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TLR2 Is a Negative Regulator of Th17 Cells and Tissue Pathology in a Pulmonary Model of Fungal Infection

Flávio V. Loures, Adriana Pina, Maíra Felonato, and Vera L. G. Calich²

To study the role of TLR2 in a experimental model of chronic pulmonary infection, TLR2-deficient and wild-type mice were intratracheally infected with Paracoccidioides brasiliensis, a primary fungal pathogen. Compared with control, TLR2²⁻/⁻ mice developed a less severe pulmonary infection and decreased NO synthesis. Equivalent results were detected with in vitro-infected macrophages. Unexpectedly, despite the differences in fungal loads both mouse strains showed equivalent survival times and severe pulmonary inflammatory reactions. Studies on lung-infiltrating leukocytes of TLR2²⁻/⁻ mice demonstrated an increased presence of polymorphonuclear neutrophils that control fungal loads but were associated with diminished numbers of activated CD4⁺ and CD8⁺ T lymphocytes. TLR2 deficiency leads to minor differences in the levels of pulmonary type 1 and type 2 cytokines, but results in increased production of KC, a CXC chemokine involved in neutrophils chemotaxis, as well as TGF-β, IL-6, IL-23, and IL-17 skewing T cell immunity to a Th17 pattern. In addition, the preferential Th17 immunity of TLR2²⁻/⁻ mice was associated with impaired expansion of regulatory CD4⁺CD25⁺FoxP3⁺ T cells. This is the first study to show that TLR2 activation controls innate and adaptive immunity to P. brasiliensis infection. TLR2 deficiency results in increased Th17 immunity associated with diminished expansion of regulatory T cells and increased lung pathology due to unrestrained inflammatory reactions. The Journal of Immunology, 2009, 183: 1279–1290.

The initial interaction between immune cells and microorganisms is mediated by several types of receptors that recognize molecular patterns of pathogens and are collectively called pathogen recognition receptors. The TLRs constitute a molecular family that recognizes a wide range of microbes and their products known as pathogen-associated molecular patterns. TLRs are expressed in diverse innate immune cells such as polymorphonuclear neutrophils (PMN), macrophages, dendritic cells, and lymphocytes. Their activation triggers a signaling cascade that results in an inflammatory response through production of proinflammatory cytokines and up-regulation of costimulatory molecules expression leading to initiation of antigen-specific adaptive immune response (1–3).

Importantly, the TLR expression was also shown to induce anti-inflammatory mediators and to discriminate the functional states of distinct T cell subsets (4, 5). Naive CD4⁺ T cells do not express significant levels of TLR2 and TLR4 mRNA and intracellular proteins, although activated and memory T cells express high levels of membrane-bound TLR2 and TLR4 (5). Besides its influence in the activation of innate immunity cells, recent evidences suggest that TLR2 signaling may regulate the expansion and function of CD4⁺CD25⁺ regulatory T cells (Tregs) (5). Indeed, the administration of TLR2 ligands to wild-type (WT) mice results in increased number CD4⁺CD25⁺ Tregs and TLR2²⁻/⁻ mice were shown to contain significantly fewer CD4⁺CD25⁺ Tregs than control mice (5–8).

As described for other microorganisms, TLRs were shown to be involved in host defense against different fungal pathogens. In vivo and in vitro studies demonstrated that Cryptococcus neoformans (9, 10), Candida albicans (11, 12), and Aspergillus fumigatus (13, 14) may signal through members of the TLR family, mainly TLR2 and TLR4. The contribution of individual TLRs to the immune response against pathogenic fungi depends on several factors such as the route of infection, the fungal morphotype, or the fungal species. Activation signals mediated by innate immunity receptors, however, are not always beneficial to the host and TLR activation can be used by pathogenic fungi to promote more severe infections (15–17).

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by the dimorphic fungus Paracoccidioides brasiliensis and constitutes the most prevalent deep mycosis in Latin America (18). Individuals from endemic areas who have inhaled mycelial fragments or fungal spores usually develop PCM infection which is characterized by positive delayed-type hypersensitive reactions but absence of specific Abs. The acute or severe form of the disease is associated with deficient cell immunity, high levels of Abs, and preferential secretion of type 2 cytokines, whereas the benign localized forms demonstrate preserved cell-mediated immunity, prevalent production of type 1 cytokines, and low levels of Abs (19, 20).

Our studies on the genetic susceptibility of hosts to P. brasiliensis infection characterized B10.A as a susceptible mouse strain due to its progressive and disseminated disease associated with impaired macrophage activation and the presence of high fungal loads in nonorganized lesions. On the other hand, A/J mice showed a regressive pattern of disease with well-organized lesions containing low numbers of yeast cells, positive cellular immunity, and
macrophage activation. These features are similar to the severe and benign forms of human PCM (20, 21).

The alveolar macrophages are the first host cells that interact with \textit{P. brasiliensis} cells and their activation is fundamental to control pathogen growth. The molecular mechanisms controlling the initial steps of \textit{P. brasiliensis} and phagocytes interaction are not well understood. It is known, however, that normal macrophages are permissive to \textit{P. brasiliensis} growth while cytokine-activated macrophages are able to restrain \textit{P. brasiliensis} multiplication (22). It was previously demonstrated that C3b, mannose receptor, and gp43, the immunomodulatory Ag of \textit{P. brasiliensis}, play an important role in the initial interaction between \textit{P. brasiliensis} cells and mouse peritoneal macrophages (23–25). Interestingly, a recent work of our laboratory demonstrated that alveolar macrophages from susceptible mice are easily activated by IL-12 and IFN-\gamma and display an efficient fungal killing associated with increased secretion of NO and proinflammatory cytokines. In contrast, pulmonary macrophages from resistant mice are poorly activated by both cytokines, present inefficient killing activity and NO secretion, and this behavior was associated with the increased activity of endogenous TGF-\beta (26). Despite their inefficient innate immunity, A/Sn mice develop a balanced Th1/Th2 immunity.

