Intercellular Adhesion Molecule-1 Is Required for the Early Formation of Granulomas and Participates in the Resistance of Mice to the Infection with the Fungus *Paracoccidioides brasiliensis*

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The migration of leukocytes to inflammatory sites elicited by *Paracoccidioides brasiliensis* is supposed to be coordinated by cytokines and chemokines. Here, we investigated the role of intercellular adhesion molecule-1 (ICAM-1) in recruiting inflammatory cells to lungs of mice infected with *P. brasiliensis* and in determining the outcome of the disease. Expression of ICAM-1 was up-regulated on T lymphocytes after infection with the fungus, and its expression was dependent on interferon- γ , tumor necrosis factor- α , and interleukin-12. Moreover, the absence of ICAM-1 resulted in high susceptibility to the infection and delayed formation of granulomatous lesions. In addition, the absence of ICAM-1 resulted in increased growth and dissemination of fungus, decreased number of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, and increased production of interleukin-4 in the inflammatory site. The organization of a granulomatous reaction in mice deficient of ICAM-1 was delayed, starting only on day 60 after infection, whereas in wild-type mice it was complete on day 30 of infection. These data show that ICAM-1 is effectively involved in cellular migration and in the organization of the granulomatous lesion caused by the fungus *P. brasiliensis*. (Am J Pathol 2006, 169:1270–1281; DOI: 10.2353/ajpath.2006.060271)

Paracoccidioidomycosis, a chronic systemic mycosis that is endemic in Latin America, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. The

disease, acquired by inhalation of the infectious form of the fungus (conidia) present in the environment, has extensive spectrum of clinical and pathological manifestations ranging from benign and localized (adult type) to severe and disseminated forms (juvenile type). Patients with the adult form of paracoccidioidomycosis usually produce low levels of specific antibodies, whereas those with the juvenile type typically show polyclonal B-cell activation and high levels of specific antibodies.^{1,2} Nevertheless, in both cases, the cell-mediated immune responses are abnormal, and absence of specific therapy leads to high mortality.¹

The main host defense against *P. brasiliensis* is the cell-mediated immune response,³ although antibodies are also deemed to be involved in the protection of infected mice.⁴ The infection induces the formation of a compact paracoccidioid granuloma, a chronic inflammatory reaction produced in an attempt to limit dissemination of the fungus. Indeed, patients with severe disease have fewer granulomas and higher numbers of viable yeast cells in the lesions. In the absence of a compact granuloma, the fungus spreads to multiple organs by means of the lymphatic and circulatory systems, resulting in disseminated lesions throughout the body.^{3,5} The mechanisms that drive the migration of cells that form and maintain granulomas around *P. brasiliensis* are not well known. Recent work has underscored the role of interferon (IFN)- γ -regulated chemokines in this process.⁶

Supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo and by The Millennium Institute for Vaccine Development and Technology (CNPq-420067/2005-1).

Accepted for publication June 22, 2006.

A.P.M. is a Ph.D. student with a scholarship from Fundação de Amparo à Pesquisa do Estado de São Paulo. J.S.S. and M.A.R. are research fellows of Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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IFN- γ induces the production of regulated on activation normal T cell expressed and secreted (RANTES)/CCL5, MCP-1/CCL2, IP-10/CXCL10, and Mig/CXCL9 in leukocytes and also the expression of the chemokine receptor CXCR3. On the contrary, the absence of IFN- γ results in production of KC and MIP-1 α , expression of CCR4, and chronic neutrophilia.⁶ IFN- γ or tumor necrosis factor (TNF)- α receptor p55-deficient mice are highly susceptible to infection, are not able to build organized granulomas, and present with great amount of fungus in the lesions and high rates of mortality.⁷ These findings suggest that precise kinetics for production of chemokines and for migration of cells to the site of infection are fundamental to control the infection. Accordingly, during the recruitment of leukocytes to inflammatory or infectious sites, regulated and organized processes involve cytokines, chemokines, and adhesion molecules.^{8,9}

