figure 6C), the IL-12 levels were 923 ± 106 and 724 ± 101 pg/ml in infected WT and p55KO mice, respectively. The levels of IL-10 produced on day 30 after infection were higher in infected WT mice (913 ± 75 pg/ml) than in infected p55KO mice (215 ± 30 pg/ml). Increased levels of IL-10 were also found on day 7 after infection in supernatants of splenocytes from infected WT mice (5080 ± 540 pg/ml) compared with GKO mice (1666 ± 28 pg/ml).

Absence of IFN- γ and p55 TNF- α Receptor Abrogates Pb-Induced T Cell Suppression

As previously described,²³ splenocytes from WT mice infected with Pb18 yeast cells proliferated less than cells from normal mice. Conversely, splenocytes from infected GKO and p55KO mice exhibited a proliferative response similar to that of noninfected mice (day zero corresponds to baseline, before inoculation; Figure 7, A and C). When we made the observation that NO could be involved in the immunosuppression observed during Pb infection,²³ we compared the NO production by spleen cells from infected knockout and WT mice. Interestingly, splenocytes from infected GKO and p55KO mice cultured with Con A produced less NO when compared with their infected littermates (Figure 7, B and D). Considering that abrogation of suppression of Con A-induced proliferate responses are concomitant to absence of IFN- γ and decrease in NO production (Figure 7), we evaluated the role of this cytokine in the control of T cell response and NO production by splenocytes of infected mice. We thus

Figure 3. Histopathology of lung sections from *P. brasiliensis*-infected WT mice. Lungs of mice at day 15 show diffuse foci of histiocytes, epithelioid cells (**wide-open arrow**), lymphocytes, and a few polymorphs aggregated around yeast cells (**arrowheads**) identifiable as epithelioid granulomas (**A, arrows**). The early structure of the granulomas are depicted by reticulum fiber silver impregnation (**B, wide-open arrows**). At day 30, well-organized granulomas with multinuclear giant cells (**wide-open arrow**) are present (**C, wide arrows**) with a normal pattern of reticulum fibers (**D, wide-open arrows**). Lung sections were stained with H&E (**A** and **C**) and impregnated with silver for reticulum fibers (**B** and **D**). Magnification, $\times 230$.

Figure 4. Histopathology of lung sections from *P. brasiliensis*-infected GKO mice. Lungs of mice at 15 days after infection show marked mononuclear cell infiltrate with incipient granuloma formation and a high number of yeast cells (**A**, **arrows**). The reticulum fiber staining shows the incipient pattern of granuloma (**B**, **wide arrows**). Lung sections were stained with H&E (**A**) and impregnated with silver for reticulum fibers (**B**). Magnification, $\times 230$.

added IFN- γ to splenocytes from normal or infected WT and GKO mice and evaluated the subsequent T-cell proliferative response and NO production. We found that the addition of IFN- γ to splenocytes from infected GKO mice cultured with Con A restored NO production (Figure 8B) to the levels detected in infected WT mice ($P < 0.001$), and led to a significant inhibition of Con A-induced T cell proliferation (Figure 8A). The addition of IFN- γ to cell cultures from noninfected GKO mice led only to a slight increase in the nitrite synthesis (from 0.2 ± 0.05 to $1.67 \pm 0.49 \mu\text{mol/L}$), that was not enough to modify the cell proliferation. To verify if NO was involved in the mechanism that mediates inhibition of T cell proliferative responses, we added the iNOS inhibitor LNMMA to cell cultures. In the absence of Con A, the addition of LNMMA did not modify the cell proliferation or nitrite production (data not shown). However, addition of LNMMA to spleen cells from infected WT mice cultured with Con A abrogated the NO production and, therefore, reversed the level of T cell proliferation to that found in cells from infected GKO, as well as uninfected WT and GKO mice. On the contrary, the addition of LNMMA to cells from normal or infected GKO mice did not result in alteration in cell proliferation, thus confirming the role of NO in suppression (Figure 8). Therefore, decreased NO production leads to increased T-cell proliferative response.