Since the contribution of TLR in \textit{P. brasiliensis} infection was never studied before, we decided to investigate the role of TLR2 in murine PCM using in vitro and in vivo models of infection. Using TLR2-normal and TLR2-deficient C57BL/6 mice, we were able to demonstrate that, both in vitro and in vivo, the presence of TLR2 causes a more severe infection. Both approaches demonstrated that TLR2 are used by \textit{P. brasiliensis} yeast to infect host cells inducing enhanced secretion of NO and cytokines. Unexpectedly, TLR2-deficient and WT mice presented similar survival times and equivalent severe lesions in the lungs. The lower fungal loads of TLR2-deficient mice were, however, associated with prevalent activation of Th17 immunity and exacerbated pulmonary inflammation containing high numbers of PMN leukocytes but diminished presence of Tregs. Altogether, our data indicate that expression of TLR2 has a beneficial effect on fungal pulmonary infection due to its negative control on Th17 immunity and tissue pathology. Furthermore, the present findings demonstrate that uncontrolled inflammatory response of hosts to \textit{P. brasiliensis} infection is as deleterious as uncontrolled fungal growth by absence or inadequate activation of immunity.

\section*{Materials and Methods}

\subsection*{Fungus}

\textit{P. brasiliensis} Pb18, a highly virulent isolate, was used throughout this investigation (27). Pb18 yeast cells were maintained by weekly subcultivation in semisolid culture medium. Washed yeast cells were adjusted to \(2 \times 10^7\) cells/ml (in vivo infection) and \(4 \times 10^7\) cells/ml (in vitro infection) based on hemocytometer counts. Viability was determined with Janus Green B vital dye (Merck) and was always higher than 85%.

\subsection*{Mice and intratracheal infection}

TLR2\(^{-/}\) mice on a C57BL/6 background were provided by S. Akira (Osaka University, Osaka, Japan), C57BL/6 control (WT) mice were obtained from our Isogenic Breeding Unit (Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo) and used at 8–12 wk of age. Mice were anesthetized and submitted to intratracheal (i.t.) \textit{P. brasiliensis} infection as previously described (28). Briefly, after i.p. anesthesia, the animals were i.t. infected with \(10^7\) \textit{P. brasiliensis} yeast cells contained in 50 \(\mu\)l of PBS. Mice were studied at 48 h, 2 wk, and 11 wk postinfection. The experiments were approved by the ethics committee on animal experiments of our institution.

\subsection*{Phagocytic and fungicidal assays}

Thiglycolate-induced peritoneal macrophages were isolated by adherence (2 h at 37°C in 5% CO\textsubscript{2}) to plastic-bottom tissue culture plates (1 \(\times 10^7\) cells/well in 24-well plates for fungicidal assays) or plated onto 13-mm round glass coverslips (1 \(\times 10^6\) cells/well in 24-well plates) for phagocytosis. Macrophages were washed to remove nonadherent cells and cultivated overnight with fresh complete medium (DME, Sigma-Aldrich) containing 10% FCS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin in the presence or absence of recombinant IFN-\gamma (20 ng/ml in culture medium; BD Pharmingen). Equivalent procedures were performed with alveolar macrophages obtained from \textit{P. brasiliensis}-infected mice at week 2 of infection by bronchoalveolar lavage with 1.5 ml of warm PBS (26). For phagocytic assays, macrophage cultures were infected with \textit{P. brasiliensis} yeast in a macrophage:yeast ratio of 50:1. The cells were cocultivated for 4 h at 37°C in 5% CO\textsubscript{2} to allow fungi adhesion and ingestion. Cells were washed twice with PBS to remove any noningested or nonadhered yeast cells and samples were processed for microscopy. Cells were fixed with methanol and stained with Giemsa (Sigma-Aldrich). Experimental conditions were performed in triplicate, and the number of phagocytosed or adhered yeast cells per 1000 macrophages was evaluated on at least five separate slides. For fungicidal assays, IFN-\gamma-primed and unprimed macrophage cultures were infected with \textit{P. brasiliensis} yeast as above described. After 48 h of culture at 37°C in a CO\textsubscript{2} incubator, plates were centrifuged (400 \(\times\) g, 10 min, 4°C), supernatants were stored at \(-70°C\), and further analyzed for the presence of nitrite and cytokines. The wells were washed twice with distilled water to lyse macrophages and suspensions were collected in individual tubes. One hundred microliters of cell homogenates were assayed for the presence of viable yeast cells. All assays were done with five wells per condition in more than three independent experiments.

\subsection*{CFU assays, mortality rates, and histologic analysis to determine severity of infections}

The numbers of viable microorganisms in cell cultures and infected organs (lungs, liver, and spleen) from experimental and control mice were determined by counting the number of CFU. Animals from each group were sacrificed at different time points, and the enumeration of viable organisms was performed as previously described (29). The numbers (log_{10}) of viable \textit{P. brasiliensis} per g of tissue (in vivo) or per ml of cell homogenates (in vitro) are expressed as the means \(\pm\) SEs. Mortality studies were done with groups of 9–11 mice inoculated i.t. with \(1 \times 10^7\) yeast cells or PBS. Deaths were registered daily for a 250-day period and experiments were repeated twice. For histology examinations, the left lung of infected mice was removed, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were stained by H&E for an analysis of the lesions and silver stained (Grocott stain) for fungal evaluation. Pathologic changes were analyzed based on the size, morphology, and cell composition of granulomatous lesions, presence of fungi, and intensity of the inflammatory infiltrates.

