PTX3 function as an opsonin for the dectin-1-dependent internalization of zymosan by macrophages

S. N. Diniz,* R. Nomizo,† P. S. Cisalpino,‡ M. M. Teixeira,§ G. D. Brown,¶ A. Mantovani,|| S. Gordon,¶ L. F. L. Reis,*,† and A. A. M. Dias*,[1]

*Ludwig Institute for Cancer Research, São Paulo, Brazil; †Hospital do Câncer, AC Camargo, São Paulo, Brazil; Departments of ‡Microbiology and §Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Brazil; ¶Sir William Dunn School of Pathology, University of Oxford, United Kingdom; and ||Department of Immunology and Cell Biology, Instituto di Ricerche Farmacologiche Mario Negri and Institute of General Pathology, Faculty of Medicine, University of Milan, Italy

Abstract: Pentraxin 3 (PTX3) is a tumor necrosis factor and interleukin-1β-stimulated gene that encodes a long PTX with proinflammatory activity. Here, we show that peritoneal macrophages derived from PTX3 transgenic (Tg) mice express higher levels of PTX3 mRNA than macrophages from wild-type (WT) mice, at basal level as well as upon stimulation with zymosan (Zy). Macrophages from Tg mice also showed improved opsonin-independent phagocytosis of Zy particles and the yeast form of the fungus *Paracoccidioides brasiliensis*. In the case of *P. brasiliensis*, an enhanced microbicidal activity accompanied by higher production of nitric oxide was also observed in macrophages from Tg mice. Using fluorescein-activated cell sorter analysis and reverse transcriptase-polymerase chain reaction, we demonstrated that basal level of Toll-like receptor-6 and Zy-induced dectin-1 expression was slightly but consistently higher in macrophages from Tg mice than in macrophages from WT mice. Recombinant (r)PTX3 protein binds to Zy particles as well as to yeast cells of *P. brasiliensis* and addition of rPTX3, to a culture of WT-derived macrophages containing Zy leads to an increase in the phagocytic index, which parallels that of Tg-derived macrophages, demonstrating the opsonin-like activity of PTX3. It is important that blockade of dectin-1 receptor inhibited the phagocytosis of Zy particles by WT and PTX3 Tg macrophages, pointing out the relevant role of dectin-1 as the main receptor involved in Zy uptake. Our results provide evidence for a role of PTX3 as an important component of the innate-immune response and as part of the host mechanisms that control fungal recognition and phagocytosis. *J. Leukoc. Biol.* 75: 649–656; 2004.

Key Words: *pentraxin · TNF · TLR · P. brasiliensis · yeast*

INTRODUCTION

The "long pentraxins" constitute an emerging family of proteins that have a conserved pentraxin (PTX) domain homologous to the prototypical acute-phase short PTX (C-reactive protein and serum amyloid P component) in their carboxy-terminal halves and novel amino-terminal domains [1, 2]. The first long PTX identified, tumor necrosis factor (TNF)-stimulated gene 14 (TSG-14), also called PTX3, was originally isolated as differentially expressed in TNF-treated human fibroblasts [2] and later as an up-regulated gene from interleukin (IL)-1β-treated human umbilical vein endothelial cells [1]. PTX3 protein consists of a 10- to 20-subunit multimer produced by fibroblasts [2], endothelial cells [1], chondrocytes, synoviocytes [3], mononuclear phagocytes, and dendritic cells (DC) [4] after stimulation with primary inflammatory mediators such as TNF [2], IL-1β [1, 2], and lipopolysaccharide (LPS) [5] or components of the mycobacterial cell wall [6]. In macrophages, PTX3 expression is down-regulated by interferon-γ (IFN-γ) and up-regulated by LPS [7]. Although TNF and IL-1β are inducers of PTX3 expression in fibroblasts, these cytokines do not modulate PTX3 expression in peritoneal macrophages [7, 8]. Upon LPS injection, the PTX3 protein level rises in the serum with kinetics similar to the short PTX, C-reactive protein and serum amyloid P component [5, 9]. It was demonstrated that immobilized PTX3 protein binds to the C1q component of the complement and activates the classical complement pathway (CCP), a characteristic function of the classic short PTX [10]. Nevertheless, in the fluid-phase, PTX3 interaction with C1q inhibits the activation of the CCP by competitive blocking of the binding of C1q with immunoglobulins (Igs) [10]. Also, in contrast to classic, acute-phase proteins, PTX3 is not expressed in the liver, and its expression is not stimulated by IL-6 [5, 9].

To investigate the physiological function of PTX3 and its role in the inflammatory response in vivo, we generated transgenic (Tg) mice overexpressing the murine gene under the control of its own promoter. PTX3 Tg mice were found to be more resistant to the systemic administration of LPS and to sepsis caused by cecal ligation and puncture, and after acti-

[1] Correspondence: Ludwig Institute for Cancer Research, Rua Prof. Antônio Prudente, 109/4° Floor, São Paulo, Brazil, CEP 01509-010. E-mail: abalen@ludwig.org.br

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vation, their peritoneal macrophages produce larger amounts of nitric oxide (NO) than wild-type (WT) cells [11]. Moreover, PTX3 Tg mice have an increased death rate with an exacerbated inflammatory response induced by ischemia and reperfusion [12]. In agreement with this proinflammatory activity, PTX3 null mice exhibit high susceptibility to invasive pulmonary aspergillosis associated with defective recognition of conidia by alveolar macrophages and DC [13]. It was suggested that PTX3 acts as a secreted pattern-recognition receptor that has a nonredundant role in resistance to selected microbial agents, in particular to the opportunistic fungal pathogen *Aspergillus fumigatus* [13, 14]. In addition to the alterations in the immune response, female PTX3 null mice show impaired fertility [13, 15].