The intercellular adhesion molecule-1 (ICAM-1), or CD54, is a cell-surface protein with five immunoglobulin-like domains that is expressed constitutively at low levels on vascular endothelial cells, lymphocytes, and monocytes.¹⁰ ICAM-1 participates in the adherence of inflammatory cells to the endothelium before diapedesis occurs¹¹ and is also related to effector functions of leukocytes, presentation of antigen, and signal transduction pathways across membranes of cells.⁸ The stimulation of a variety of cells, such as endothelial, mesangial, and bronchial epithelial cells, with inflammatory cytokines [interleukin (IL)-1, TNF- α , and IFN- γ] increases expression of ICAM-1¹² and favors the transendothelial migration of leukocytes through the interaction with β 2 integrins.^{11,12} The levels of inflammatory cytokines TNF- α , IL-1, IL-6, IL-12, and IFN- γ ^{6,7,13–20} are increased during the infection with *P. brasiliensis*. Expression of ICAM-1 is also greatly increased in the lungs of mice infected with conidial forms of *P. brasiliensis*²¹ and is a probably a consequence of the production of proinflammatory cytokines. However, the exact role of ICAM-1 in the recruitment of leukocytes to the primary site of infections with the yeast form of *P. brasiliensis*, in the formation of granulomas, and in the resistance against the pathogen is not known. We therefore analyzed its role in the resistance to infection with *P. brasiliensis*. Our results show that ICAM-1 participates in the formation of granulomas that confer resistance against infection with this pathogen.

Materials and Methods

Animals

Male C57BL/6 wild-type mice (WT; ICAM-1^{+/+}) and mice deficient in ICAM-1 (ICAM-1KO; ICAM-1^{-/-}), IFN- γ (IFN- γ KO), TNFR1 (p55KO), and IL-12 (IL-12KO), 6 to 8 weeks old in the beginning of the experiments, were used. The mice were bred and maintained in microisolator cages in the animal housing facility of the Department of Biochemistry and Immunology, School of Medicine-Universidade de São Paulo. Mice with targeted disruption of ICAM-1, IFN- γ , TNFR1, and IL-12 were obtained from Jackson Laboratories (Bar Harbor, ME). All knockout mice were

originally generated in a mixed 129-B6-DBA background and then backcrossed to the C57BL/6J background for more than eight generations. All animal experiments were performed in accordance with protocols approved by the School of Medicine of Ribeirão Preto Institutional Animal Care and Use Committee.

Fungus, Mice Infection, and Mortality

Yeast cells of *P. brasiliensis* strain (Pb18 and Pb339) were cultured at 35°C in Fava-Netto's medium²² for 7 to 14 days, harvested, washed three times in phosphate-buffered saline (PBS), pH 7.2, and the viability determined as previously described.²³ The animals were anesthetized by intraperitoneal injection with 100 μ l of PBS with 2.5% of tribromoethanol and infected via trachea with 1×10^6 viable yeast cells of Pb18 suspended in 100 μ l of PBS. Deaths of 45 WT and 50 ICAM-1KO mice were registered daily until 120 days after the infection.

Antigens

Surface antigens of yeast cells of *P. brasiliensis* (Pb18) were used. The yeast cells were carefully removed from the culture medium and submitted to agitation in a vortex in PBS, for 30 seconds. The suspension was centrifuged for 10 minutes ($1400 \times g$), the supernatant obtained, and the protein concentration determined.

Assay for Organ Colony-Forming Units

To determine the growth and dissemination of *P. brasiliensis*, the amounts of colony-forming units recovered from the tissues were analyzed at 30, 60, and 90 days after infection. The organs were aseptically removed, weighed, homogenized in sterile PBS using tissue grinders, and 100 μ l of the samples diluted in 900 μ l of PBS. Aliquots of 100 μ l of each sample were dispensed into Petri dishes containing brain heart infusion agar (Difco Laboratories, Detroit, MI), supplemented with 4% normal horse serum, and 5% Pb339 broth yeast culture filtrate from 2-week-old cultures. The plates were incubated at 37°C, the colonies counted 14 days later, and the numbers of colony-forming units per g of tissue calculated.

Morphology

Animals selected randomly from each group were sacrificed at 30, 60, and 90 days after infection. The lungs, spleen, and liver were obtained, fixed in PBS/10% formalin for 24 hours, and embedded in paraffin. Tissue sections (5 mm) were stained with hematoxylin and eosin (H&E), impregnated with silver for demonstration of reticulum fibers, or silver methenamine (Grocott) to detect the mycotic structures using standard protocols.

Immunocytochemical Analyses

Five- μ m frozen tissue sections were obtained and fixed with ice-cold acetone for 10 seconds. The slides were

placed in a humidified chamber, and the endogenous peroxidase activity blocked with 3% hydrogen peroxide for 20 minutes, followed by incubation with PBS plus 5% (P/V) of nonfat milk (Nestle, San Paulo, Brazil). The slides were washed with PBS and incubated overnight with rabbit IgG anti-mice CD54 (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (controls) diluted 100 times in PBS with 3% (w/v) of nonfat milk. After successive rinsing with PBS, the sections were incubated for 30 minutes with biotin-labeled goat anti-rabbit antibody and washed three times in PBS. Next, the sections were incubated with avidin-biotin-peroxidase complex, the color developed with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, ON, Canada), the slides counterstained with Mayer's hematoxylin, dehydrated, and mounted with Canada Balsam.