\subsection*{Measurement of cytokines and NO}

Supernatants from lung homogenates or cell cultures were separated and stored at \(-70°C\). Cytokine (IL-2, IL-4, IL-5, IL-23, IL-17, IL-12, IL-10, IL-6, TNF-\alpha, and IFN-\gamma) and chemokine (MCP-1 and KC) levels were measured by capture ELISA with Ab pairs purchased from BD Pharmingen. Active and acid-activatable latent TGF-\beta forms were also measured (kits from R&D Systems) in our biological fluids. The ELISA procedure was performed according to the manufacturer’s protocol and absorbances were measured with a Versa Max Microplate Reader (Molecular Devices). NO production was quantified by the accumulation of nitrite in the supernatants from in vitro and in vivo protocols by a standard Griess reaction. All determinations were performed in duplicate and expressed as \(\mu\)M NO.

\subsection*{Assessment of leukocyte subpopulations in lung inflammatory exudates}

After 2 and 11 wk of infection, lungs from each mouse were digested enzymatically for 30 min with collagenase (1 mg/ml) and DNase (30 mg/ml) in culture medium (Sigma-Aldrich). Lung cell suspensions were centrifuged in the presence of 20% Percoll (Sigma-Aldrich) to separate leukocytes from cell debris. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer; viability was \(\geq85\%\). For differential leukocyte counts, samples of lung cell suspensions were stained (Shandon Cytospin) onto glass slides and stained by the Diff-Quik blood stain (Baxter Scientific). A total of 200–400 cells was counted from each sample. For flow cytometry, lung leukocytes were resuspended at \(10^6\) cells/ml in staining buffer (PBS plus 0.1% Na2, plus 1%
FACS) FCRs were blocked by unlabeled anti-CD16/32 Abs (BD Biosciences) and cells were stained for 20 min at 4°C. PE-labeled anti-CD40, anti-CD86, anti-CD11b, anti-CD25, and anti-CD69 and FITC-labeled anti-IAα, anti-CD80, anti-CD4, and anti-CD8 mAbs (BD Biosciences) were used. Cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) and stored in the dark at 4°C until analyzed in a flow cytometer. The acquisition and analysis gates were restricted to the lymphocytes or macrophages. Tregs were characterized by intracellular staining for Foxp3 using a Treg staining kit from BD Biosciences. Surface staining of CD69 and intracellular Foxp3 expression were back-gated on the CD4+ T cell population. For intracellular cytokine staining, cells were stimulated for 6 h in complete medium in the presence of 50 ng/ml PMA, 500 ng/ml ionomycin (both from Sigma-Aldrich), and monensin (3 mM; eBioscience). After surface staining for CD4 and CD8, cells were fixed, permeabilized, and stained by PerCP-Cy5 anti-IL-17 Abs (eBioscience). The cell surface expression of leukocyte markers as well as intracellular expression of Foxp3 and IL-17 in lung-infiltrating leukocytes were analyzed in a FACS Calibur flow cytometer (BD Pharmingen) using the FlowJo software (Tree Star).

**Fungal ability of neutrophils**

To study the role of neutrophils in the control of fungal growth, PMN leukocytes from WT and TLR2 KO mice were in vivo depleted by i.p. administration of 0.5 ml of saline-diluted (1/5) rabbit anti-mouse PMN polyclonal Ab (Accurate Chemical & Scientific) at days −1, −3, and +5 of i.t. infection (0 time) with 1 million *P. brasiliensis* yeast cells. Control mice received equivalent amounts of normal rat IgG. The number of blood PMN cells was evaluated before treatment (at day −2) and 24 h after the mAb inoculation. Groups of IgG-treated and PMN-depleted mice (n = 4–6) were sacrificed 2 and 6 days after *P. brasiliensis* infection and severity of disease evaluated by CFU counts. Mortality studies were done with similar groups of PMN-depleted groups of eight to nine mice inoculated i.t. with 1 × 106 yeast cells. Deaths were registered daily for a 10-day period and experiments were repeated twice. Alveolar neutrophils were obtained by bronchoalveolar lavage of mice infected 2 wk earlier with 1 million yeast cells. The number of PMN leukocytes in the nonadherent fraction of lung cells was adjusted to 2 × 105 cells, plated in 96-well plates, challenged with 1 × 105 yeast cells, and cocultivated for 1 h at 37°C. One hundred microliters of cell homogenates was assayed for the presence of viable yeast cells. All assays were done with three wells per condition in more than two independent experiments.

**Lymphoproliferation assay**

Cells were assayed for proliferation using an in vitro fluorescence-based assay. Briefly, 1 × 105 cells from spleens were stained with 1 μl (5 mM) of CFSE (Molecular Probes) in PBS and 5% FCS. FcRs were blocked by unlabeled anti-CD16/32 Abs (BD Biosciences), P. brasiliensis-soluble Ag (100 μg/ml), or Con A (1 μg/ml; Sigma-Aldrich). A minimum of 20,000 events was acquired on a FACSCalibur flow cytometer using CellQuest software (BD Pharmingen). The proliferation index (PI) was calculated as the mean fluorescence intensity (MFI) of unstimulated cultures/MFI of stimulated cultures.

**Statistical analysis**

Data were analyzed by Student’s t test or two-way ANOVA depending on the number of experimental groups. Values of p < 0.05 were considered significant.