Macrophages are important players in innate immunity against pathogenic fungi and bacteria. To determine the impact of PTX3 overexpression in macrophage functions, we investigated the phagocytic capacity of peritoneal macrophages from PTX3 Tg mice. Here, we provide evidence for a role of PTX3 as an important component of the opsonin-independent phagocytosis of zymosan (Zy) A and the yeast cell form of the fungal pathogen *Paracoccidioides brasiliensis*. Macrophages from PTX3 Tg mice have an improved phagocytosis activity. It is important that recombinant (r)PTX3 binds to Zy and *P. brasiliensis* and functions as an opsonin, by increasing the phagocytic index (PI) of WT-derived macrophages to levels comparable with those of Tg-derived macrophages. Our results provide evidences for a role of PTX3 in the opsonization of yeast, leading to macrophage recognition, phagocytosis, and fungicidal responses.

MATERIALS AND METHODS

Animals

The generation and initial characterization of PTX3 Tg mice (CD1 background) were described earlier [11]. Two strains of Tg mice having two (Tg2 line) or four (Tg4 line) extra copies of the murine *PTX3* gene under the control of its own promoter in heterozygosity were used in this study. Mice were housed in a clean, temperature-controlled room and received sterile food and acidic water ad libitum. For all experiments, mice were used at 8–10 weeks of age, and WT controls had the same background as the mutant line (CD1).

Peritoneal macrophages

Resident peritoneal macrophages (RMø) and thioglycollate-elicited peritoneal macrophages (EMø; 5 days after 2 ml intraperitoneal injection) were isolated from male or female CD1 WT and PTX3 Tg mice. Cells were harvested from the peritoneal cavity with ice-cold phosphate-buffered saline (PBS) and washed twice with PBS, and their viability was evaluated by trypan blue dye exclusion. Cells were cultured in RPMI 160 supplemented with 10% heat-inactivated fetal bovine serum and 50 mg/ml gentamycin sulfate (R10 medium) at 37°C in 5% CO_2. After 60 min of incubation, media and nonadherent cells were removed by aspiration, and fresh R10 medium was added. In all experiments, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay evaluated macrophage viability and cell density [16]. Briefly, MTT (Sigma Chemical Co., St. Louis, MO) was added to the cultures to a final concentration of 500 μg/ml and was incubated for 4 h at 37°C in 5% CO_2. The medium was removed, and MTT–formazan was solubilized by adding 10% sodium dodecyl sulfate (SDS) prepared in dimethylformamide/H_2O (1:1 v/v) and measured spectrophotometrically at 550 nm.

Phagocytosis of Zy particles

Pools of RMø and EMø (3×10^5/well) from WT or PTX3 Tg mice (n=4 mice per group) were cultured in an eight-well LabTek chamber (Nunc, Rochester, NY, Cat. No. 177402) for 1 h at 37°C in 5% CO_2. Nonopsonized or opsonized Zy A particles (Sigma Chemical Co.) were added to the culture at a ratio of five particles per cell. Opsonized Zy was obtained by incubating the particles for 30 min at 37°C with normal murine serum. After 2 h of incubation, the wells were washed several times with sterile PBS, fixed with cold absolute methanol, and stained with giemsa (Sigma Chemical Co.). The PI was calculated as PI = PMø × nZy, where PMø is the percentage of phagocytic macrophages and nZy is the average number of Zy particles per macrophage. Experiments were done in triplicate, and in each one, 200 cells were analyzed for each parameter in at least five different fields. For inhibition assays, laminarin (100 μg/ml; Sigma Chemical Co.), 2A11 antidectin-1 rat antibodies [17], and control rat serum (100 μg/ml) were incubated for 20 min and 1 h at 4°C, respectively, before the addition of Zy. WT-derived macrophages were treated with 20 μg/ml of a human rPTX3 protein [18] just before the addition of Zy. PTX3 was expressed in Chinese hamster ovary (CHO) cells and purified under endotoxin-free conditions by immunoaffinity [18]. Purified PTX3 was checked for purity by SDS-polyacrylamide gel electrophoresis and for LPS contamination by *Limulus amebocyte* lysate assay (BioWhittaker, Walkersville, MD) [13].

Phagocytosis and fungicidal activity

Pools of RMø isolated from WT and PTX3 Tg mice (n=4 animals per group) were plated in an eight-well LabTek (Nunc; for phagocytosis assays) or in a six-well plate (Nunc; for fungicidal assays) at a density of 3×10^5 and 3×10^6 cells/well, respectively. Adherent macrophages were kept untreated or treated with 1000 U/ml of recombinant murine IFN-γ (R & D Systems, Minneapolis, MN) for 16 h followed by stimulation with 10 ng/ml LPS for 1 h at 37°C in 5% CO_2.

P. brasiliensis, virulent strain AP [19], was cultured on yeast extract, casein peptone, D-glucose, and agar medium. Before use, yeast cells were washed three times with PBS and gently agitated, and larger cells were allowed to sediment for 5 min. The supernatant, containing small cells, was harvested. Trypan blue dye exclusion assessed the viability of the fungal cells. *P. brasiliensis* were mixed with macrophages at a ratio of one fungus for five cells [20].

The RMø/*P. brasiliensis* cultures were incubated at 37°C in 5% CO_2 for 6 h (phagocytosis test) or for 24 h (NO production and fungicidal test). LabTek cultures were washed with PBS, fixed with methanol, and stained with giemsa. PI was calculated using the formula already described. After 24 h of incubation, nitrite (NO_2^-) concentration in the culture supernatants was determined as a measure that reflects NO production according to the Griess method [21]. Fungicidal activity was determined by plating 100 fungal units in brain infusion heart medium agar supplemented with 4% fetal bovine serum, 5% of *P. brasiliensis*-conditioned media, and 40 mg/ml gentamicin (Sigma Chemical Co.) recovered from the cell lysate. Colony-forming units (CFU) were determined after 7–10 days of incubation at 35°C [20].

