Role of complement and Fcγ receptors in the protective activity of the long pentraxin PTX3 against Aspergillus fumigatus

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Running title: PTX3-FcγR-CR3 axis in Aspergillus phagocytosis
Abstract

PTX3 is a soluble pattern recognition molecule playing a non-redundant role in resistance against *Aspergillus fumigatus*. The present study was designed to investigate the molecular pathways involved in the opsonic activity of PTX3. The PTX3 N-terminal domain was responsible of conidia recognition, but the full-length molecule was necessary for opsonic activity. The PTX3-dependent pathway of enhanced neutrophil phagocytic activity involved: Complement activation via the alternative pathway; Fcγ receptor (FcγR) IIA/CD32 recognition of PTX3-sensitized conidia and Complement receptor 3 (CR3) activation; CR3 and CD32 localization to the phagocytic cup. Gene targeted mice (ptx3, FcR common γ chain, C3, C1q) validated the *in vivo* relevance of the pathway. In particular the protective activity of exogenous PTX3 against *A. fumigatus* was abolished in FcR common γ chain-deficient mice. Thus, the opsonic and antifungal activity of PTX3 is at the crossroad between Complement, CR3- and FcγR-mediated recognition.

Since short pentraxins (e.g. C-reactive protein) interact with Complement and FcγR, the present results may have general significance for the mode of action of these components of the humoral arm of innate immunity.
Introduction

Innate immunity plays a key role as a first line of resistance against pathogens and in the activation and orientation of adaptive immunity. Similarly to adaptive immunity, innate immunity is based on cellular and humoral mediated mechanisms. The humoral pattern recognition receptors include collectins, ficolins and pentraxins.

Pentraxins are phylogenetically conserved proteins characterized by a multimeric structure and divided in short (C-reactive protein (CRP) and serum amyloid P component (SAP)) and long pentraxins. The long pentraxin 3 (PTX3) is the prototype of the long pentraxin sub-family: it shares similarities with the short pentraxins but differs for the presence of an unrelated, long N-terminal domain, as well as for gene organization, cellular source, inducing stimuli and ligands recognized. PTX3 is produced and released by a variety of cell types, including phagocytes, dendritic cells (DC), fibroblasts, and endothelial cells in response to primary inflammatory signals and Toll-like receptor (TLR) engagement. In neutrophils (PMN), PTX3 is stored in a ready-made form in secondary granules, and is secreted in response to recognition of microbial moieties and inflammatory signals localizing in neutrophil extracellular traps (NETs).

Recombinant PTX3 binds a variety of fungi, bacteria and viruses, including several species of *Aspergillus*, and has opsonic activity, facilitating phagocytosis, innate immune cell activation in terms of cytokine and nitric oxide production and orienting the development of adaptive immune response. Consistently, studies in vivo in *ptx3*-deficient mice suggest that the role played by PTX3 in innate resistance is non-redundant and relevant in selected fungal, bacterial and viral infections, in particular *Aspergillus fumigatus* and recombinant PTX3 has therapeutic effects in murine models of fungal infections in immunocompetent and immunodeficient mice. PTX3 has also been observed to have a regulatory role on inflammation by acting as a feedback mechanism of inhibition of leukocyte recruitment.

PTX3 binds also the first component of the classical complement cascade C1q, interacting with the C1q globular head (gC1q). This interaction results in activation of the classical
complement cascade when C1q is immobilized, a situation which mimics C1q bound to a microbial surface, or in inhibition of C1q haemolytic activity, when interaction occurs in the fluid phase, by competitive blocking of relevant sites 13. In addition, similarly to CRP 15,16, PTX3 interacts with Factor H, the main soluble regulator of the alternative pathway of complement activation, promoting Factor H deposition on PTX3-coated surfaces and preventing an exaggerated complement activation 17. Finally, PTX3 interacts with ficolin-2, enhancing complement deposition on Aspergillus fumigatus conidia 18. PTX3 recognition of the bacterial component OMP-A from Klebsiella pneumoniae triggers a pro-inflammatory response, based on complement activation 19,20. All these properties suggest that this long pentraxin behaves as a bona fide predecessor of antibodies.

Aspergillus fumigatus, an opportunistic ubiquitary fungus, is associated with a wide spectrum of diseases in humans, ranging from severe infections to allergy in immunocompromised and atopic subjects, respectively 21. In particular, aspergillosis is a major life-threatening infection in patients with defective phagocytosis, for instance during chemotherapy or radiotherapy-induced neutropenia and monocytopenia 21. The innate immune system represents the first line of defense against A. fumigatus 22. The complement system is activated on inhaled conidia via the alternative pathway and results in C3 deposition and cleavage 23,24. Recently, a MBL-dependent C2 bypass mechanism, which directly activates C3 and the alternative pathway on A. fumigatus conidia, has been proposed 25,26. Opsonization with complement proteins leads to phagocytosis by PMN and macrophages, which are major players in the innate resistance towards this fungus 24,27. PMN represent a ready-to-use reservoir of PTX3 and release it in response to microbial or inflammatory signals, and macrophages or DC rapidly produce it in NFkB-dependent manner. In vitro and in vivo data indicate that PTX3 expressed by PMN is essential to control phagocytosis and fungal growth 2.

PMN interaction with foreign microbes is facilitated by the recognition of opsonized material via the complement receptors CR1 (CD35), CR3 (CD11b/CD18, αMβ2), and CR4 (CD11c/CD18, αXβ2), which recognize antigen-bound complement components such as C3b or
C4b. PMN also express Fcγ receptor (FcγRIIA (CD32) and FcγRIIIB (CD16), and can be induced to express FcγRI (CD64) FcγRs are also involved in the mobilization and activation of CD11b/CD18 in the phagocytic cup.