Discussion

Disseminated PCM in GKO and p55KO mice as well as the kinetics of lesion development in lungs after Pb infection were described. The pattern of cytokine response in the absence of IFN- γ and TNF- α receptor p55 in infected mice was also evaluated. Our results suggest that IFN- γ and TNF- α are essential for the resistance and survival of Pb-infected mice. Although the role of IFN- γ has been extensively studied in fungal infections such as *Candida albicans*^{18,24,25} and *Histoplasma capsulatum*,^{26,27} the role of this cytokine in mediating resistance or susceptibility to Pb infection is poorly understood. *In vitro*, IFN- γ plays a pivotal role against several pathogens,^{28–30} including Pb yeast cells,¹³ through macrophage activation. Treatment of infected mice with very high amounts of anti-IFN- γ resulted in increased fungal burden, although the animals did not die until 8 weeks after infection.¹⁴ In the present study the infection of GKO mice resulted in 100% mortality after only 16 days of infection as a consequence of early fungal dissemination to liver, spleen, and lungs, whereas WT mice were resistant to infection. Histological examination of lungs from infected GKO mice confirmed the yeast cells were widespread throughout the major organs. These data suggest that mice lacking a functional IFN- γ gene were unable to contain and control a virulent Pb infection, besides the intense inflammatory

Figure 5. Histopathology of lung sections from *P. brasiliensis*-infected p55KO mice. Lungs of mice at day 15 after infection show foci of tissue consolidation characterized by intra-alveolar exudation of mononuclear cells and a few polymorphs correlated with a great number of yeast forms of the fungus (**A**, arrows). The reticulum fiber pattern of the lung parenchyma is preserved (**B**, arrows). At day 30, the pulmonary lesions are focal and diffuse characterized by mononuclear cells and a few epithelioid cells and multinuclear giant cells (**wide arrow**) in association with abundant yeast forms without granuloma formation (**C**). The reticulum fiber pattern of the lung is fairly well preserved (**D**). Lung sections were stained with H&E (**A** and **C**) and impregnated with silver for reticulum fibers (**B** and **D**). Magnification, $\times 230$.

Figure 6. TNF- α and IFN- γ production by spleen cells from infected WT (open bars), GKO (black bars), and p55KO (hatched bars) mice. Spleno-cytes were harvested from GKO (0, 7, and 15 days after infection) and p55KO mice (0, 15, 30, and 60 days after infection), cultured for 48 hours with Con A (2 μ g/ml, A and C) and with the F1 fraction (40 μ g/ml, B and D) and the supernatants assayed for IFN- γ (A and C) and TNF- α (B and D). The amount of IFN- γ and TNF- α found in splenocytes cultured without stimulus was below the limit of detection of our enzyme-linked immunosorbent assay. Each scale bar represents the mean $\pm$ SE of three mice per group in an experiment representative of two separate experiments. *, $P < 0.001$ compared with infected WT mice.

reaction and formation of incipient granulomas associated with a great number of yeast cells. Moreover, our data could also show that TNF- α , acting through the p55 receptor, is also important to control parasite dissemination, parasite growth, and the inflammatory response. Infection of p55KO mice, whose only abnormality is a lack

Figure 7. Absence of the IFN- γ and TNF- α receptor p55 results in lower NO production and normal T-cell proliferative response of Pb-infected mice. Spleen cells from noninfected (day zero) or infected WT (open bars), GKO (black bars), and p55KO (hatched bars) mice were cultivated with Con A (2 μ g/ml) and T cell proliferation was evaluated (A and C). Nitrite (B and D) was assayed in supernatants of splenocytes (2×10^5 cells/ml) cultured with Con A for 48 hours. Each scale bar represents the mean $\pm$ SE of three mice per group in an experiment representative of two separate experiments. *, $P < 0.001$ compared with infected WT mice.

Figure 8. Treatments that restore or abrogate NO production by splenocytes from Pb-infected mice inhibit and reverse T cell proliferation, respectively. Spleen cells from noninfected (NI) WT (striped bars) and GKO (hatched bars) mice or WT (open bars) and GKO (black bars) mice at 15 days after infection (I) were cultured with Con A (2 μ g/ml) with or without IFN- γ (100 U/ml) or LNMMA (200 μ mol/L) and the T-cell proliferative response (A) and NO production (B) were evaluated (for details see Materials and Methods). Splenocytes cultured with medium only were used as a control. Each scale bar represents the mean $\pm$ SE of three mice per group in an experiment representative of two separate experiments. *, $P < 0.001$ compared with infected WT mice. **, $P < 0.01$ compared with GKO infected mice in absence of IFN- γ .

of p55 receptor,¹⁶ resulted in increased fungal burden and absence of granuloma formation. In fact, the granulomatous inflammatory reaction, a specialized and efficient tissue response against certain parasites,³¹ requires TNF- α , which is supposed to be responsible for attracting and activating effector cells, as well as for macrophage accumulation and differentiation into epithelioid cells.³² Our results agree with recent studies showing that TNF- α activities mediated by the p55 receptor are important in granuloma formation in response to bacteria^{16,33} and protozoa.³⁴ Accordingly, we could not detect typical granuloma formation in either GKO or p55KO, suggesting that both TNF- α , signaling via the p55 receptor, and IFN- γ are involved in the mediation of granuloma formation in response to Pb. The lesions observed in the lungs of infected p55KO mice showed a small number of apparently typical epithelioid cells, in accordance with