Delayed-Type Hypersensitivity (DTH) Assay

To evaluate the DTH reactions of infected mice, the left hind footpad of the animals were inoculated subcutaneously with 2 μ g/ml *P. brasiliensis* antigen in 25 μ l of PBS and the footpad thickness measured using a dial caliper (Mitutoyo Corp., Tokyo, Japan) 24 hours later. The differences among the thickness of the footpad injected with antigen and the contralateral paw, injected with PBS, were calculated.

*Measurement of Serum *P. brasiliensis*-Specific Isotypes*

Specific anti-*P. brasiliensis* antibody IgG, IgG1, and IgG2 were measured by two-site sandwich enzyme-linked immunosorbent assay (ELISA) using the surface antigens of yeast cells of *P. brasiliensis* (Pb18). The sera were obtained from uninfected mice (controls) and from infected mice at 30 and 60 days after infection. Briefly, the surface antigen (5 μ g/ml) was dispensed into a 96-well plate and incubated overnight at 4°C. The wells were washed and blocked with 5% (P/V) of nonfat milk (Nestle) by incubation for 1 hour at room temperature. After three washes, serial dilutions of each serum sample were added to the well and incubated for 2 hours at 37°C. After, rabbit anti-mouse total immunoglobulins (Ig), immunoglobulin G1 (IgG1), and IgG2b (1:2000) (Zymed, San Francisco, CA) were added and incubated for 1 hour at 37°C. Goat anti-rabbit IgG conjugated to peroxidase (1:2000) (Pierce, Rockford, IL) was dispensed into each well, and the plates were incubated for 1 hour at 37°C. The reaction was developed with a substrate solution containing 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD). The reaction was stopped with 4 N of sulfuric acid, and the optical densities were measured at 490 nm with an ELISA reader (EMAX; Molecular Devices Corp., Sunnyvale, CA). The average of the optical densities obtained with sera from control mice, diluted 1:50, was considered the cutoff for each respective isotype. Optical densities for each dilution of experimental sera were compared with control values.

Cytokine Detection in Organ Supernatants

Cytokine concentrations in the tissues were measured by two-site sandwich ELISA. To evaluate IFN- γ and IL-4 cytokines secreted in the lungs, the organs were removed, and a piece weighed and homogenized in 1 ml of complete inhibitor cocktail buffer (Boehringer, Mannheim, Germany) using a tissue homogenizer. The samples were centrifuged at 5000 $\times$ g for 10 minutes, and 100 μ l were dispensed into 96-well plates containing anti-IFN- γ (XMG1.2) or anti-IL-4 (BVD6) (all from BD Pharmingen, San Jose, CA) as capture monoclonal antibodies (mAbs). The ELISA procedure was performed according to the manufacturer's protocol. The IFN- γ bound to the mAb was detected with polyclonal rabbit anti-IFN- γ (Santa Cruz Biotechnology) followed by goat biotinylated anti-rabbit IgG (Life Technologies Inc., Gaithersburg, MD). The IL-4 was detected using biotinylated mAbs (11B11). The avidin-biotin peroxidase technique was used to reveal the presence of the cytokines. Standard curves were constructed for serial twofold dilutions of murine recombinants (R&D Systems, Minneapolis, MN), and the minimal detectable concentration was 100 pg/ml for both assays. Optical densities were measured at 450 nm, by use of a microplate ELISA reader (EMAX; Molecular Devices).

Isolation of Leukocytes from Lungs

Leukocytes were obtained from lungs of infected or normal mice as previously described.²⁴ Briefly, the lungs were deposited in medium with Liberase C1 enzymes blend (0.5 mg/ml; Roche, Indianapolis, IN) and incubated for 40 minutes at 37°C. Afterward, the lungs were dissociated in medium containing 10% fetal bovine serum and 0.05% DNase I (Sigma Aldrich, St. Louis, MO) with a medmachine (BD Bioscience, Rockville, MD) according to the manufacturer's protocol. The tissue homogenates were filtered using a 50- μ m syringe filter and then centrifuged, and the leukocytes were thus obtained.