**Results**

**TLR2 deficiency leads to less severe fungal infection of macrophages associated with decreased synthesis of NO, IL-10, and MCP-1**

Before performing fungicidal studies, we asked whether the initial interaction between *P. brasiliensis* yeast cells and peritoneal macrophages from TLR2−/− and WT mice was equivalent. Macrophage cultures (1 × 105/well), performed in round glass coverslips, were preactivated or not with IFN-γ (20 ng/ml) and infected with 2 × 104 viable yeast cells (1:50 fungus:macrophage ratio). After a 4-h incubation, supernatants were aspirated, the monolayers were gently washed with PBS, and the cells were stained with Giemsa. An average of 1000 macrophages was counted and the number of ingested and/or adherent yeast cells was determined. Compared with WT macrophages, a lower number of yeast cells associated (ingested/adhered) with TLR2−/− macrophages was observed (Fig. 1A). Peritoneal macrophages were cultivated with *P. brasiliensis* yeast cells for an additional 48-h period. Supernatants were removed and assayed for the presence of NO and cytokines and cell homogenates were plated for CFU determinations. As shown in Fig. 1B, the presence of functional TLR-2 led to increased recovery of viable yeast cells from untreated and IFN-γ-primed macrophages (20 ng/ml). In addition, higher levels of NO were produced by macrophages from WT mice than those of TLR2−/− mice (Fig. 1B). Additional experiments were performed with alveolar macrophages obtained from bronchoalveolar lavage fluids of normal, uninfected, WT, and TLR2-deficient mice (Fig. 1C). Lower CFU counts were recovered from untreated TLR2-deficient cells, although no significant differences were seen with IFN-γ-activated macrophages. As observed with peritoneal cells, TLR2-deficient alveolar macrophages synthesized lower levels of NO.

To better characterize the role of TLR2 in *P. brasiliensis* infection, culture supernatants of peritoneal macrophages were evaluated for the presence of some macrophage-activating cytokines (IL-12 and TNF-α), a deactivating cytokine (IL-10), and for a chemokine involved in mononuclear cell chemotaxis, MCP-1. As depicted in Fig. 2, IFN-γ-primed and untreated macrophages from TLR2−/− mice secreted decreased levels of IL-10 and MCP-1 than those from WT mice. IL-12 and TNF-α, however, appeared in similar levels.

**In vivo, absence of TLR2 induces lower fungal loads but increased lung pathology**

We infected groups (n = 6–8) of TLR2−/− and WT mice i.t. with 1 million *P. brasiliensis* yeast cells and evaluated parameters of infection and local inflammatory pathology over the course of infection. Diminished fungal burdens were detected in the lung tissue of TLR2−/− mice at all postinfection periods (48 h and 2 and 11 wk) assayed (Fig. 3A). These lower fungal burdens were accompanied by lower NO levels in lung homogenates (Fig. 3B). Equivalent fungal counts were detected in the liver and spleen tissue (our unpublished data).

To assess the influence of TLR-2 deficiency in disease outcome, the mortality of *P. brasiliensis*-infected TLR2−/− and WT mice was registered daily for a 250-day period and the median survival time was calculated for each strain. Unexpectedly, despite the significant differences in fungal burdens, no differences between mortality data (p = 0.104) were detected (Fig. 4A). The mean survival times of TLR-2−/− and WT mice were 221 and 190 days, respectively. To better understand this result, histopathological examination of lungs was done at weeks 2 and 11 of infection. As can be seen in Fig. 4, B–E, an equivalent and severe pattern of inflammatory reactions was detected in the lungs of both mouse strains. The pulmonary lesions replaced almost all of the normal parenchyma and were composed of confluent granulomas of various sizes containing many fungal cells surrounded by a small ring of mononuclear cells. Some lesions were also surrounded by an evident fibrotic layer. Multinucleated cells were scarcely seen. Lesions of TLR2-deficient mice showed a more prominent presence of inflammatory polymorphonuclear cells and decreased numbers of *P. brasiliensis* yeast cells (Fig. 4, D and E).

**TLR2−/− deficiency determines a sustained recruitment of PMN cells to the lungs**

To better characterize the inflammatory reaction at the site of infection, leukocyte recruitment to the lung tissue of *P. brasiliensis*-infected TLR2−/− and WT mice was studied at weeks 2 and 11 of infection. As can be seen in Fig. 5, A and B, at both postinfection
periods, increased numbers of polymorphonuclear cells (PMN) were observed in the lungs of TLR2−/− mice than in their normal controls. Consistent with these findings, 72 h after infection, significantly increased influx of PMN cells were detected in the bronchoalveolar lavage fluids of TLR2−/− mice (our unpublished data). At both postinfection periods, lower numbers of macrophages were recovered from lungs of TLR2-deficient mice, but only at week 2 was this difference significant. We further characterized the presence of neutrophil- and macrophage-mobilizing chemokines in the lung homogenates of WT and TLR2−/− mice. In good agreement with cell phenotypes detected at weeks 2 and 11 of infection, increased levels of KC were detected in

**FIGURE 1.** Macrophages (Mφ) from TLR2-deficient mice have a decreased ability to interact with fungal yeast cells. A. For phagocytic assays, IFN-γ-primed (20 ng/mL, overnight) and unprimed peritoneal macrophage cultures were infected with P. brasiliensis yeast cells in a macrophage:yeast ratio of 50:1. The cells were cocultivated for 4 h at 37°C in 5% CO2, to allow fungi adhesion and ingestion. Cells were washed, fixed, and stained with Giemsa; an average of 1000 macrophages was analyzed and the number of macrophages with adhered or ingested yeast cells was determined. B. For fungicidal assays, IFN-γ-primed and unprimed peritoneal macrophages were infected with yeast cells as above described. After 48 h at 37°C in 5% CO2, plates were centrifuged and supernatants were used to determine the levels of nitrite and cytokines. The monolayers were washed with distilled water to lyse macrophages and 100 μL of cell homogenates was assayed for the presence of viable yeast cells by a CFU assay. Supernatants from fungicidal assays were used to determine the levels of nitrites using the Griess reagent. C. Alveolar macrophages were collected from bronchoalveolar lavage fluids of normal mice and studied as in B for fungicidal activity and NO production. Data are the mean ± SEM of quintuplicate samples from one experiment representative of three independent determinations. *p < 0.05 between strains.