The present study was designed to investigate the mechanisms of PTX3 as an endogenous neutrophil-stored opsonin and as an exogenously administered therapeutic agent. Therefore we investigated the involvement of complement components, complement receptors and FcγRs, which have been proposed as pentraxin receptors. Here we report that PTX3 acts as an opsonin, facilitating conidia recognition and phagocytosis in an FcγR- and C-dependent manner. In particular, our results in vitro and in vivo suggest that the opsonic activity of PTX3 is mediated through FcγRII-dependent CR3 activation, which is involved in the phagocytosis of C3-opsonized A. fumigatus conidia.

Materials and Methods

Reagents:
Recombinant human PTX3 and its C- and N-terminal fragments were purified from CHO cells, as described. Recombinant PTX3 contained <0.125 endotoxin units/ml as checked by the Limulus amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD).

Antibodies, complement depleted sera, complement components and animals used are listed in Supplemental Methods.

A. fumigatus was obtained from a fatal case of pulmonary aspergillosis. Heat inactivated or viable conidia were used as specified.

PMN isolation and phagocytosis assay
Human PMN were isolated from fresh whole blood obtained from healthy volunteers, as described. 2.5x10⁶ PMN (95% pure, based on morphology) were plated in 24 well-plate in 0.5 ml RPMI with 1, 3, 10% normal human serum (NHS), heat inactivated serum (HHS) (30 min at 56°C) or...
complement depleted sera and 2.5x10^7/well A. fumigatus conidia. When mentioned, the experiment was performed in the absence of NHS, but in the presence of C3 (125µg/ml), Factor B (20µg/ml), Factor H (50µg/ml), Factor I (3.4µg/ml) and Factor D (0.14µg/ml), e.g. physiological concentrations present in 10% of NHS. In selected experiments, PMN were pre-incubated for 1h on ice in the presence of blocking antibodies to CD11b, CD11c, CD32, CD16 or isotype controls (10µg/10^6 cells). Opsonization was performed by incubating A. fumigatus conidia in Ca^{2+}/Mg^{2+} PBS with 50µg/ml PTX3 (1.1µM, assuming a molecular mass of 45 kDa for the PTX3 protomer), or the N-terminal-PTX3, or the cross-linked C-terminal-PTX3 (1.1µM) for 1 or 2h at room temperature. In some experiments, after opsonization conidia were washed to remove unbound PTX3. Following a 30 min incubation at 37°C on an orbital shaker at 150 rpm, phagocytosis was blocked by adding NaF (Sigma, final concentration 0.2 M). Cytospins were stained with Diff Quick (Dade, Biomap, Italy). At least 200 PMN per sample were counted under oil immersion microscopy (100X). Results are expressed as Phagocytic Index (PI), the average number of conidia phagocytosed per 100 neutrophils, or as percentage of phagocytosis (% phagocytosis), the percentage of neutrophils containing at least one conidium. Normality of data was assessed where necessary. The statistical analysis (mean, SEM and Student’s paired t test) was performed on the PI values obtained from 3-6 different donors for experimental condition and from independent experiments.

PTX3 released by PMN (8 x 10^6/ml) was measured in the supernatant after incubation for 30, 60 or 120 min with conidia (3 x 10^8) in the presence of 0%, 3% or 10% NHS, as described 2.

**Phagocytosis assay and PMN activation in whole blood**

For phagocytosis assays in vivo or in whole blood or with murine bone marrow cells, conidia were labeled with Fluorescin 5(6)-isothiocyanate (FITC) (Sigma) (5mg/ml in DMSO). 10^8 FITC-conidia eventually opsonized with PTX3 (50 or 100 µg/ml) were added to 1ml of whole blood and incubated for 30 min at 37°C. Samples were placed on ice to block phagocytosis and red cells were
lysed by adding 10ml of cold ACK lysis solution pH 7.2. 100µl of each sample were fixed with 1% PFA and analyzed by flow cytometry.

PTX3 binding to *A. fumigatus* conidia, PMN activation and C3 deposition assays are described in Supplemental Methods.

**Confocal microscopy**

After activation in whole blood, human PMN were treated and incubated with anti-PTX3 rabbit polyclonal antibody and biotin-conjugated anti-CD32 (Serotec) or anti-activated CD11b mAb (BioLegend) followed by Alexa® Fluor 488-goat anti-rabbit IgG and Alexa® Fluor 647-streptavidin, or Alexa® Fluor 647-goat anti-mouse IgG (Molecular Probes), as described in Supplemental Methods.

Blood collected from C57BL/6J, ptx3- or FcR common γ chain- (FcRγ) deficient mice was treated as described in Supplemental Materials and incubated with anti-mouse CD11b (BD), followed by anti-rat 488 (Alexafluor). Cells were analyzed with a laser scanning confocal microscope (FluoView FV1000; Olympus). Images (1,024 x 1,024 pixels) were acquired with an oil immersion objective (100x 1.4 NA Plan-Apochromat; Olympus).

**In vivo phagocytosis and infection**

Mice were injected intratracheally with 8x10^7 heat inactivated FITC-labelled conidia opsonized with PTX3 and sacrificed 4h later. All procedures involving animals conformed with institutional guidelines in compliance with national and international law and policies on the care and use of laboratory animals. BAL leukocytes were analyzed by FACS with PerCP anti-CD45, PE anti-Ly6G, APC anti-CD11b, or cytospun and stained with Diff Quick for microscopic analysis.