observations in p55KO mice inoculated with live Calmette-Guérin bacillus.³⁵

The survival of infected p55KO mice was longer in comparison with infected GKO mice probably because TNF- α , acting through the p75 receptor, produces higher amounts of NO than infected GKO mice (Figure 7), and, therefore, are more resistant to infection (Figure 1), thus reinforcing the hypothesis that NO controls Pb infection.²³ Similarly, it has been shown that the TNF- α p55 receptor-independent pathway involved TNF- α p75 receptor signaling, because neutralization of TNF- α abrogated the ability of p55KO macrophages to produce sufficient NO to kill *Leishmania major*.³⁶ Moreover, the elimination of *L. major* was delayed when compared with control animals, suggesting that this pathway may be less efficient than signaling via the p55 receptor.³⁶ Also, it was observed that mice genetically deficient in TNF- α p55 and p75 receptors succumbed significantly earlier to the infection with *Toxoplasma gondii* as compared with TNF- α p55KO mice.³⁴ Taken together, these observations agree with our hypothesis that in the absence of the TNF- α p55 receptor, part of the function of this receptor is taken over by the TNF- α p75 receptor.

It is well known that IFN- γ promotes TNF- α synthesis by murine macrophages³⁷ and that IFN- γ and TNF- α act synergistically to induce antimicrobial activity in many infectious diseases.^{38,39} In our model, the greatly reduced capacity of Pb-infected GKO mice to produce TNF- α may be an important factor in their inability to resist Pb infection, because this cytokine is important in granuloma formation.^{12,34} The lungs of GKO mice showed formation of incipient granulomas probably related to partial production of TNF- α ; whereas infected p55KO mice produced high levels of IFN- γ , which may help to control parasite growth and dissemination even in the absence of the TNF p55 receptor, resulting in higher resistance to infection than that observed in GKO mice. The low IFN- γ production by infected WT mice was not due to decreased IL-12 synthesis, because it did not change during the whole infection course. Also, low levels of IL-10 (data not shown) and high levels of IFN- γ , known to inhibit and to activate the iNOS, respectively,²⁹ may contribute to induce NO synthesis and to control fungus replication.

We have previously shown that mice infected with the protozoan *Trypanosoma cruzi* produce high levels of IFN- γ and TNF- α , which lead to activation of iNOS and NO production.^{40,41} Despite its importance as a microbicidal agent, NO has been shown to be involved in the establishment and maintenance of lymphocyte unresponsiveness in mice infected with several parasites,^{30,40} including fungi.²⁸ Similarly, in Pb infection associated with a classical granuloma reaction there is a marked suppression of cell proliferative response to the mitogen Con A (Figure 7) and IFN- γ , TNF- α (Figure 6), and NO production (Figure 7). In the absence of the IFN- γ and TNF- α receptor p55, however, the proliferative response to Con A was similar to that verified in cells from normal mice. Interestingly, production of high levels of NO suppressed the proliferative response to Con A. This observation was reinforced by the addition of IFN- γ to spleno-

cytes from infected GKO mice, which resulted in decreased proliferative response and increased NO production (Figure 8). Moreover, inhibition of the iNOS by the addition of LNMMA to the cultures of splenocytes from infected WT mice abrogated NO production and reverted the proliferative response to values similar to those found in cells from infected GKO and p55KO mice (Figure 8). Indeed, NO has been associated with a decreased proliferative response to Con A^{42,43} and a recent report demonstrated that treatment of Pb-infected mice with a specific inhibitor of NO synthesis prevented failure of the proliferative capacity of spleen cells in response to Con A and Pb antigen.²³ The low NO production in GKO and p55KO mice may also contribute to the observed decrease in granuloma formation, because inhibition of NO metabolism caused a loss of granulomatous architecture in lesions induced by Calmette-Guérin bacillus.⁴⁴ Taken together, these data suggest that NO may modulate the immune response to Pb antigens in infected mice and may contribute to the organization of the granulomatous lesion.

In summary, a congenital deficiency in IFN- γ or the TNF- α receptor p55 results in a pronounced loss of protective immunity in mice infected with Pb. In association with this impairment of immunity, generation of NO is compromised, suggesting that IFN- γ and TNF- α participate in the generation of this mediator that acts as an immunosuppressor in infections caused by Pb18 and also in controlling dissemination of the fungus. In accordance with our results, patients with PCM have been demonstrated to produce lower levels of IFN- γ and TNF- α , suggesting that these cytokines are important to control the development of the disease.⁴⁵ The knowledge of the regulatory mechanisms that lead to the control of Pb infection in resistant individuals may contribute to a future immunotherapy for PCM that affects thousands of people in South and Central America.

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