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from resident peritoneal cells and adherent macrophages treated with Zy using Trizol reagent (Invitrogen, Carlsbad, CA) according the manufacturer's instructions. For RT-PCR, aliquots of 1 μg total RNA were reverse-transcribed using oligo dT (Amersham Pharmacia, Little Chalfont, UK) and Superscript II RT (Invitrogen). Amplification was performed for 18 cycles [Toll-like receptor-2 (TLR2) and dectin-1] and 25 cycles (TLR6 and PTX3), and the cDNA corresponding to hypoxanthineguanine phosphoribosyl transferase (HGPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were amplified from the same samples as references. The number of cycles for each amplification was determined in separate experiments to avoid saturation. RT-PCR products were fractionated through agarose gel, blotted onto nylon membranes, and hybridized [22] with $^{32}P\alpha$-deoxycytidine 5′-triphosphte (dCTP)-labeled, specific probes corresponding to TLR2, TLR-6, dectin-1, or PTX3 genes, cloned with primers described below. The primers used for PCR amplification were: TLR2 gene 5′-CCTGTTGATCTTGCTCGTAGGTGCC-3′ and 5′-TTCATCCAAGGGCCACTCCAGGTAG-3′; TLR6 gene 5′-CTTAATAGTCGGAAGCATGACCCCG-3′ and 5′-AAGGTTGGACCTCTGGTGAGTTCTG-3′; dectin-1 gene 5′-AGGCCCTATGAAGAACTACAGACA-3′ and 5′-TGGCCAGACAGCATAAGGAA-3′; PTX3 gene 5′-CCTGCTTTGTGCTCTCTGGT-3′ and 5′-TCTCCAGCATGATGAACAGC-3′; HGPRT gene 5′-ATATAATTGACACTGGTAAAAC-3′ and 5′-GTATCAACT-

TGCGCTCATCTTAGG-3′; and GAPDH gene 5′-TTGTGGAAGGGCTCAT-GACCA-3′ and 5′-CGTATTCATTGTCATACCAGG-3′.

Expression of dectin-1 protein on macrophages from WT and PTX3 Tg mice

To measure the levels of dectin-1 protein produced by macrophages, cells derived from WT or PTX3 Tg mice were incubated for 2 h at 37ºC with five Zy particles per cell. After incubation, cells were harvested with the aid of a cell scraper, washed twice with PBS, and permeabilized with IntraPrep (Immunotech, Marseille, France) following the manufacturer's instructions. Cells were then incubated with 1 μg/ml monoclonal rat anti-mouse dectin-1 IgG2b antibody (2A11) [17] or with control rat normal serum for 1 h at 4°C. After incubation, cells were washed three times and incubated with rabbit anti-rat IgG fluorescein isothiocyanate (FITC) conjugate (1:100; Sigma Chemical Co.) for 30 mim at 4°C.

Analyses were performed using a FACScan cytometer (BD Biosciences, San Jose, CA), and data acquisition from 10,000 cells were performed by the Consort 32 system, Lysis II software (BD Biosciences). Mean fluorescence value (MFV) of 2A11-stained cells was obtained by subtracting the fluorescence from control normal serum.

Binding rPTX3 protein to Zy and *P. brasiliensis*

Zy particles (1 mg), *P. brasiliensis* (10^6), or *Escherichia coli* (10^8) were washed twice in PBS (pH 7.0) with calcium (1 mM) and magnesium (0.7 mM), resuspended in 100 μl RPMI containing biotin-labeled PTX3 [13] (100 μg/ml), and incubated at room temperature for 30 min. After washing twice with excess PBS, the pellet was resuspendend in 100 μl PBS containing FITC-conjugated streptavidin (Calbiochem, San Diego, CA; 1 μl) and incubated for 30 min at 4°C. The samples were then washed twice and resuspended in 500 μl PBS containing 1% formaldehyde (pH 7.0) and analyzed in a BD Biosciences FACScan.

RESULTS

Macrophages from PTX3 Tg mice express augmented levels of PTX3 mRNA

We described earlier that expression of the mouse PTX3 Tg in our Tg2 and Tg4 lines appeared to be tightly regulated by its own promoter, and no leakage was observed in the lungs, heart, or liver of Tg mice, even after stimulation with LPS [11]. To characterize the impact of PTX3 overexpression on macrophage functions, we first determined the levels of PTX3 mRNA in macrophages and its induction by Zy. As observed in **Figure 1**, macrophages from PTX3 Tg mice have higher basal levels of PTX3 mRNA, which remains higher than that of macrophages from WT mice throughout all time points.

Enhanced phagocytosis of Zy by macrophages from PTX3 Tg mice

RMø and EMø from WT and Tg mice were used to determine their PI when incubated with nonopsonized or opsonized Zy particles. As observed in **Figure 2**, when nonopsonized Zy was used, the PI for Tg-derived macrophages was higher for RMø (Tg2=265.2; Tg4=265.75; Fig. 2A) or EMø (Tg2=337; Tg4=296; Fig. 2B) than those for WT-derived macrophages (112.3 and 155.85). However, when serum-opsonized Zy particles were used, no statistically significant differences were observed between macrophages from Tg and WT mice (Fig. 2, A and B).