C57BL/6 and FcRγ-deficient mice were anesthetized with 2.5% avertin (Sigma Chemical Co, St. Louis, MO) before intranasal infection with 2x10^7/20 µl viable, unopsonized *A. fumigatus* conidia
and treated with 1mg/kg/intranasally of PTX3 as previously described 9,10 or sterile saline for 5 consecutive days, starting the day of the infection. Mice were sacrificed one day after PTX3 treatment to count CFU in the lung and brain. For histology, paraffin–embedded sections were stained with Periodic acid–Schiff.

RESULTS

The PTX3 opsonizing activity is serum dependent

As shown in Fig. 1A, PTX3 (1.11 µM) amplifies PMN phagocytic activity in a serum dependent manner. In the absence of serum, phagocytosis of *A. fumigatus* conidia occurred at very low levels (PI 50) and increased in the presence of NHS. The effect was dose-dependent, as the PI increased to 153 ± 3, 175 ± 2 and 183 ± 2 with 1, 3 or 10 % NHS, respectively. The opsonization of conidia with PTX3 only marginally or inconsistently increased the PI in the absence of serum (PI 72 ± 9). In the presence of 1, 3 or 10% NHS, PTX3 significantly increased the PI to 237 ± 42, 294 ± 11 and 254 ± 20, respectively (p=0.03 and p=0.007 with 3 and 10% NHS, respectively). Similar results were obtained when the % of phagocytosis was analyzed.

Conidia phagocytosis in the presence of HHS was higher than in the absence of serum, but the opsonic activity of PTX3 was abolished (Fig. 1A). By contrast, depletion of IgG from serum did not modify PTX3 activity (p=0.04 and p=0.007 before or after IgG depletion) (Fig.1B).

Based on dose-response experiments (0.27 to 2.22µM), we observed that the PI increased starting from 0.27µM, reaching a plateau at 1.11µM (Fig.1C). Even at 1.11µM, PTX3 had no effect in the absence of NHS. The following experiments were performed with 1.11µM.

Similar stimulation by PTX3 (Fig.1D, p=0.0005) was observed when FITC-labeled *A. fumigatus* phagocytosis in whole blood was analyzed.
We then mapped the domain(s) involved in the opsonic activity of PTX3 (Fig. 1E). The N-terminal domain of PTX3 binds to *A. fumigatus* conidia but has no opsonic activity. Thus recognition is mediated by the N-terminal domain but phagocytosis requires the whole molecule.

As shown in Fig.1G, the addition of PTX3 in the assay (0 min pre-incubation) marginally modified the PI in a 30 min phagocytosis assay (231 vs 184 ± 2, in the presence and absence of PTX3, respectively), while pre-incubation of conidia with PTX3 increased the PI from 231 (without pre-incubation) to 283 ± 15 (60 min pre-incubation) and 328 ± 6 (120 min pre-incubation). When conidia were washed after pre-incubation to eliminate unbound PTX3, the PI was comparable to the PI in the presence of unbound PTX3 (296 ± 12 vs 328 ± 6, p=ns). These results suggest that conidia opsonization by PTX3 and not phagocyte activation by unbound PTX3 is responsible of increased phagocytosis.

**Role of Complement**

The following experiments were performed with commercially available sera depleted of specific complement components. Fig.2A shows that the amplification mediated by PTX3 occurred independently of the presence of C1q in the assay (p=0.04 in the absence and p=0.005 in the presence of C1q). PTX3 activity was also independent of the presence of C4, which is implicated in both the classical and lectin pathways (p=0.006 in the absence and p=0.03 in the presence of C4). To address the role of the alternative pathway, Factor B-depleted serum was used (Fig. 2A): the amplification of PI and % of phagocytosis were completely abrogated and were restored by the addition of recombinant Factor B (p=0.004). In the presence of C3-depleted serum, the phagocytosis was compromised, consistently with the fact that the three pathways of C activation were inactive and C3 fragments (C3b, iC3b) are major opsonins recognized by C receptors (CR3 and CR4). As shown in Fig.2A, in the absence of C3, the PI was very low and was increased to normal levels by the reconstitution of serum with recombinant C3. PTX3-mediated amplification was abrogated in the absence of C3 and restored after the addition of recombinant C3 (p=0.04).
Finally, to address the role of the C terminal pathway C5-9 and of the C5a anaphylotoxin in PTX3-mediated activity, C5-depleted serum was used. As shown in Fig. 2A, PTX3 activity occurred independently of the absence of C5 (p = 0.04 in the absence and p = 0.01 in the presence of C5). Finally, we performed the phagocytosis assay in the absence of serum, but in the presence of the recombinant components of the alternative pathway (C3, Factor B, Factor H, Factor D and Factor I) at the concentration present in 10% NHS. As shown in Fig. 2B, alternative pathway components reconstituted a phagocytic activity comparable to NHS, and PTX3 maintained its opsonizing activity (p = 0.04 with NHS, p = 0.01 with alternative pathway components).