**FIGURE 2.** Macrophages (Mφ) from TLR2−/− mice secrete diminished levels of IL-10 and MCP-1. IFN-γ treated (20.00 ng/ml) or untreated macrophages of TLR2−/− and TLR2+/+ C57BL/6 mice were challenged with viable P. brasiliensis yeast cells (1:50, fungus:macrophage ratio) during 4 h, washed, and further cultivated for 48 h at 37°C in 5% CO2. Plates were then centrifuged and supernatants used for cytokine measurements using ELISA. Data are the means ± SEM of triplicate samples from one experiment representative of three independent determinations. *p < 0.05 between strains.
By days 2 and 6 after infection, PMN-depleted TLR2 deficient mice were in vivo depleted of PMN cells by a polyclonal Ab and infected i.t. with the fungus. The bars represent means ± SEM of log_{10} CFU obtained from groups of six to eight mice at 48 h and 2 and 11 wk after infection. The results are representative of three experiments. * p < 0.05 between strains.

**FIGURE 3.** In vivo, absence of TLR2 induces lower fungal loads and NO synthesis. Depicted is the recovery of CFU (A) and NO (B) from the lungs of TLR2^{−/−} and WT control mice infected i.t. with 1 × 10^{6} yeast cells. The bars represent means ± SEM of log_{10} CFU obtained from groups of six to eight mice at 48 h and 2 and 11 wk after infection. The results are representative of three experiments. **WT** and **TLR2^{−/−}**.

Depletion of PMN leukocytes increases fungal loads and mortality rates of TLR2-deficient but not normal mice

The finding that PMN cells were abundantly recruited to the lungs of TLR2^{−/−} mice in the presence of diminished fungal growth led us to verify whether these cells had a significant antifungal effector function in our model of pulmonary PCM. Thus, TLR2 deficiency resulted in increased KC production and PMN recruitment while in TLR2-normal mice a prevalent MCP-1 synthesis led to enhanced macrophage chemotaxis to the site of infection.

**FIGURE 4.** Despite the different fungal burdens, TLR2^{−/−} and TLR2^{+/+} mice present equivalent survival times and lung histopathology. A, Survival times of TLR2^{−/−} and WT control mice after i.t. infection with 1 × 10^{6} P. brasiliensis yeast cells was determined in a period of 250 days. No significant differences were seen in the median survival times of both mouse strains; the results are representative of two independent experiments. Photomicrographs of pulmonary lesions of WT (B and C) and TLR2^{−/−} (D and E) mice at week 11 of infection with 1 million P. brasiliensis yeast cells. At this period, despite the higher fungal loads detected in WT mice, no differences in the severity of lesions between TLR2-deficient and control mice were noted. Both mouse strains presented extensive and confluent lesions occupying almost all lung parenchyma which presented an elevated number of yeast cells. H&E (B and D; ×100) and Grocott-stained lesions (C and E; ×100).

TLR deficiency determines diminished T cell reactivity and influx to the lungs

We have further analyzed the phenotype and activity of lung inflammatory cells of TLR2^{−/−} and WT mice infected i.t. with 1 million P. brasiliensis yeast cells. To determine the activation profile of pulmonary macrophages, the expression of CD11b, MHC class II (I-A^k), CD80, CD86, and CD40 Ags was assayed by flow cytometry. As can be seen in Fig. 7, no differences in lung macrophages were detected at weeks 2 and 11 of infection. The phenotypic analysis of lymphocyte subsets was also performed. Thus, the expression of CD4, CD25, CD8, and CD69 molecules was studied in lung-infiltrating lymphocytes. As depicted in Fig. 7A, at week 2 of infection, no differences in the number and activation of T cells were observed. However, a decreased frequency of activated CD4^{+} T lymphocytes expressing CD25 (CD4^{+}CD25^{+}) was detected in TLR2^{−/−} animals at this period of infection (data not shown). By week 11, however, significantly decreased numbers of CD4^{+}, CD8^{+}, and CD8^{+}CD69^{+} T cells were detected in TLR2^{−/−} animals (Fig. 7B).

To characterize the lymphoproliferative activity of spleen cells obtained from WT and TLR2^{−/−} mice at week 4 of infection, CFSE-labeled lymphocytes were in vitro stimulated with P. brasiliensis Ag,
anti-CD3 plus anti-CD28 mAbs, or Con A and cultivated for 72 h. As depicted in Table I, compared with TLR2-deficient mice, control mice developed higher lymphoproliferative activity with all stimuli used, including the polyclonal T cell activator Con A. A similar result was detected with unstimulated control lymphocytes, probably due to the presence of yeast cells in the spleens of infected mice at this postinfection period. Together, these experiments demonstrated that TLR2-deficient mice mount a deficient T cell response as revealed by the diminished migration of T cells to the site of infection and the impaired lymphoproliferative activity.

FIGURE 5. TLR2−/− deficiency determines a sustained increased recruitment of PMN cells to the lungs. Number of leukocyte subsets (macrophages, PMN neutrophils, and lymphocytes) in the lung-infiltrating leukocytes (LIL) from TLR2−/− and WT mice inoculated i.t. with 1 million P. brasiliensis yeast cells. By 2 (A) and 11 (B) wk after infection, lungs of TLR2−/− and WT mice (n = 6–8) were excised, washed in PBS, minced, and digested enzymatically. Lung cells suspensions were centrifuged in the presence of 20% Percoll (Sigma-Aldrich) to separate leukocytes from cell debris. Cell suspensions were cytospun onto glass slides and stained by the Diff-Quik blood stain. Lung cell homogenates were also obtained after 2 and 11 wk of infection and analyzed by ELISA for the content of KC (C) and MCP-1 (D) chemokines. Data are expressed as means ± SEM. *, p < 0.05 between strains.