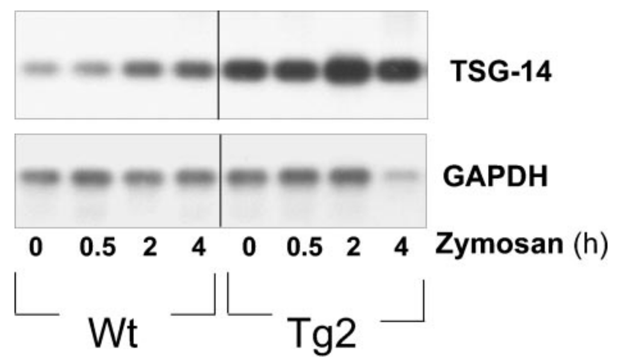

Fig. 1. Analysis of PTX3 mRNA expression by macrophages from WT and PTX3 Tg mice after Zy stimulation. RMø isolated from WT or PTX3 Tg2 mice were cultured in the absence or in the presence of Zy (five particles to one cell) for the indicated period of time. Total RNA was extracted, and PTX3 mRNA expression was evaluated by RT-PCR. PCR products were fractionated through a 1.2% agarose gel, blotted onto nylon membranes, and hybridized with a $^{32}P\alpha$-dCTP-labeled, specific probe corresponding to PTX3 and GAPDH cloned with primers described in Materials and Methods.

Efficient phagocytosis and killing of *P. brasiliensis* by PTX3 Tg macrophages

To further verify the effect of PTX3 overexpression in the antifungal response, we evaluated the phagocytosis of *P. brasiliensis* by RMø from WT and PTX3 Tg2 mice. Our results demonstrate that as in the case of Zy, macrophages derived from Tg mice have a higher PI ($P<0.05$) as compared with WT-derived macrophages (**Fig. 3A**). Moreover, macrophages from Tg mice showed an increased fungicidal activity leading to a significant reduction in the number of CFU recovered after 24 h as compared with the number of CFU recovered from cultures of WT-derived macrophages (Fig. 3B). It is interesting that this increased fungicidal activity is paralleled by an augmented production of NO (Fig. 3C). Treatment of Tg- or WT-derived macrophages with IFN-γ and/or LPS abolished the differences, and both groups of macrophages were equally efficient in phagocytosis, killing, and NO production. (Fig. 3, A–C).

Effects of Zy on the expression of TLRs and dectin-1

Exposure of macrophages to components of yeast cell walls activates signaling pathways triggering the inflammatory response [23, 24]. As a result of the ascribed role of TLR2, TLR6, and dectin-1 receptors in the recognition of Zy particles by macrophages [17, 25–27], we determined the levels of these receptors in RMø from WT and PTX3 Tg after exposure to Zy. For TLR2, no difference in the basal levels of mRNA was observed (**Fig. 4A**). Similarly, TLR2 mRNA decreased after 4 h of treatment with Zy to a comparable extent in WT and PTX3 Tg mice (Fig. 4A). When we measured the levels of dectin-1, we observed a small but reproducible difference in the Zy-induced expression, and Tg mice showed higher levels than WT mice (Fig. 4A). For TLR6, we again observed a discreet but reproducible difference in the basal levels of mRNA, and macrophages from Tg mice had higher levels than WT mice (Fig. 4B). The increased expression of dectin-1

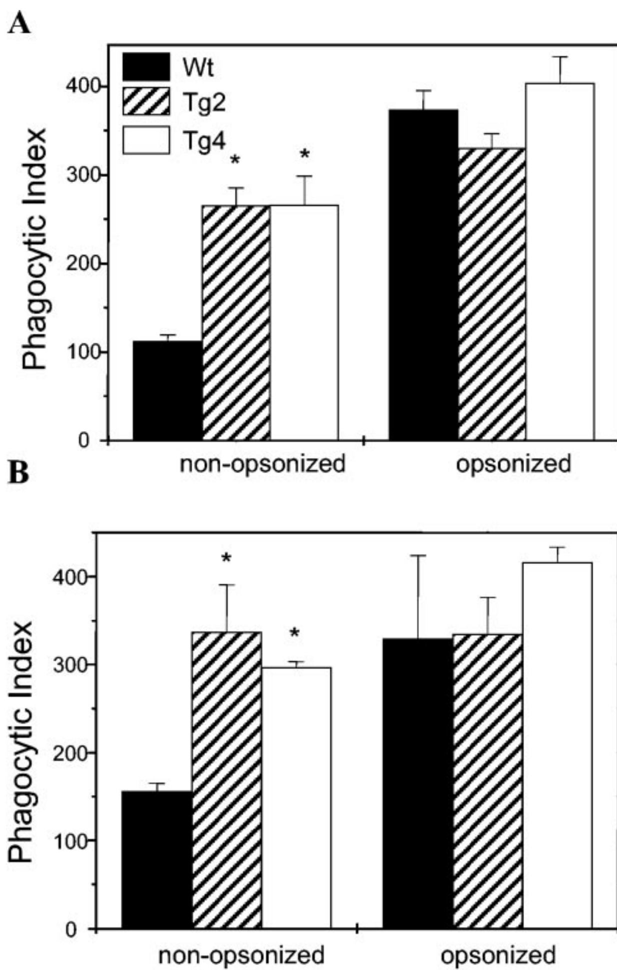

Fig. 2. Phagocytosis of Zy in vitro by macrophages derived from WT and PTX3 Tg mice. RMø (A) and EMø (B) from WT and PTX3 Tg2 or Tg4 mice were cultured in the presence of nonopsonized or opsonized Zy (five particles/cell) for 4 h. PI was calculated as described in Materials and Methods. *, $P < 0.05$, for comparisons between WT and Tg mice. Data represent the average value of four independent measurements, and error bars denote SD.

mRNA by PTX3 Tg macrophages could also be confirmed by fluorescein-activated cell sorter (FACS) analysis as indicated by the comparison of the observed MFV (**Fig. 5**). It is interesting that the augmented expression of dectin-1 was more pronounced when we performed intracellular staining (Fig. 5).