Role of CD11b

Given the role of C3 in PTX3 activity, we next analyzed the role of iC3b and C3b receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18). To this aim, the phagocytosis assay was performed in the presence of blocking antibodies to CD11b or CD11c. As shown in Fig. 3A, in the presence of anti-CD11b, PTX3 activity was abolished (p = ns), whereas the anti-CD11c had no effect (p = 0.04 in the presence versus absence of PTX3). Irrelevant isotype controls did not influence PTX3 activity. Next we analyzed C3 and iC3b deposition on A. fumigatus conidia by FACS analysis in the presence of NHS at different concentrations (from 3% to 50%) and PTX3 (1.11 µM) at different time points (2-40 min). Under these experimental conditions we did not observe a PTX3-dependent increase of C3 fragment deposition using available reagents (not shown), although we cannot exclude a different kinetics of deposition or a minor but functionally important increase. Increased activity or affinity of CR3 for conidia might also be responsible of the increase in phagocytic efficiency mediated by PTX3. To address this point, we analyzed the active form of CD11b on PMN in the presence of conidia opsonized or not by PTX3, by using the CBRM1/5 antibody recognizing an activation epitope of CD11b. In nine different donors analyzed, the active form of CD11b decreased on the cell membrane in the presence of PTX3-opsonized conidia compared to the absence of PTX3 (MFI w/o PTX3: 555 ± 65; MFI with PTX3: 453 ± 55; p = 0.009). However, the total activated CD11b,
evaluated after permeabilization of cells, was significantly higher in the presence of PTX3-opsonized conidia compared to non-opsonized conidia (MFI w/o PTX3: 1473 ± 205; MFI with PTX3: 1639 ± 228; p=0.006), suggesting increased internalization. Figure 3B shows FACS analysis results on activated CD11b on the membrane and in permeabilized cells of one representative donor out of nine tested. The addition of PTX3 alone did not modify CD11b activation (see also Fig. 4B). Figure 3C and supplemental Figure 1 show the ratio between activated and total CD11b MFI PE/APC and percentage, respectively, in permeabilized cells in further six different donors. This analysis suggests that expression of the CD11b activation epitope is induced by conidia and that location of the activated integrin on the cell membrane or inside the cell (in the phagocytic cup) reflects the process of translocation in the phagocytic cup.

**Role of FcγRs**

It has been reported that pentraxins, including PTX3, interact with FcγRs, and mediate part of their biological activity through the activation of these receptors 33,34. Several studies demonstrated that FcγR are also involved in the mobilization and activation of CD11b/CD18 in the phagocytic cup 32. Thus, we addressed the involvement of PMN FcγRs in CD11b activation by PTX3-opsonized conidia, by adding blocking anti-FcγRs antibodies in the phagocytic assay. As shown in Fig.4A, PTX3 pro-phagocytic activity was inhibited by the presence of anti-CD32 (p=ns in the presence versus absence of PTX3), whereas in the presence of anti-CD16 or irrelevant antibodies, PTX3 activity was maintained (p=0.04 and 0.03, respectively). Moreover, in seven different donors analyzed, the increase in the activated CD11b in permeabilized cells observed in the presence of PTX3-opsonized conidia (MFI w/o PTX3: 556 ± 139; MFI with PTX3: 715 ± 167; p=0.02) was significantly inhibited by the presence of F(ab')2 blocking anti-CD32 (MFI with PTX3 and anti-CD32: 563 ± 134; p=0.0004) but not by anti-CD16 (MFI with PTX3 and anti-CD16: 633 ± 141; p=ns). Figure 4B shows FACS analysis results on activated CD11b of one representative donor out
of seven tested and figure 4C shows the ratio between activated and total CD11b MFI PE/APC in permeabilized cells in further three different donors. All together these results suggest that PTX3-dependent activation of CD11b could be mediated by inside-out signaling through FcγRIIA/CD32.

**Recruitment of CR3 and CD32 in the phagocytic cup**

To visualize the cellular localization of CR3 and CD32 during the phagocytic process, highly resolved confocal images were acquired on plated (Fig. 5A, B and C) or cyto spun PMN (Fig. 5D). Resting PMN or PMN undergoing *A. fumigatus* conidia phagocytosis were permeabilized and stained for the active form of CD11b, CD32 and PTX3. As shown in Fig.5A, in resting PMN, endogenous PTX3, CD11b and CD32 were localized in granules. As expected, activated CD11b immunostaining was very faint in resting PMN 37. In the presence of unopsonized conidia (Fig. 5B, left panels), endogenous PTX3 was localized in granules and in part in the phagocytic cup. CD32 and CD11b were distributed in the cell and in part in the phagocytic cup, where they co-localized with endogenous PTX3. When conidia were opsonized with PTX3 (Fig.5B, C and D, right panels), both CD32 and CD11b were mostly localized in the phagocytic cup around PTX3-opsonized conidia. Therefore, interaction with PTX3-sesitized conidia results in an increase in total activated CD11b, with localization and sequestration of activated CD11b and CD32 to the phagocytic cup.

**Mode of action of PMN-stored PTX3**

Fig. 6A shows the kinetics of PTX3 release by human PMN during phagocytosis. PTX3 levels in PMN supernatant were already significantly increased by the presence of conidia and 10% NHS upon 30 min incubation (11.6 ± 1.2 ng/ml vs 34.7 ± 2.4 ng/ml, p= 0.001). In the absence of the pathogen, we did not observe spontaneous release of PTX3. Moreover, PTX3 release was favored by the combined presence of conidia and NHS, which suggests that facilitated recognition, due to the concerted effect of Complement and PTX3, further increases degranulation (Fig. 6B).
We next analyzed conidia phagocytosis by murine wild type and ptx3-deficient bone marrow PMN in the presence of 10% NHS or HHS (Fig. 6C). In agreement with previous studies, we observed defective phagocytosis by ptx3-deficient PMN compared to wild type PMN in the presence of NHS ($p<0.0001$). In the presence of HHS, the defect was marginal ($p=0.11$). Phagocytosis by wild type PMN was impaired in the presence of HHS, compared to NHS ($p=0.0002$). When conidia were preopsonized with recombinant PTX3, phagocytosis was significantly increased in wild type cells ($p=0.004$ and $p<0.0001$, with 50 µg/ml and 100 µg/ml, respectively) and the defect of ptx3-deficient PMN was completely rescued ($p<0.0001$ with 50 µg/ml and 100 µg/ml) in the presence of NHS, but not HHS.