Adoptive Cell Transfer Experiments

Leukocytes were obtained from lungs of infected WT mice at the day 10 after infection as described above. CD4 and CD8 T cells were isolated using anti-mouse IgG Dynabeads (Dyna, Oslo, Norway) according to the manufacturer's protocol and labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE), as previously described.²⁵ In brief, leukocytes (1×10^7 cells/ml) were stained with 5 μ mol/L CFSE (5 minutes, 37°C, darkness). Staining was stopped by adding complete culture medium, the cells were centrifuged (5 minutes, 490 $\times$ g) and suspended in the same medium. Next, 1×10^6 CD4⁺ and CD8⁺ cells were intravenously injected into uninfected or infected WT and ICAM-1KO mice (day 10 after infection). After 12 hours, leukocytes from lungs were obtained, and the migration of CFSE⁺ cells was investigated. Multivariate data

Figure 1. ICAM-1 is up-regulated in the lungs of *P. brasiliensis*-infected mice. **A** and **B:** C57BL/6 (WT) mice were infected with *P. brasiliensis*; the lungs were harvested 30 days later; and the presence and localization of CD4, CD8, and CD54 were detected by immunohistochemistry. **C:** Intensity of positive fluorescence of ICAM-1 (MIF) in lymphocytes isolated from the lungs of mice at day 15 after infection. The cells were gated on CD4 (white bars) and CD8 (black bars) and the intensity of ICAM-1 expression determined using FACS analysis. **D:** The percentage of reduction of ICAM-1 expression in lymphocytes isolated from lungs of mice deficient of IFN- γ (GKO), p55TNF- α (TNF- α P55KO), and IL-12 (IL-12KO) at day 15 after infection. The data shown represent the mean $\pm$ SEM of the results obtained from at least three animals and are representative of three independent experiments. * $P < 0.05$ compared with uninfected. Scale bars = 50 μ m.

analysis was performed by flow cytometer on a FAC-
Sort using Cellquest software (Becton-Dickinson Co.,
Mountain View, CA) by setting a gate on the live cells
on side scatter versus forward scatter dot plot and
determining the expression of CFSE. The results show
the percentage of stained cells in lungs of three to four
mice.

Flow Cytometry Analysis

The cell surface expression of CD3, CD4, CD8, and
ICAM-1 (CD54) was assessed by flow cytometry. Leu-
kocytes isolated from lungs of infected and normal
mice were washed in ice-cold PBS and incubated for
30 minutes at 4°C with 0.5 μ g of anti-CD16/CD32 mAb

Figure 2. ICAM-1 mediates the migration of T lymphocytes to the site of *P. brasiliensis*-infection. The numbers of CD3⁺CD4⁺ (A) and CD3⁺CD8⁺ (B) T cells in the lungs of WT (white bars) and ICAM-1KO mice (black bars) were determined by counting the leukocytes by hemocytometer and the percentage of each subpopulation by FACS. The data shown represent the mean $\pm$ SEM of the results obtained from at least three animals and are representative of three independent experiments. * $P < 0.05$ compared with WT infected mice. C and D: CD4 and CD8 T cells from infected WT mice (10 days after infection) were purified, labeled with CFSE, and adoptively transferred to uninfected (white bars) or infected (black bars) WT and ICAM-1KO mice, and the stained cells in the lungs were evaluated 12 hours later by FACS. In C, the values indicate the percentages of CFSE⁺ cells, being that the isotype control and CFSE⁺ are shown as light and dark lines, respectively. In D, the data shown represent the mean $\pm$ SEM of the results obtained with three (uninfected) or four (infected) animals. * $P < 0.05$ compared with WT infected mice.

(Fc block, clone 2.4G2; BD Pharmingen, San Diego, CA), followed by the addition of 0.5 μ g per 1×10^6 cells of the following antibodies: fluorescein isothiocyanate-labeled anti-CD3, phycoerythrin-labeled anti-CD4 or anti-CD8, fluorescein isothiocyanate-labeled anti-CD4 or anti-CD8, and phycoerythrin-labeled anti-CD54 (all from BD Pharmingen). Subsequently, cells were washed twice and suspended in 300 μ l of PBS-bovine serum albumin (Sigma Aldrich). Multivariate data analysis was performed in a FACSsort Flow cytometer using the Cellquest software (Becton-Dickinson) by setting a gate on the live cells on side scatter versus forward scatter dot plot and determining the expression of CD4⁺ and CD8⁺ on the CD3⁺ cell population or ICAM-1⁺ on the CD4⁺ or CD8⁺ cell population. The results show mean of intensity of fluorescence (MIF) and numbers of cells in lungs of mouse, of at least three independent experiments.