rPTX3 binds specifically to Zy particles and to yeast *P. brasiliensis*

It was reported by Garlanda and co-workers [13] that recombinant human PTX3 (rhPTX3) could bind conidia from *A. fumigatus* as well as *Pseudomonas aeruginosa* and *Salmonella typhimurium*. To determine whether differences observed in phagocytosis could be explained by the binding of PTX3 to Zy and *P. brasiliensis* during our assays, Zy particles, *E. coli*, and *P. brasiliensis* were incubated with biotinylated CHO-derived rhPTX3 [13]. By FACS analysis, we observed a specific binding of PTX3 to Zy particles and yeast cells of *P. brasiliensis* (**Fig. 6, A** and **B**). However, PTX3 failed to bind to *E. coli* (Fig. 6C).

rPTX3 enhances phagocytosis of Zy by WT macrophages, and dectin-1 is the main receptor involved

Having observed that PTX3 could bind to Zy and *P. brasiliensis*, we next asked whether addition of exogenous PTX3 would function as an opsonin, enhancing phagocytosis of macrophages derived from WT mice. Macrophages derived from WT or Tg mice were incubated with Zy in the presence or absence of rhPTX3, and the PI was determined (**Fig. 7A**). The addition of rPTX3 enhanced uptake of Zy by WT cells, increasing the PI from 84.6 to 199.6 (P value <0.05) comparable with that of Tg-derived macrophages not treated with exogenous PTX3.

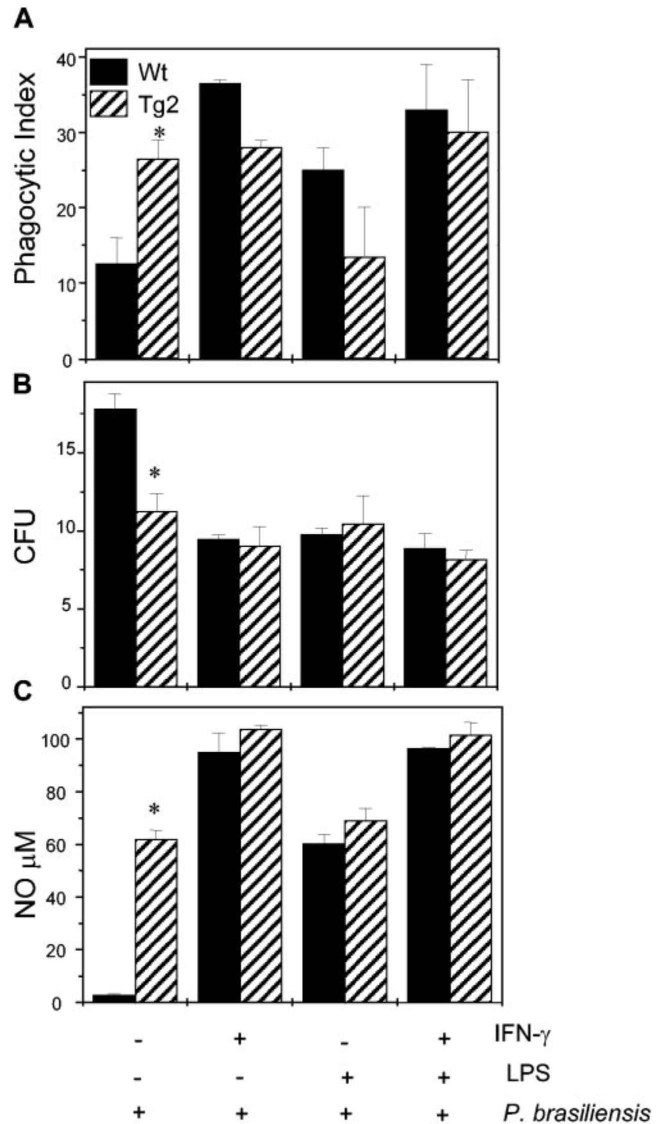

Fig. 3. Macrophages response to *P. brasiliensis*. RMø were recovered from WT or PTX3 Tg mice and incubated with yeast cells of *P. brasiliensis* at a ratio of one fungal unit per five macrophages. PI (A), killing (CFU; B), and NO production (C) by unstimulated (–) or IFN-γ- and/or LPS-stimulated (+) macrophages from WT and PTX3 Tg2 mice were determined. CFU was determined by plating 100 fungal units from each treatment. NO was determined by the Griess method as described in Materials and Methods. *, $P < 0.05$, for comparisons between WT and Tg mice. Bars denote the average of two independent measurements, and error bars denote SD.

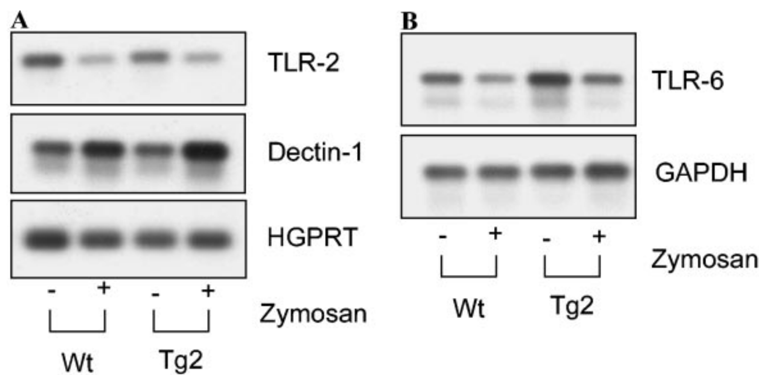

Fig. 4. Expression of TLR2, TLR6, and dectin-1 in macrophages from WT and PTX3 Tg mice after Zy stimulation. RMø derived from WT and PTX3 Tg2 mice were kept untreated (–) or were stimulated for 4 h with nonopsonized Zy (+). Total RNA was isolated, and 1 μg was reverse-transcribed using oligo dT primers. PCR was performed for 18 cycles (TLR2 and dectin-1; A) or 25 cycles (TLR6; B). Amplified products were fractionated through a 1.2% agarose gel, blotted onto nylon membranes, and hybridized with $^{32}P\alpha$-dCTP-labeled, specific probes. HGPRT and GAPDH probes were used as loading control.