Finally, we analyzed by confocal microscopy CD11b localization in wild type, ptx3-deficient and FcRGamma-deficient PMN upon interaction with conidia. As shown in Fig. 6D, in wild type PMN, immunostaining for CD11b was localized on the cell membrane and around unopsonised phagocytosed conidia; in ptx3- and FcRGamma-deficient PMN, immunostaining for CD11b remained localized on the membrane and no immunostaining was observed around phagocytosed conidia. When conidia were preopsonized by recombinant PTX3, CD11b immunostaining around phagocytosed conidia or in the phagocytic cup was increased in wild type as well as in ptx3-deficient PMN, but not in FcRGamma-deficient PMN.

All together, these results indicate that the pro-phagocytic activity of endogenous PMN-stored PTX3, which is released by PMN during the phagocytosis assay, depends on the presence of fresh serum and thus of Complement, and is associated to the recruitment of CD11b in the phagocytic cup.

**In vivo mechanism of action**

To demonstrate the molecular mechanisms involved in PTX3-mediated activity, wild type, C1q-, C3- and FcRGamma-deficient mice, as well as sCR1-treated mice were injected intratracheally with FITC-labeled *A. fumigatus* conidia and sacrificed 4h later to assess phagocytosis by BAL Ly6G⁺CD11b⁺
cells by FACS analysis and microscopic count. As shown in Table I, showing one out of 2-3 experiments performed with similar results, in wild type mice both the MFI and the percentage of FITC positive Ly6G⁺CD11b⁺ cells and the PI of PMN were significantly increased by pre-opsonization of conidia with PTX3 (p=0.03, p=0.03 and p=0.04, respectively). PTX3 activity was conserved in C1q-deficient mice (p=0.03, p=0.04 and p=0.007, for MFI, % and PI, respectively), whereas it was lost in sCR1-treated mice, in C3-deficient mice and in FcRγ-deficient mice. Finally, PTX3 activity was conserved in SCID and Rag2-deficient mice (p=0.03 and p=0.004 for MFI, respectively), which suggests that immunoglobulins are not involved in the interplay among PTX3, FcγRs, complement and CR3.

Role of FcRγ in vivo

Finally, we analyzed the therapeutic potential of PTX3 in an in vivo model of pulmonary aspergillosis in wild type mice compared to FcRγ-deficient mice. FcRγ-deficient mice are more susceptible than wild type mice to the inflammatory pathology associated with the pulmonary infection (Fig.7A). Few infiltrates of inflammatory mononuclear cells scattered in an otherwise intact lung parenchyma were present in C57BL/6 mice as opposed to the abundant infiltration of inflammatory cells and signs of diffuse interstitial pneumonia observed in FcRγ-deficient mice. This may have compensated for the defective phagocytic activity of FcRγ-deficient mice and thus explain the relative ability of these mice to restrain the fungal growth (Figure 7B). PTX3 limited inflammation (Figure 7A) and restrained the fungal growth (Figure 7B) both in lung and brain in wild type mice, but was totally ineffective in FcRγ-deficient mice.

All together, these results confirm in vitro data indicating that PTX3-mediated phagocytic activity depends on complement but not on the classical pathway. Moreover, they demonstrate the non-redundant role of FcγRs in PTX3 opsonizing activity and therapeutic potential in aspergillosis.
Discussion

The present study was designed to investigate the mechanisms of PTX3 as an endogenous neutrophil-stored opsonin and as an exogenously administered therapeutic agent. Therefore we investigated the molecular mechanisms underlying PTX3-mediated opsonic activity in a model of *A. fumigatus* conidia phagocitosis by human PMN in vitro. In particular, we addressed the involvement of complement components, complement receptors and FcγRs. Specifically, we now report the role of the alternative pathway of the complement, CD11b activation induced by PTX3 opsonized conidia, the functional involvement of FcγRs in PTX3-dependent activities, and finally that PTX3 activity in innate defense is lost in C3- or FcγR-deficient mice.

PTX3 activates different effector pathways possibly involved in innate resistance to this opportunistic pathogen, including the classic, the alternative and the lectin pathway of complement activation by binding C1q, Factor H an ficolin-2, respectively or the promotion of phagocytosis by interacting with an as yet unidentified cellular receptor(s) possibly FcγRs, which have been proposed as pentraxin receptors.