FIGURE 6. Fungicidal activity of PMN neutrophils. A and B, PMN leukocytes were in vivo depleted from WT and TLR2-deficient mice, control mice developed higher lymphoproliferative activity with all stimuli used, including the polyclonal T cell activator Con A. A similar result was detected with unstimulated control lymphocytes, probably due to the presence of yeast cells in the spleens of infected mice at this postinfection period. Together, these experiments demonstrated that TLR2-deficient mice mount a deficient T cell response as revealed by the diminished migration of T cells to the site of infection and the impaired lymphoproliferative activity.

A and B, PMN leukocytes were in vivo depleted from WT and TLR2−/− mice by i.p. administration of 0.5 ml of a 1/5 dilution of a rabbit anti-mouse PMN polyclonal Ab at days −1, +3, and +5 of i.t. infection (0 time) with 1 million P. brasiliensis yeast cells. Control mice received equivalent amounts of normal rat IgG. This treatment resulted in 75 and 87% of PMN depletion at days 0 and 6 of infection, respectively. IgG-treated and PMN-depleted mice (n = 4–6) were sacrificed at days 2 and 6 days after fungal infection and severity of disease evaluated by CFU counts in lung homogenates. C, Mortality data of PMN-depleted mice. Groups of mice (n = 8–9) were treated as above described and increased mortality was observed only in the PMN-depleted TLR2−/− group. Data were obtained from two independent experiments. D, Alveolar neutrophils were obtained by bronchoalveolar lavage of mice infected 2 wk earlier with 1 million yeast cells. The number of PMN leukocytes in the nonadherent fraction was adjusted to 2 × 105 cells, challenged with 1 × 105 yeast cells, and cocultivated for 1 h at 37°C. One hundred microliters of cell homogenates were assayed for the presence of viable yeast cells. All assays were done with three wells per condition in over two independent experiments. *, p < 0.05 compared with WT mice; #, p < 0.05 compared with IgG controls.
TLR2-deficient mice develop a Th17-skewed T cell response

Because the equilibrium between pro- and anti-inflammatory cytokines determines the efficiency and cellular composition of inflammatory reactions (30) and because recent investigations have demonstrated an increased presence of PMN cells associated with prevalent IL-17 secretion (31), cytokines associated with Th1 (IL-12, TNF-α, IL-2, and IFN-γ), Th2 (IL-4, IL-5, and IL-10), and Th17 (TGF-β, IL-6, IL-17, and IL-23) immunity were assessed in lung homogenates obtained at different periods of infection (Figs. 8 and 9A). However, 48 h after infection, at the innate phase of immunity, higher levels of TGF-β and IL-17 were detected in the lungs of TLR2-deficient mice. At week 2, this strain produced higher amounts of IL-6, TGF-β, IL-17, and IL-23. By week 11, TLR2−/− mice showed a sustained increase of IL-23 associated with lowered concentrations of IL-10 and IL-12. We have further defined the phenotype of IL-17-producing cells. As shown in Fig. 9B, after 72 h and 2 wk of P. brasiliensis infection, significantly increased numbers of IL-17+CD4+ T cells were detected in the lungs of TLR2−/− mice. No differences were observed in the total numbers of CD8+ T cells, although the frequency of this T cell subset was significantly augmented in TLR2-deficient mice. These findings demonstrate that TLR2 deficiency induces, since the early phase of pulmonary infection, a Th17-skewed immune response, without severely impairing Th1 and Th2 immunity.

TLR2 deficiency results in lower expansion of CD4+ CD25+FoxP3+ Tregs

In murine candidiasis, TLR2 deficiency was associated with decreased IL-10 production and deficient expansion of CD4+ Tregs (4, 32). In addition, differentiation of Tregs has an inhibitory effect on Th17 expansion (33, 34). Although Th17 cells and Tregs require TGF-β to their differentiation, the concomitant presence of some proinflammatory cytokines such as IL-1 or IL-6 results in preferential development of Th17 cells and impaired Treg expansion (34). These findings led us to ask whether the prevalent Th17 immunity developed by TLR2-deficient mice was associated with decreased expansion and recruitment of Tregs to the lungs. Thus, TLR2−/− and WT mice were sacrificed at weeks 2 and 11 after infection and the presence of CD4+CD25+FoxP3+ T cells was characterized by flow cytometry in the CD4+ subpopulation of lung-infiltrating lymphocytes. Surface staining of CD25+ and intracellular FoxP3+ expression was back-gated on the CD4+ T cell population. As can be seen in Fig. 10, no differences were detected at week 2 but, at week 11 a lower number of CD4+CD25+FoxP3+ T cells was observed in the lung inflammatory lymphocytes of TLR2-deficient than in WT mice.

Discussion

Recent studies have established the central role of TLRs in the innate immune recognition of a wide variety of microorganisms, including fungal pathogens (12, 15, 17). Since the contribution of TLR in P. brasiliensis infection was never investigated, the present report assessed the role of TLR2 in the innate and acquired phases of immunity to this pulmonary fungal pathogen. Data here presented demonstrate that TLR2 promotes fungal infection, but it has a concomitant beneficial effect to the host due to its inhibitory effect on the development of inflammatory reactions associated with prevalent Th17 immunity. Furthermore, it was also shown that TLR2 activation positively controls the expansion of Tregs, which modulate innate and adaptive immune cells and restrain lung inflammatory pathology.