When Tg-derived macrophages were used, addition of rhPTX3 did not alter the PI. Phagocytosis of Zy in the presence or in the absence of rhPTX3 was dramatically inhibited by antidectin-1 antibody as well as by laminarin, a soluble form of β-glucan (Fig. 7A).

The increased PI obtained by addition of exogenous PTX3 seems to be a result of two additive pathways. First, in the presence of rhPTX3, the number of Zy particles internalized by macrophages from WT mice is significantly higher than that observed in the absence of rhPTX3 (*P* value=0.0001; Fig. 7B). Second, addition of exogenous PTX3 also increased the number of macrophages from WT mice that internalized Zy particles (*P* value=0.0013; Fig. 7B; black and gray bars). Nevertheless, the number of Tg-derived macrophages that internalized Zy was still higher than that of WT mice, even after exposure to rhPTX3 (*P* value=0.00085; Fig. 7C; gray and hatched bars). In Figure 7D, there is a representative photomicrograph of the internalization of Zy by macrophages from WT mice treated or not with rhPTX3 and by untreated macrophages from Tg mice.

DISCUSSION

The antimicrobial host-immune response relies on innate and adaptive mechanisms [28]. Macrophages are major players of the innate response, and they not only phagocytose and kill the pathogens but also coordinate additional host responses by synthesizing inflammatory mediators and cytokines that in turn modulate the adaptive response [29]. The phagocytosis of pathogens by macrophages is based on the recognition of pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents but not on the host cells. Hence, molecules that are involved in the recognition of PAMPs have a central role in mediating innate-immune response.

A large body of evidence in different animal and human models of disease now supports the recognition of PTX3 as a central molecule in the inflammatory response. It has been demonstrated that PTX3 has an important role in defense against bacterial [11] and fungal infection [13] as well as in the extent of tissue damage by noninfectious agents [12, 30]. Here, we demonstrate that PTX3 expression is up-regulated by Zy

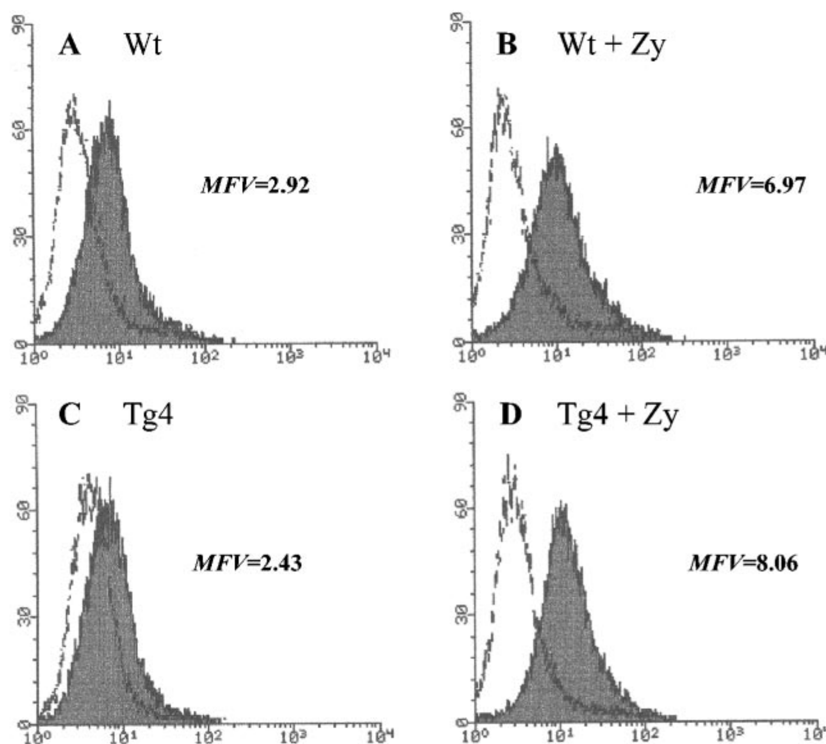

Figure 5. Detection of dectin-1 protein expression on macrophages after Zy stimulation. RMø derived from WT (A and B) or PTX3 Tg4 (C and D) mice were kept untreated (A and C) or were stimulated with nonopsonized Zy (five particles/cell; B and D) for 4 h. Cells were permeabilized with IntraPrep and then incubated with antidectin-1 monoclonal antibody (2A11; see Materials and Methods) or with normal rat serum for 1 h. Cells were washed and incubated with rabbit anti-rat IgG conjugated to FITC. Open curves show fluorescence with control serum, and MFV of 2A11-stained cells was obtained by subtracting the fluorescence from control normal serum.