The data presented here confirm previous reports on the role of complement in conidia phagocytosis. Actually, phagocytosis was significantly increased in the presence of fresh serum as source of complement compared to the absence of serum or in the presence of HHS. The experiments performed in the presence of complement components-deficient sera indicate that C3 in particular plays a key role in *Aspergillus* conidia phagocytosis, whereas the deficiency of C5 is less relevant, suggesting that C3-mediated opsonization of conidia and not the C5a-mediated activation of phagocytes is mainly involved in this process. Dumestre-Pérard et al. demonstrated that the alternative pathway is activated by different *Aspergillus* species, whereas neither the classical nor the lectin pathways through C4 and C2 cleavage are activated. In our experimental conditions, C1q-deficiency marginally reduced the phagocytic activity in vitro, but severely impaired resistance to lung infection, and the reconstitution of C4-, C5- and Factor B-deficient sera with the recombinant proteins marginally increased the phagocytic index, suggesting that...
possibly all pathways are implicated in conidia opsonization, at least of this clinical strain. In this scenario, the opsonization of conidia with recombinant PTX3 in *in vitro* assays amplified the complement-dependent effects on phagocytosis. Actually, PTX3-facilitated phagocytosis was observed only in the presence of NHS, and in particular, in the presence of C3-sufficient serum. Similarly, in *in vivo* phagocytosis, Complement inhibition with sCR1 treatment abolished PTX3 effects. Phagocytosis assays performed with wild type and ptx3-deficient PMN in the presence of fresh serum as source of Complement indicate that also the pro-phagocytic activity of endogenous PTX3 depends on Complement. Concerning the pathways of complement activation relevant for PTX3 activity, we observed that in the absence of C1q or C4 *in vitro* and C1q *in vivo*, PTX3 maintained its facilitating activity. Only Factor B, thus the alternative pathway, was necessary for PTX3 activity. Actually, the *in vitro* reconstitution of the alternative pathway (C3, Factor B, Factor I, Factor H) was sufficient for PTX3 activity. Finally, C5-deficiency was irrelevant, suggesting that PTX3 acts through amplification of C3-dependent opsonization and not through C5a-dependent cell activation. In agreement with the *in vitro* and *in vivo* data presented here, recombinant PTX3 played a therapeutic role in aspergillosis in C1q-deficient mice rescuing their defective resistance to the infection 3, thus suggesting that PTX3 plays its role in facilitating conidia phagocytosis independently of the interaction with C1q. *Aspergillus* immune evasion through interaction with Factor H and C4b binding protein has been recently demonstrated 39,40. Given the PTX3 interaction with both *Aspergillus* conidia and Factor H, we speculate that PTX3 could play a role in counterbalancing these immune evasion systems on Complement.

Experiments performed with integrin blocking antibodies, as well as FACS and confocal analysis indicated that in the presence of PTX3-opsonized conidia, CD11b activation, internalisation, recruitment to the phagocytic cup and CD11b-dependent phagocytosis were increased. Previous studies have provided evidence that FcγR-derived signals induce activation of CR3 41. Specifically, FcγR stimulation in macrophages promotes CR3 clustering into high-avidity complexes in phagocytotic cups by a mechanism involving release of integrins from their
cytoskeletal constraints, thus enhancing their lateral diffusion. A similar mechanism of inside-out activation of CR3 integrin by CD44 ligation has recently been demonstrated\(^{42}\). In particular, CD44 ligation led to increased mobility of CR3, increased recruitment of the high-affinity state of CR3 to the phagocytic cup, and finally increased phagocytosis. The experiments performed here in the presence of FcγR blocking Abs as well as confocal and FACS analysis strongly suggest that upon opsonization of conidia with PTX3, FcγRIIA/CD32 mediates inside-out activation of CD11b and consequently phagocytosis of C3b-opsonized \textit{A. fumigatus} conidia (Supplemental figure 2). Human and murine FcγRs differ in many aspects including the affinity for the antibody Fc-fragment and the signalling pathway induced. Thus it is difficult to extrapolate data from animal studies to the human system. However, the results obtained with FcRγ-deficient mice, which lack signalling from any functional activating FcRs, demonstrate their involvement in PTX3 therapeutic activity towards \textit{Aspergillus}. On the same line, CD11b recruitment in the phagocytic cup was defective in ptx3- and FcRγ-deficient PMN and was rescued by recombinant PTX3 in ptx3-deficient PMN but not FcRγ-deficient PMN. These results suggest that both endogenous, neutrophil-stored, and exogenous (e.g. in a therapeutic setting) PTX3 acts via the same Complement/CD11b/FcγR-dependent pathway.

As shown in previous studies\(^2\) and confirmed here, endogenous PTX3 stored in neutrophil granules plays an essential role in recognition and disposal of conidia. Therefore the margin for increased efficiency in the phagocytosis of conidia by PMN in the presence of exogenous PTX3 is limited. This could explain the significant but far from spectacular enhancement observed in the presence of recombinant PTX3 in vitro, compared to the more impressive effect observed in wild type vs ptx3-deficient mice.

Moreover, additional mechanisms could be involved in PTX3-dependent protection from fungal infection in vivo. These include PTX3-dependent amplification of the antifungal role of other cell types, such as macrophages, dendritic cells, epithelial cells; induction of other mediators such as β-defensins and cathelicidins; development of protective Th1 adaptive responses through up-
regulation of IL-12 in dendritic cells, modulation of inflammatory responses \textsuperscript{3,9-11}. Therefore, mechanisms other than increased phagocytosis contribute to the antifungal activity of PTX3 in vivo. PMN may actually feed on some of these (for instance activation of Th1 and Th17 responses) \textsuperscript{43}.

Finally, the \textit{in vivo} experiments in SCID and Rag2-deficient mice, which lack T and B cells, indicate that low affinity natural antibodies, which could bind conidia or Fc\(\gamma\)Rs, do not contribute to the interplay among PTX3, complement, CR3 and Fc\(\gamma\)R-mediated recognition described here.