Although TLR usually do not mediate fungal uptake (35), our in vitro studies suggested that P. brasiliensis yeast cells use TLR2 to interact with macrophages and to activate innate immune cells. P. brasiliensis yeast cells appear to be recognized by TLR2 molecules, resulting in increased adherence/ingestion and augmented production of NO and cytokines (IL-10 and MCP-1). Despite this increased activation, 48 h after infection an increased number of viable fungi were recovered from TLR2-normal cells. This finding...
appears to be paradoxical, since the killing ability of murine macrophages was previously reported to be associated with NO production by cytokine-stimulated phagocytes (36). Although the killing mechanisms were not here addressed, it appears that the increased NO secretion by TLR2-normal macrophages was not sufficient to abolish the increased fungal interaction mediated by TLR2 expression. Furthermore, the concomitant increase in IL-10 production could have inhibited the fungicidal ability of TLR2-normal macrophages. Along the same line, we could hypothesize that the lower IL-10 synthesis by TLR2/−/− macrophages would enhance IDO activity which would control P. brasiliensis growth and inhibit inducible NO synthase activity. This possibility is under investigation in our laboratory and would accommodate the lower fungal loads and NO production observed with TLR2/−/− macrophages (16, 30). Similarly to our results, TLR2-deficient macrophages exhibited an increased ability to contain another fungal pathogen, C. albicans, but this fact was not associated with different patterns of cytokine production (37).

The in vivo infection of TLR2-deficient mice resulted in decreased fungal burdens in all postinfection periods assayed. The less severe infection was always associated with low NO production. Again, the differences in fungal burdens could be explained by the different fungal interaction with alveolar macrophages at early phases of infection and not by differences in NO secretion. Forty-eight hours after infection, production of pulmonary cytokines was similar in both mouse strains, except for the augmented synthesis of TGF-β and IL-17 by TLR2-deficient mice. Interestingly, IL-17 is a cytokine involved with proinflammatory activity and increased PMN chemotaxis due to its ability to promote the synthesis of CXC chemokines (38–40). Consistent with the augmented IL-17 production, 72 h after infection increased PMN influx was detected in the alveolar spaces of TLR2/−/− mice (our unpublished data) and these cells could have efficiently controlled fungal growth but also sustained the synthesis of IL-23 and IL-17.

**FIGURE 8.** Type 1 and type 2 cytokines in lung homogenates of TLR2/−/− and WT mice. At 48 h and 2 and 11 wk after i.t. infection with 10⁶ yeast cells of P. brasiliensis, lungs from TLR2/−/− and WT control mice were collected and disrupted in 5.0 ml of PBS and supernatants were analyzed for cytokine content by capture ELISA. The bars depict means ± SEM of cytokine levels (6–8 animals per group). *, p < 0.05 between strains.  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TLR2/−/− (PI)</th>
<th>WT (PI)</th>
</tr>
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<tbody>
<tr>
<td>Control lymphocytes</td>
<td>0.78</td>
<td>2.82</td>
</tr>
<tr>
<td>Lymphocytes + AgPb</td>
<td>0.99</td>
<td>2.43</td>
</tr>
<tr>
<td>Lymphocytes + anti-CD28</td>
<td>0.66</td>
<td>2.66</td>
</tr>
<tr>
<td>Lymphocytes + Con A</td>
<td>1.3</td>
<td>6</td>
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* Spleen suspensions were labeled with CFSE and cultured for 3 days in the presence of anti-CD3 (0.3) Ab, anti-CD28 (2.5 µg/ml) Ab, P. brasiliensis-soluble Ag (100 µg/ml), or Con A (1 µg/ml). The intensity of CFSE was assessed by flow cytometry.

* The PI was calculated as the MFI of unstimulated cultures/MFI of stimulated cultures. Data are representative of two independent experiments.
Consistent with this possibility, lung homogenates of PMN-depleted TLR2−/− mice showed diminished levels of IL-23 (data not shown), suggesting that neutrophils could have a positive influence in the differentiation and expansion of Th17 cells. Although IL-17 production is usually associated with the Th17 subset of CD4+ T cells, this cytokine was also described to be produced by innate immune cells such as γδ T cells, dendritic cells, PMN cells, or NKT lymphocytes (41–45). In our model, CD4+ T cells were shown to be the main source of IL-17 because increased numbers of IL-17+CD4+ T cells were present in the lung inflammatory infiltrates of TLR2−/− mice since the early phase of infection. This fact led us to suppose that TLR2 signaling could impair interactions of macrophages with fungal agonists that preferentially induce cytokines associated with the Th17 pathway of T cell differentiation, a fact not previously described in PCM.

At week 2 of infection, TLR2-deficient mice produced low levels of MCP-1 associated with augmented synthesis of pulmonary TGF-β, IL-6, IL-17, and IL-23. This pattern of cytokine secretion points to a preferential activation of Th17 cells without affecting other Th1/Th2 cytokines. Indeed, Th17 cell development occurs in the presence of TGF-β and IL-6 and is maintained in the presence of IL-23, an IL-12-related cytokine (46, 47). Interestingly, all of those mediators involved in the development and maintenance of Th17 immunity were found in lung homogenates of TLR2-deficient mice and increased numbers of
Materials and Methods. The cell surface expression of leukocyte markers after infection cell suspensions were obtained and stained as described in Materials and Methods. The cell surface expression of leukocyte markers as well as intracellular FoxP3 expression in lung-infiltrating leukocytes were analyzed by flow cytometry. Surface staining of CD25 and intracellular FoxP3 expression were back-gated on the CD4+ T cell population. The data represent the mean ± SEM of the results from six to eight mice per group and are representative of two independent experiments. * p < 0.05 compared with WT mice.