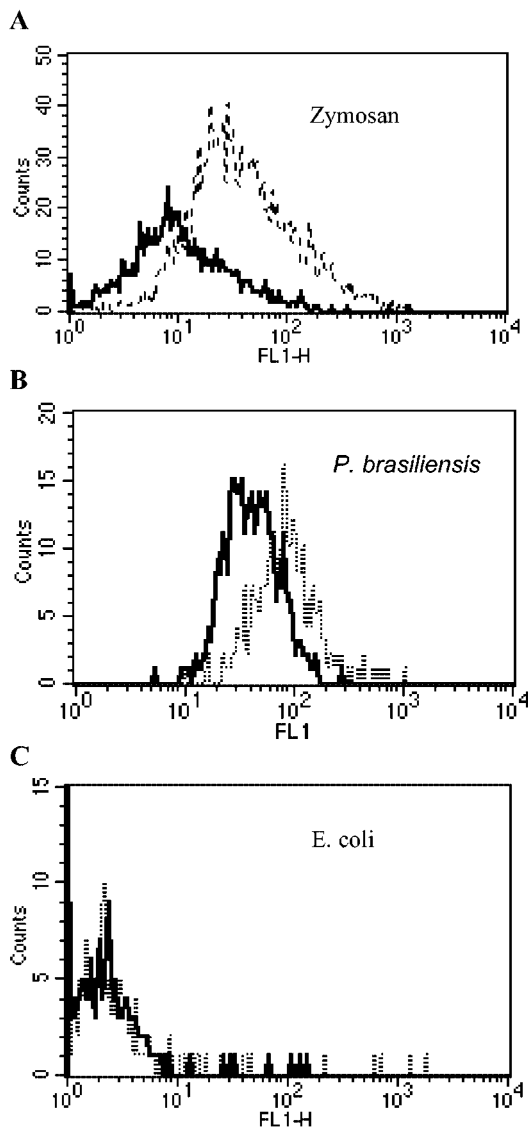

Fig. 6. Binding of rPTX3 to Zy and *P. brasiliensis*. Biotin-labeled rhPTX3 (100 μg/ml) was incubated with Zy (A), *P. brasiliensis* (B), and *E. coli* (C) for 30 min at room temperature. After washing and incubation with FITC-conjugated streptavidin, Zy, *E. coli*, or yeast cells were submitted to FACScan analysis. Solid line curves represent control fluorescence in the absence of PTX3.

and plays a critical role in phagocytosis as well as in killing of intracellular pathogens, by functioning as an opsonin, and by enhancing production of NO.

Zy, a yeast-derived particle composed mainly of polysaccharides, of which β-glucan and mannan are the major constituents [31], is known as a powerful activator of macrophages stimulating their phagocytic, cytotoxic, and antimicrobial activities, including NO production (reviewed in ref. [24]). The interaction of Zy with macrophages is mediated primarily by dectin-1 [17, 25]. Zy also interacts and activates TLR2, and such interaction leads to activation of a TLR2-mediated signaling cascade and of nuclear factor (NF)-κB [32, 33]. Our data show that in the presence of Zy, macrophages expressed higher levels of PTX3 mRNA (Fig. 1), which is in agreement with the notion that PTX3 gene expression is mediated via NF-κB [34]. As macrophages from PTX3 Tg mice showed higher efficiency in internalizing Zy (Fig. 2) as well as *P. brasiliensis* (Fig. 3) in an opsonin-independent manner, it is possible that during natural infection by the fungus, the gene is activated, and secreted PTX3 would make a positive loop enhancing the clearance of the pathogen. In the case of *P. brasiliensis*, we also found that macrophages from PTX3 Tg mice are more efficient

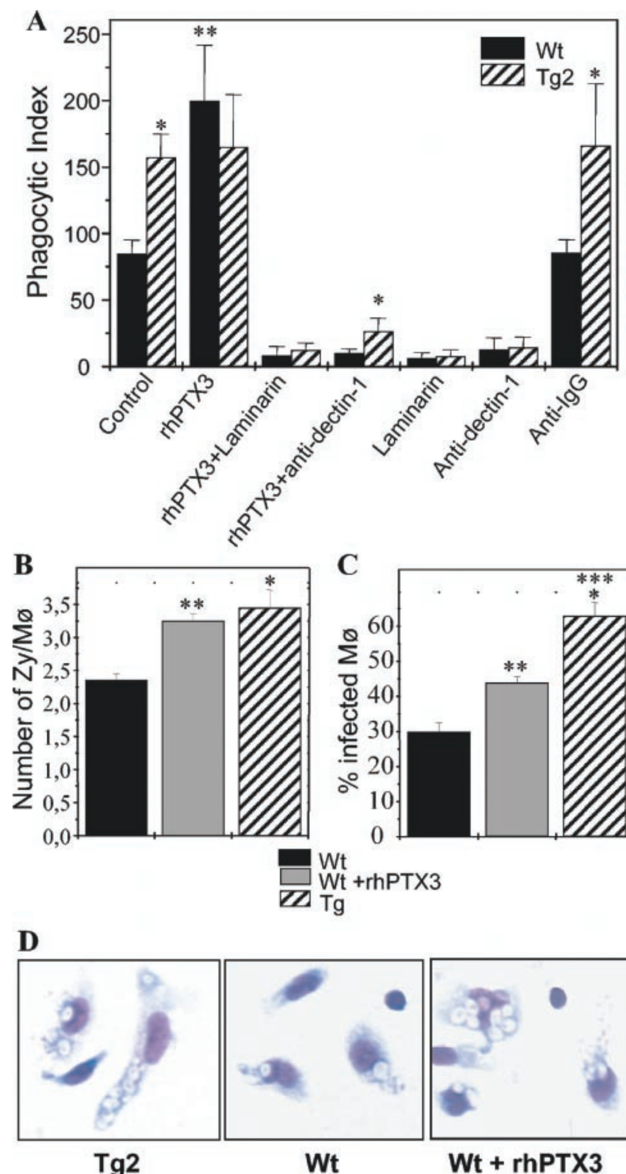

Fig. 7. Uptake of PTX3-opsonized Zy is primarily mediated by dectin-1. Macrophages from WT and PTX3 Tg mice were pretreated with laminarin (20 min), antidectin-1 antibody (1 h), or rat IgG2b isotype (1 h) at 4°C. rhPTX3 (20 μg/ml) and Zy (five particles/cell) were added to the culture and incubated for 1 h at 4°C followed by 20 min at 37°C for internalization. Cells were washed with PBS, fixed, and stained with giemsa. (A) PI for the indicated cultures; (B) average number of Zy particles per macrophages; (C) percentage of infected macrophages. Results are the average of three independent pools of macrophages derived from four mice in each pool. *, $P < 0.05$, for Tg × WT comparison; **, $P < 0.05$, for WT × PTX3-treated WT; ***, $P < 0.05$, for Tg × PTX3-treated WT. (D) Representative fields of giemsa-stained cells from Tg2-derived macrophages (left panel) or WT-derived macrophages untreated (center panel) or treated with rhPTX3 (right panel) in light microscopy (original magnification, 400×).