Several phagocyte PRR cooperate when they sense whole organisms through different PAMPs or with particular PAMPs able to interact with multiple PRR. For instance, CD36 mediates binding and internalization of Gram-positive bacteria and cooperates with TLR2 and TLR6, which induce cytokine production \textsuperscript{44}. Similarly, OMP-A interacts with scavenger receptors LOX-1 and SREC-I, activates phagocytes through TLR2 and binds to PTX3, thus leading to amplification of the innate response to this moiety \textsuperscript{19,20}. This cooperation among simultaneously engaged PRR leads to activation of diverse cellular signalling pathways and of the humoral arm of the innate immune system resulting in synergy and amplification of the innate responses to pathogens. The results presented here demonstrate that coexistence of Fc\(\gamma\)R ligands (PTX3) and CR3 ligands (C3bi) on opsonized conidia initiates Fc\(\gamma\)RII-dependent CR3 mobilization and activation leading to amplification of \textit{A. fumigatus} conidia recognition and phagocytosis. Thus, PTX3 is a fluid phase PRM whose opsonic activity is at the crossroad between complement, CR3 and Fc\(\gamma\)R-mediated recognition. Since classic short pentraxins, such as CRP, interact with complement components (Factor H and C1q) and with Fc\(\gamma\)Rs \textsuperscript{34}, the results reported here may have broad implications for the mode of action of this ancient class of functional ancestors of antibodies.

\textbf{Acknowledgments}
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**Authorship contributions and Disclosure of Conflict of Interest**

F. M. performed research, interpreted data; A.D. performed research; L. D. performed research; T. Z. performed research; S. Z. performed research; B. B. analyzed data; L. R. analyzed data; A.M. designed research, interpreted data, wrote the manuscript; C. G. designed research, analyzed and interpreted data, wrote the manuscript.

All authors declare that they do not have financial conflict of interest.
References


Table I: *In vivo* phagocytosis of FITC-conidia by alveolar neutrophils.

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<th>MFI in Ly6G&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt; (%)</th>
<th>MFI in Ly6G&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt; CD45&lt;sup&gt;+&lt;/sup&gt; PI in PMN</th>
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FACS analysis (MFI and %) and microscopic analysis (PI) of internalized FITC-conidia. Student’s t test. Data show one out of 2-3 experiments performed for each group with similar results.
Figure legend

Figure 1. PTX3-mediated increase of *A. fumigatus* conidia phagocytosis is serum and complement dependent, but not Ig-dependent. (A) For the phagocytic assay, *A. fumigatus* conidia were pre-incubated or not (CTR) with 1.1µM PTX3 and then incubated for 30 min with neutrophils in the presence of 1, 3, or 10% normal human sera (NHS) or heat inactivated human sera (HHS). Results are mean ± SEM of 3–6 pooled experiments. (B) The phagocytosis assay was performed with 10% NHS or Ig-depleted human serum (NHS-Ig). Results are mean ± SEM of 3 pooled experiments. (C) The phagocytosis assay was performed with *A. fumigatus* conidia pre-incubated with different doses of PTX3 (0.27-2.22µM) in the presence or not of 10% of NHS. Results are representative of one out of two experiments. (D) FACS analysis of PTX3-opsonized FITC-conidia phagocytosis. MFI results are mean ± SEM (n=7 donors). (E) Full-length PTX3 (PTX3\text{full}), N-terminal domain (PTX3\text{N-term}) and C-terminal domain of PTX3 (PTX3\text{C-term}) were incubated with *A. fumigatus* conidia at different concentrations (6.9-444 nM). The binding was detected as described in Material and Methods. MFI results are representative of one out of three independent experiments. (F) FACS analysis of FITC-conidia phagocytosis after opsonization with full-length PTX3, N-terminal or C-terminal domains. Results (neutrophil MFI) are representative of one out of three independent experiments. (G) Conidia were pre-incubated (white and grey bars) or not (black bar) with 1.1µM PTX3 for the indicated time (0, 60, 120 min) and eventually washed to eliminate unbound PTX3 before the phagocytosis assay (grey bar) in the presence of 10% NHS. Results are mean ± SEM of 2-6 pooled experiments. *, p ≤ 0.05; **, p < 0.01; ***, p = 0.0005, Student’s paired t test.

Figure 2. PTX3-mediated increase of *A. fumigatus* conidia phagocytosis depends on the alternative pathway of complement. (A) The phagocytosis assay was performed in the presence of 10% C1q-, C3-, C5- or Factor B-depleted human serum (C1qDHS, C3DHS, C5DHS and FBDHS), or C4-depleted guinea pig serum (C4DGS). Sera were reconstituted with the respective
purified human proteins (C1q, C3, C4, C5, Factor B). (B) The phagocytosis assay was performed in the presence of C3, Factor B, Factor H, Factor D, Factor I at the concentration present in 10% NHS. Results are paired PI values of independent donors in the absence or presence of recombinant PTX3. *, p ≤ 0.05; **, p < 0.01; ***, p < 0.0001, Student’s paired t test.

**Figure 3. PTX3-mediated increase of *A. fumigatus* conidia phagocytosis depends on CD11b and is associated to CD11b activation.** (A) The phagocytosis assay was performed in the presence of 10% NHS and anti-CD11b (M1/70) or anti-CD11c (3.9) blocking antibodies or control IgGs. Results are mean ± SEM of 3-5 pooled experiments. *, p ≤ 0.05; **, p < 0.01; Student’s paired t test. (B) FACS analysis of activated CD11b associated to the membrane or present in permeabilized cells (membrane and intracellular) on neutrophils after incubation of whole blood with PTX3-opsonized conidia for 15 min. The histogram shows one out of nine donors analyzed with similar results. (C) Ratio of activated and total CD11b MFI PE/APC in permeabilized cells after incubation for 15 min with conidia or PTX3-opsonized conidia (n=6).