Statistical Analysis

The data are reported as mean $\pm$ SEM. Statistical analysis was performed using analysis of variance followed

by the parametric Tukey-Kramer test (INSTAT software; GraphPad, San Diego, CA). P values ≤ 0.05 were considered statistically significant.

Results

The Expression of ICAM-1 Is Increased in the Lungs of *P. brasiliensis*-Infected Mice

Because T cells are critical for efficient resolution of fungal infections, we studied the recruitment of CD4⁺ and CD8⁺ T cells into the lungs of mice on days 15 and 30 after infection with *P. brasiliensis*. Immunocytochemical staining revealed that CD4⁺ and CD8⁺ cells were very abundant (Figure 1A), being that the positive cells were found predominantly around the granulomatous lesions. We next investigated the expression of ICAM-1 and whether it is modulated in the lungs after intratracheal infection with yeast forms of *P. brasiliensis*. Immunohistochemical staining showed increased immunoreactivity to ICAM-1 in the lungs of WT mice compared with the uninfected controls (Figure 1B). The

Figure 3. The expression of ICAM-1 is necessary for resistance against the infection with *P. brasiliensis*. **A:** The WT (○) and ICAM-1KO (●) mice were infected with *P. brasiliensis* and the survival observed daily during 120 days. **B:** Colony-forming units in the lungs, liver, and spleen of WT (white bars) and ICAM-1KO (black bars) mice were determined on days 30, 60, and 90 after infection. The data represent the mean $\pm$ SEM of the results of three animals and are representative of three independent experiments. * $P < 0.05$ compared with WT infected mice. ND, not detected.

positive staining was found in endothelial and inflammatory cells, as soon as day 15 after infection. Flow cytometry of cells from the lesions confirmed that the intensity of expression of ICAM-1 on CD4⁺ and CD8⁺ T cells increased 36 and 54%, respectively, after infection with *P. brasiliensis* (Figure 1C). Because the expression of ICAM-1 is up-regulated by proinflammatory cytokines, we examined the presence of ICAM-1 on lymphocytes recovered from lungs of mice genetically deficient in IFN- γ , TNFR1, and IL-12, on day 15 after infection. The results showed a significant ($P < 0.05$) inhibition of the expression of ICAM-1 on leukocytes from GKO, p55KO, and IL-12KO mice (Figure 1D). Therefore, the *P. brasiliensis*-induced expression of ICAM-1 is dependent on the action of proinflammatory cytokines.

ICAM-1 Is Involved in the Mechanism of T-Cell Migration and Resistance to Infection with *P. brasiliensis*

To study the role of ICAM-1 in the mechanism of cell migration and in the resistance of the host against infection, we used mice deficient in ICAM-1. We found that the migration of both CD3⁺CD4⁺ (Figure 2A) and CD3⁺CD8⁺ T cells (Figure 2B) to the lungs of ICAM-1KO mice at day 30 after infection is significantly lower when compared with littermate controls. To finally determine the role of ICAM-1 expressed on pulmonary vascular endothelium in the cell migration to the lungs, CD4⁺ and CD8⁺ T cells from lungs of WT mice were purified, labeled with CFSE, and adoptively transferred to unin-

fected or infected WT and ICAM-1KO mice, and the cell migration into the lungs was evaluated 12 hours later. The results, analyzed by flow cytometry (Figure 2C), showed a significantly ($P < 0.05$) increased migration of CFSE⁺ lymphocytes into the lungs of infected WT and ICAM-1KO mice (Figure 2D). Finally, the cell migration to the lungs of infected WT mice was significantly higher ($P < 0.05$) than that observed in infected ICAM-1KO mice (Figure 2D). These results indicate that ICAM-1 expression on the vascular endothelium of *P. brasiliensis*-infected mice favors the establishment of a pulmonary inflammatory reaction during the early phase of infection. We also observed that the absence of ICAM-1 results in decreased resistance to the infection. In fact, whereas all control mice survived during the period of this study (120 days), 20% of the ICAM-1KO mice succumbed by day 65 of infection (Figure 3A). Moreover, the amount of fungus recovered from the organs at days 30 and 60 after infection was higher when compared with that of WT mice (Figure 3B). Although by day 90 after infection the WT mice had controlled the infection, the amount of fungus in the lungs of ICAM-1KO mice was still very high at this same time point (Figure 3B). These results suggest that ICAM-1 is involved in the control of the replication and dissemination of *P. brasiliensis* to the organs of infected mice.