in killing, as revealed by the reduced number of CFU recovered from these macrophages (Fig. 3B), a phenomenon that could be related to the higher production of NO from these cells (Fig. 3C) [35]. This observation is in agreement with the hypothesis that PTX3 plays an important role in antifungal activity [13].

The observation that macrophages from PTX3 Tg mice showed higher PI than the WT controls prompted us to further investigate the mechanism of Zy internalization and how PTX3 would influence such a mechanism. As mentioned earlier, β-glucan and mannan are the major components of Zy [31], and the interaction of this particle with macrophages is mediated primarily by dectin-1 but also involves other cellular receptors such as TLR2 and TLR6 (reviewed in ref. [24]). As shown in Figures 4 and 5, Zy modulates the expression of these receptors. Whereas TLR2 and TLR6 mRNAs are down-regulated, dectin-1 mRNA is augmented upon stimulation of macrophages with Zy. As Zy acts via TLR2 and TLR6 triggers cytokine and chemokine production [26, 36], down-regulation of TLR2 and TLR6 by Zy could function as an important negative feedback to avoid exacerbation of the inflammatory reaction. Conversely, the up-regulation of dectin-1 could be critical to a more efficient clearance of the pathogen.

As demonstrated by Garlanda and co-workers [13], PTX3 recognizes PAMPs in *A. fumigatus* conidia and in some enterobacteriaceae. It is important that these authors demonstrated that addition of rPTX3 could augment conidial internalization by WT-derived macrophages and more relevant, completely restored this activity by otherwise incapable PTX3$^{-/-}$ -derived macrophages. Similarly, we showed here that rhPTX3 also binds to Zy and to a lesser extent, to *P. brasiliensis* but not to *E. coli* (Fig. 6) and likewise, improved the PI of WT-derived macrophages to the levels of that obtained with macrophages derived from PTX3 Tg mice.

What is not clear is whether PTX3 acts as an opsonin or modulates cytokine gene expression [11–13] via a specific cellular receptor. To determine the mechanism by which PTX3 improves internalization of Zy, we took advantage of an anti-dectin-1 antibody as well as of laminarin, a short β-glucan, and determined the impact of these reagents on the PI from macrophages derived from WT or PTX3 Tg mice. Our data demonstrated that PTX3 acts as an opsonin, but internalization of PTX3-opsonized Zy is dependent on dectin-1 as demonstrated by the inhibitory effect of antidectin 1 antibody and by laminarin. This observation indicates that even if a PTX3-specific cellular receptor does exist, the opsonin-like activity of PTX3 is not dependent on its receptor.

Based on the evidence available in the literature and presented here, we propose a model for the opsonin-like activity of PTX3 (**Fig. 8**). It has been shown that PTX3 forms multimers corresponding to decamers [18]; we demonstrated here that PTX3 binds to Zy. Based on this, we hypothesize that PTX3 leads to the aggregation of Zy, presenting higher numbers of particles to activated macrophages. Based on the data presented in Figures 4 and 5, the presence of PTX3 also leads to a rather modest but consistent increase of dectin-1 at the mRNA and protein levels. Hence, in the presence of PTX3, macrophages would have a higher number of cell-surface receptors to internalize Zy that are presented at higher concentrations as aggregates.

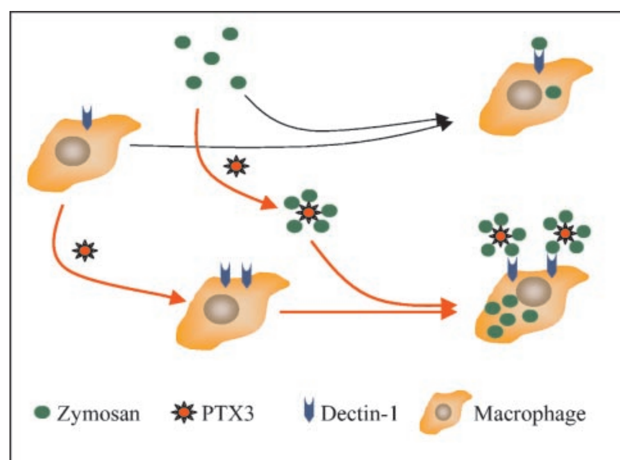

Fig. 8. A model for the opsonin-like activity of PTX3: In the absence of PTX3, macrophages do internalize Zy in a dectin-dependent manner (as reviewed in ref. 24 and corroborated by data from Figure 7A). In the presence of PTX3, particles to be internalized are aggregated by the binding of monomeric units of PTX3 to individual particles, leading to the internalization of a higher number of particles per each individual macrophage. At the same time, macrophages exposed to PTX3 appear to express higher levels of dectin-1, as suggested by data from Figures 4 and 7C. This latter activity of PTX3 would imply the existence of an as-yet unidentified PTX3-specific cellular receptor, and moreover, augmented expression of dectin-1 might be an indirect effect of PTX3. These two pathways would lead to an improvement of the phagocytic capacity of macrophages.

Altogether, our data corroborate previous results pointing to a pivotal role of PTX3 as a mediator of the innate-immune response during fungal infection and further substantiate its therapeutic potential.

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