**Figure 4. PTX3-mediated increase of *A. fumigatus* conidia phagocytosis depends on CD32 which regulates CD11b activation.** (A) The phagocytosis assay was performed in the presence of 10% NHS and anti-CD16 (LNK16) or anti-CD32 (AT10 or IV.3) blocking antibodies or control IgGs. Results are mean ± SEM of 3-5 pooled experiments. *, p ≤ 0.05; Student’s paired t test. (B) FACS analysis of membrane and intracellular activated CD11b in permeabilized neutrophils defined as FSC-A<sup>high</sup>/SSC-A<sup>high</sup>, after incubation of whole blood with PTX3-opsonized conidia for 15 min, in the presence of F(ab')2 blocking anti-human CD16 (3G8) and CD32 (7.3). The histogram shows one out of seven donors analyzed with similar results. (C) Ratio of activated and total CD11b MFI PE/APC in permeabilized cells after incubation for 15 min with conidia or PTX3-opsonized conidia in the presence of anti-CD16 (3G8) or anti-CD32 (IV.3) blocking antibodies.
Figure 5. The opsonization of conidia with PTX3 increases the co-localization of CD11b and CD32 in the neutrophil phagocytic cup. (A, B, C) Confocal microscopy analysis (FluoView FV1000; Olympus) of PTX3, CD11b, and CD32 in resting cells (A); CD11b and PTX3 (B) or CD32 and PTX3 (C) colocalization by double staining. After phagocytosis of non-opsonized conidia (B, C, left panels) or PTX3-opsonized conidia (B, C, right panels), cells were fixed with 4% PFA and stained for PTX3 (green) and CD11b (red) (B) or PTX3 and CD32 (red) (C). DNA labelling is also shown (DAPI). Panels from top to bottom show single staining for CD11b (A left panel, B) or CD32 (A right panel, C); for PTX3; double fluorescence for PTX3 and CD11b (A left panel, B) or PTX3 and CD32 (A right panel, C); double fluorescence and differential interference contrast (Nomarski) (inset: Normaski and DAPI). (D) Triple staining for CD11b, CD32 and PTX3 in cytospun neutrophils by confocal microscopy. After phagocytosis of non-opsonized conidia (left panels) or PTX3-opsonized conidia (right panels), cells were cytospun and fixed with 4% PFA and stained for human PTX3 (green), CD11b (blue) and CD32 (red). Panels from top to bottom show double fluorescence for PTX3 and CD11b; CD11b and CD32; PTX3 and CD32; triple staining for PTX3, CD11b and CD32; triple fluorescence and differential interference contrast (Nomarski). Images (1,024 x 1,024 pixels) were acquired with an oil immersion objective (100x 1.4 NA Plan-Apochromat; Olympus). One or more internalized conidia per cell are indicated by asterisks or arrows. Bars indicate magnification.

Figure 6. Endogenous PTX3 is released by PMN during conidia phagocytosis and contributes to internalization through the molecular mechanisms used by recombinant PTX3. (A) Kinetics of PTX3 release from human PMN in the presence of 10% NHS and conidia. PTX3 levels was measured in the supernatants of 8 x 10⁶/ml PMN at different time points of incubation (0, 30, 60, 120 min) with conidia and 10% NHS or NHS alone. (B) PTX3 levels were measured in PMN supernatants upon 2h of incubation with or without conidia and different concentrations of NHS (0, 3, 10%). (C) FACS analysis of FITC-conidia phagocytosis by wild type (ptx3+/+) and
px3-deficient (-/-) bone marrow PMN (CD45+, Ly6G<sub>high</sub>, CD11b<sub>high</sub>) in the presence of 10% NHS or HHS. When indicated, conidia were pre-opsonized with recombinant PTX3 (50 or 100 µg/ml). Experiments were performed in duplicate with PMN collected from 4-10 ptx3+/+ and ptx3-/- mice. Data were normalized and expressed as percentage of the mean in wild type PMN. *, p ≤ 0.05; **, p < 0.01; ***, p < 0.0001; Student’s unpaired t test. (C) Confocal analysis of total CD11b location in wild type, ptx3-/- and FcRγ-deficient (FcRγ-/-) PMN during phagocytosis of conidia eventually opsonised with recombinant PTX3. Upper panels: CD11b (red) and nucleus (DAPI). Middle panels: CD11b (red) and Normarski. Lower panels: CD11b in pseudocolor scale. Two or three internalized conidia per cell are indicated with asterisks. Note that location of CD11b in the phagocytic cup is impaired in ptx3-/- and FcRγ-/- PMN and is rescued by recombinant PTX3 in ptx3-/- PMN, but not in FcRγ-/- PMN.

**Figure 7. The therapeutic potential of PTX3 in aspergillosis is abrogated in FcRγ-deficient mice.**

(A) Periodic acid-Schiff-stained medial sections of lungs from C57BL/6 or FcRγ-/- mice intranasally infected with 2x10<sup>7</sup>/20µl viable, unopsonized *Aspergillus fumigatus* conidia and treated with 1mg/kg/intranasally of PTX3 or saline for 5 consecutive days and assessed one day after the end of treatment. Note the presence of few infiltrates of inflammatory mononuclear cells scattered in an otherwise intact lung parenchyma in C57BL/6 mice as opposed to the abundant infiltration of inflammatory cells and signs of diffuse interstitial pneumonia in FcRγ-/- mice and its amelioration by PTX3 treatment in C57BL/6 but not in FcRγ-/- mice. Bars indicate magnification. (B) CFU (mean ± SE, n=6) in the lung and brain of C57BL/6 or FcRγ-/- mice treated with PTX3 or saline.
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Role of complement and Fc\(\gamma\) receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*

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