Humoral and Cellular Immune Responses against *P. brasiliensis* in Infected ICAM-1KO Mice

To verify how immune effector responses are affected by ICAM-1, we measured the production of *P. brasiliensis*-

Figure 4. The humoral and cellular immune response in ICAM-1-deficient mice. Sera from WT (white bars) and ICAM-1KO (black bars) mice were analyzed for levels of immunoglobulin IgG (A), IgG2 (B), and IgG1 (C) by using an isotype-specific ELISA. The bars represent the mean $\pm$ SEM of the OD obtained for sera, diluted 50 times, of three animals and are representative of two independent experiments. D: The DTH assay was measured on days 15, 30, and 60 days after infection. The mice were challenged by footpad injections ($2 \mu\text{g}/\text{ml}$) of fungal antigens (right footpad) or PBS (left footpad). The bars represent the mean $\pm$ SEM of the difference between the right and left footpad of four mice per group and are representative of three experiments. * $P < 0.05$ compared with WT mice. Lungs from WT (white bars) and ICAM-1KO (black bars) mice at 15 days after infection with *P. brasiliensis* were collected, weighed, and homogenized in sterile PBS, and the amount of IFN- γ and IL-4 was determined by ELISA. Each scale bar represents the mean $\pm$ SEM of the concentration of IFN- γ (E) and IL-4 (F) of three mice and is representative of two separate experiments. * and **, $P < 0.05$ compared with uninfected WT mice or with infected and uninfected WT mice, respectively.

specific antibodies and the DTH responses to an extract of fungal antigens in *P. brasiliensis*-infected WT and ICAM-1KO mice. Relative to the preinfection serological status, by day 30 after infection ICAM-1KO mice presented with significantly ($P < 0.05$) higher levels of *P. brasiliensis*-specific IgG1 (Figure 4C), but there were no differences in the levels of specific IgG2 (Figure 4B) or total IgG (Figure 4A). By day 60, infected ICAM-1KO mice presented a significant increase in the levels of fungus-specific total IgG, IgG1, and IgG2 antibodies, whereas WT-infected mice presented an increase in fungus-specific total IgG and IgG1 antibodies (Figure 4, A–C). However, no differences between both infected groups were observed. These results suggest that a defect in the antibody response does not account for the striking susceptibility of ICAM-1-deficient mice to infection with *P. brasiliensis*. In contrast to the humoral response, on day 15 after infection, the DTH response was significantly ($P < 0.05$) decreased in ICAM-1KO mice when compared with that elicited in WT controls. On days 30 and 60 after infection, the DTH reactions were similar in both groups (Figure 4D). These results suggest that infected ICAM-1KO mice present a delay in the recruitment and organization of the anti-*P. brasiliensis* cellular immune response.

We next assayed the levels of IFN- γ and IL-4 in homogenates of lungs from *P. brasiliensis*-infected WT and ICAM-1KO mice. In both groups, the levels of IFN- γ were significantly higher than the levels found in homogenates from uninfected lungs and had increased by a similar amount in both groups (Figure 4E). Conversely, signifi-

cantly higher levels of IL-4 were detected in the homogenate obtained from lungs of infected ICAM-1KO mice when compared with that of WT-infected mice (Figure 4F).

ICAM-1 Modulates the Development and Organization of Granulomas in *P. brasiliensis*-Infected Mice

We next studied the evolution of granulomas formed in the absence of ICAM-1. The results show that on day 30 after infection, the lungs of WT mice exhibited diffuse foci of well-organized granulomatous lesions composed of histiocytes intermixed with a few neutrophils, epithelioid cells, and a few multinuclear giant cells surrounded by a rim composed of a great number of lymphocytes and few fibroblasts (Figure 5A). In addition, the normal pattern of reticulin fibers in granulomas could be seen (Figure 5C). Specific staining for yeast forms of the fungus demonstrated that they were confined within the granulomatous structure (Figure 5E). In contrast, the lungs of ICAM-1KO mice showed incomplete organization of the granulomas, with diffuse extensive foci of consolidated parenchyma as revealed by intra-alveolar exudation of lymphomononuclear cells and a few polymorphs (Figure 5B). These findings correlated with the presence of a greater number of yeast forms of the fungus (Figure 5F). The reticulin structure of the pulmonary tissue was relatively preserved (Figure 5D). By day 60 after infection, the lungs of WT