IL-17+CD4+ T cells were found in the lungs of these mice after 72 h and 2 wk of 
P. brasiliensis infection. This Th17-biased immunity resulted in altered cellular influx to the site of infection. At weeks 2 and 11 of infection, increased production of KC, a CXC chemokine involved in PMN chemotaxis, and augmented influx of neutrophils to the lungs of TLR2-KO mice were detected; these findings are in agreement with previous reports showing increased participation of PMN leukocytes in inflammatory exudates governed by Th17 immunity (38–40, 48, 49). Consistent with our results, association of IL-17 synthesis and increased PMN influx to the site of infection was seen in bacterial pneumonia (50), inflammatory lung disorders (51), and other fungal infections (31, 52). In addition, the diminished presence of MCP-1, a chemokine chemoattract for macrophages, was associated with decreased numbers of these cells in the lung cell infiltrates of TLR2-deficient animals.

The Th17-induced PMN accumulation in inflammatory reactions can play antagonistic effects to the hosts, being protective due to its microbicidal ability or deleterious due to the release of tissue-damaging components (41). In our model, the higher number of lung-infiltrating PMN associated with Th17 immunity was responsible, at least partially, by the better fungal clearance observed in TLR2−/− mice. This was shown by in vivo depletion of PMN cells that led to increased fungal burdens only in TLR2−/− mice. The immunoprotective effect of neutrophils was also verified by the early mortality of PMN-depleted TLR2−/− mice. These data are consistent with previous findings in murine candidiasis describing a more severe systemic infection associated with deficient IL-17 production and impaired influx of neutrophils to infected organs (53) However, as described in other models of prevalent Th17 immunity, we believe that PMN cells exerted a dual effect in murine PCM. The protection granted by the increased influx and efficient fungicidal ability was probably accompanied by tissue damage caused by the release of protein-degrading enzymes such as neutrophil elastase, metalloproteinases, and other cytotoxic compounds as reactive oxygen species (31, 41, 50–54). Since equivalent fungicidal ability was detected in airway neutrophils from TLR2−/− and control mice, we believe that the increased number of PMN cells that migrate to the lungs and not the intrinsic activation of neutrophils was responsible for the control of fungal growth.

Despite the control of fungal growth, our data also suggest that Th17 immunity and PMN cells have also exerted a deleterious effect in TLR2−/− mice. Indeed, the diminished fungal burdens of TLR2−/− mice were concomitant with pulmonary lesions as severe as those displayed by WT mice. In both mouse strains nonorganized inflammatory reactions replaced almost all lung parenchyma, suggesting that high fungal loads (developed by WT animals) or prevalent Th17 immunity (mounted by TLR2−/− mice) have similar deleterious effects to P. brasiliensis-infected mice.

The phenotypic analysis of lymphocyte subsets performed after 2 and 11 wk of infection showed a decreased frequency of CD4+ and CD8+ T cells in TLR2−/− mice. This fact could be attributed to the lower fungal burden detected in this mouse strain or to the Th17-skewed immunity developed by the deficient animals. The early secretion of TGF-β and IL-6 by TLR2−/− mice appeared to have induced a precocious and prevalent Th17 immunity which resulted in diminished macrophage and T cell migration to the lungs. These cells were, however, partially replaced by PMN neutrophils which characterize Th17-mediated inflammatory exudates.

By week 11 of infection, TLR2−/− mice appear to maintain their prevalent Th17 immunity, whereas WT mice presented increased fungal burdens associated with augmented production of IL-10, IL-12, and significant expansion of Tregs. It can be supposed that these Tregs exerted an enhancing effect on fungal growth but an inhibitory activity on inflammatory reactions by down-modulating innate and adaptive immunity. Consistent with this interpretation, a recent work elegantly demonstrated the association of Tregs and increased fungal burdens in an experimental model of PCM using CXCR5 KO mice (55). The absence of TLR2 associated with decreased differentiation of Tregs here observed is also consistent with the increased secretion of TGF-β, which, associated with IL-6 (or other proinflammatory cytokine), could have inhibited FoxP3 production and expansion of Tregs (30).

In murine candidiasis, TLR2-deficient mice were shown to develop a more severe infection (56), a fact not subsequently confirmed (4, 16). The increased resistance of TLR2-deficient mice was associated with diminished IL-10 production and a decreased expansion of Tregs, which led to a more efficient Th1 immunity (4, 16). Similarly to the candidiasis model, PCM in TLR2−/− mice results in lower fungal loads and decreased expansion of Tregs. However, expression of TLR2 appears to be protective to pulmonary PCM because of its inhibitory activity on the development of Th17 immunity and tissue pathology. In addition, the decreased expansion of Tregs could have contributed to the maintenance of pathogenic inflammatory reactions.

The prevalent Th17 immunity developed by TLR2-deficient mice could be explained by the use of different pattern recognition receptors at the initial phase of infection. Other experimental models have implicated the use of Dectin-1 or TLR4 in the induction of Th17 immunity (57–59). Despite the initial pattern recognition receptor used, our results add a new mechanistic pathway which can lead to deleterious Th17 immunity in fungal infections in the absence of TLR2 signaling.

In summary, we have demonstrated for the first time that TLR2 are important innate receptors for P. brasiliensis which appears to use TLR2 as a virulence mechanism, which facilitates the access of fungal cells into macrophages. Most important, this is the
first study to demonstrate that TLR2 has a protective effect in pulmonary fungal infections due to its ability to deviate T cell responses from pathogenic Th17 immunity to a balanced Th1/Th2 response modulated by Tregs.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

References