Figure 5. ICAM-1 contributes to the development and organization of *P. brasiliensis*-induced granulomas. Lung tissue of WT (**A**, **C**, **E**) and ICAM-1KO (**B**, **D**, **F**) mice at day 30 after infection with *P. brasiliensis* were obtained, fixed in formalin, paraffin embedded, cut into 2- μ m sections, stained with H&E (**A**, **B**), impregnated with silver for reticulum fibers (**C**, **D**) or with Grocott to detect mycotic structures (**E**, **F**), and analyzed by light microscopy. Scale bars = 50 μ m.

mice showed quite well-defined granulomatous lesions composed of histiocytes, a few polymorphs, epithelioid cells, and a few multinuclear giant cells with a clear-cut delimited rim of lymphocytes and fibroblasts (Figure 6A). The pattern of reticulin fibers observed in these mature granulomas could be seen clearly surrounding the yeast forms of the fungus (Figure 6C). In contrast, the pulmonary lesions seen in infected ICAM-1KO mice were diffuse and characterized by coalescent foci of incipient granulomatous structures composed of histiocytes, a few epithelioid cells, and occasional giant cells surrounded by an ill-defined rim of lymphocytes (Figure 6B). An in-

cipient pattern of reticulin fibers was observed in the granulomas (Figure 6D), and specific staining for yeast forms of the fungus demonstrated that they were constrained within these incipient granulomatous lesions (Figure 6F). At day 90 after infection, we observed an evolution in the formation of granulomas in ICAM-1-deficient mice. At this point, these animals were finally able to restrict the fungus and presented with diffuse foci of well-organized giant cell granulomas and strikingly few yeast forms (Figure 7B). The lungs of WT mice showed diffuse occasional foci of mononuclear cells associated with thickening of the interalveolar septa,

Figure 6. The formation and resolution of granulomas is dependent of ICAM-1. Lung sections from WT (A, C, E) and ICAM-1KO (B, D, F) mice at day 60 after infection with *P. brasiliensis* were obtained and stained with H&E (A, B), impregnated with silver for reticulum fibers (C, D), and stained with Grocott to detect mycotic structures (E, F). Scale bars = 50 μ m.

and no yeast forms of the fungus could be detected (Figure 7A).

Discussion

The events that result in the formation of granulomas induced by *P. brasiliensis* remain poorly understood. Although cytokines such as IFN- γ and chemokines such as CCL5, CCL2, CXCL10, and CXCL9 are implicated,⁶ we do not know the sequential steps in which they are involved and the cells and adhesion molecules that must take part in these events, the end of which is the mature

granuloma. Herein, we investigated the role of ICAM-1 in the formation of granulomas and in the resistance of the host against infection with *P. brasiliensis*. We were prompted to examine this aspect because initial studies revealed that expression of ICAM-1 was markedly increased in CD4⁺ and CD8⁺ cells present in the lungs of *P. brasiliensis*-infected mice. Expression of ICAM-1 also increases in cells from lung tissue of mice infected with *Bordetella pertussis*²⁶ and *Klebsiella pneumoniae*²⁷ and results in the accumulation and activation of leukocytes at the inflammatory sites elicited by these pathogens. Experiments of adoptive transfer of lym-

Figure 7. The granulomas are organized later in mice deficient of ICAM-1. Lung sections from WT (A) and ICAM-1KO (B) mice at day 90 after infection with *P. brasiliensis* were obtained and stained with H&E. Scale bars = 50 μ m.

phocytes strongly suggest that the ICAM-1 expression on the vascular endothelium of lungs of *P. brasiliensis*-infected mice favors the establishment of a pulmonary inflammatory reaction.

During the *P. brasiliensis* infection, the expression of ICAM-1 is regulated by IFN- γ , TNF- α , and IL-12, because in the absence of these cytokines, there was a marked decrease in the expression of the adhesion molecule, a finding associated with a deficient migration of T cells to the lungs and with an ineffective control of fungus in the absence of ICAM-1. IFN- γ is critical in modulating expression of ICAM-1,^{28–30} being that TNF- α ^{28–30} and IL-12^{11,31,32} are also involved in the up-regulation of expression of this adhesion molecule. Accordingly, mice deficient in the TNF- α -p55 receptor or in IFN- γ ,⁷ mice treated with anti-IL-12,¹⁷ and patients deficient in the β -1 subunit of the IL-12 receptor³³ all present with increased susceptibility to infection and disseminated disease. These findings suggest that ICAM-1 could be involved in the mechanism of resistance against this fungus. Indeed, in the present work, ICAM-1KO mice were more susceptible to infection than the littermate WT controls. Higher mortality and decreased migration of T lymphocytes to target organs were also found in animals deficient in ICAM-1 when infected with *Trypanosoma cruzi*³⁴ or *Chlamydia trachomatis*.³⁵

The greater susceptibility to infection in the absence of ICAM-1 could be attributable to the production of low levels of IFN- γ and/or antibodies. IFN- γ is involved in the activation of macrophages that are the effectors of the anti-fungal immunity and protect against *P. brasiliensis*^{3,36} and other fungi such as *Candida albicans*³⁷ and *Cryptococcus neoformans*.^{38,39} On day 15 after infection the levels of IFN- γ in the lungs of WT and ICAM-1KO mice were similar, suggesting that the lower resistance against *P. brasiliensis* seen in ICAM-1KO mice is not because of insufficient IFN- γ . The absence of ICAM-1 also did not change the levels of fungus-specific IgG, clearly showing that the greater susceptibility is not attributable to the levels of antibodies. Accordingly, minimal effects on the generation of specific T-cell responses and humoral immunity were observed in the absence of ICAM in mice infected with the protozoa *T. cruzi*.³⁴ However, in infection

with *P. brasiliensis*, its absence results in a delay of the DTH response to the specific antigens and increased production of IL-4 on day 15 after infection. The IL-4 could be produced by alveolar macrophages⁴⁰ and histiocytes, which are present in the lungs at this time of infection. Therefore, the absence of ICAM-1 significantly hampers the migration of cells toward the target tissue, which could lead to a reduction of the local production of factors that attract T cells, such as chemokines and cytokines, resulting in increased fungal load. The progression and dissemination of this infection is inversely correlated with the DTH response¹³ and directly proportional with production of IL-4,⁴¹ indicating, as shown before,^{7,13,20} that the type-1 T-cell response is crucial to control the infection. Indeed, IL-4 is related with susceptibility and deficient formation of granulomas in *P. brasiliensis*-infected mice.^{41,42} Altogether, these findings suggest that the expression of ICAM-1 in the lungs of mice infected with *P. brasiliensis* contributes not only to the recruitment and adhesion of circulating T cells but also to the formation and organization of granulomas and with the adequate balance of cytokine production. Together these events orchestrate the generation of effector immune responses and the host defenses responsible for preventing the fungus to escape and disseminate.

In the absence of ICAM-1, the morphology of the pulmonary inflammatory infiltrate changes markedly in comparison to that of WT hosts: a greater number of well-delimited and confluent lesions appear and lead to extensive damage of the lung in the absence of ICAM-1. An evolution of the inflammatory response occurs only after day 60 after infection, together with a partial organization of the granulomas. By day 90, fewer amounts of viable yeast cells are found in the organs and the now well-formed granulomas suggest that a more efficient immune response and control of infection was finally able to take place. These results and the ultimate ability to mount a DTH response suggest that compensatory mechanisms of cellular migration are established in ICAM-1KO mice at later times points of the infection, as observed in mice infected with visceral leishmaniasis.²⁹ The possible compensatory mechanisms involved in the migration of inflammatory cells to the site of infection

could be explained by the interactions established among other adhesion molecules, such as VCAM-1, ICAM-2, ICAM-3, and MadCAM-1, that participate in the interaction between endothelial cells and leukocytes during cellular migration.⁹ These ICAM-1-independent pathways as previously described in other model,²⁹ could contribute toward the re-establishment of migration of leukocytes and to the defense of the host against *P. brasiliensis*. Supporting this possibility, the cell migration into the lung tissue was only partially abrogated when activated leukocytes from *P. brasiliensis*-infected WT mice were adoptively transferred to infected ICAM-1KO mice. However, despite these compensatory mechanisms, our data showed that the outcome is, nevertheless, significantly worse in ICAM-1-deficient hosts. By the time this compensation effectively occurs, the greater load of fungus that was able to become established initially demands a correspondingly greater magnitude of effector responses that, in the case of the 20% of ICAM-1KO mice that succumbed to the infection, were not met.

The results presented here suggest that during experimental infections with *P. brasiliensis*, ICAM-1 is essentially playing an irreplaceable role in the migration of CD4⁺ and CD8⁺ T lymphocytes to the lungs. The deficit in lymphocyte migration was associated with growth and dissemination of the fungus, indicating the importance of the local action of lymphocytes to control the fungal load. Finally, based on these results, ICAM-1 can be considered as a potential immunotherapeutic target to prevent the growth and dissemination of fungus.

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