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The Therapeutic Potential of the Humoral Pattern Recognition Molecule PTX3 in Chronic Lung Infection Caused by *Pseudomonas aeruginosa*

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Chronic lung infections by *Pseudomonas aeruginosa* strains are a major cause of morbidity and mortality in cystic fibrosis (CF) patients. Although there is no clear evidence for a primary defect in the immune system of CF patients, the host is generally unable to clear *P. aeruginosa* from the airways. PTX3 is a soluble pattern recognition receptor that plays nonredundant roles in the innate immune response to fungi, bacteria, and viruses. In particular, PTX3 deficiency is associated with increased susceptibility to *P. aeruginosa* lung infection. To address the potential therapeutic effect of PTX3 in *P. aeruginosa* lung infection, we established persistent and progressive infections in mice with the RP73 clinical strain RP73 isolated from a CF patient and treated them with recombinant human PTX3. The results indicated that PTX3 has a potential therapeutic effect in *P. aeruginosa* chronic lung infection by reducing lung colonization, proinflammatory cytokine levels (CXCL1, CXCL2, CCL2, and IL-1β), and leukocyte recruitment in the airways. In models of acute infections and in vitro assays, the phagocytotic effect of PTX3 was maintained in C1q-deficient mice and was lost in C3- and Fc common γ-chain–deficient mice, suggesting that facilitated recognition and phagocytosis of pathogens through the interplay between complement and FcγRs are involved in the therapeutic effect mediated by PTX3. These data suggested that PTX3 is a potential therapeutic tool in chronic *P. aeruginosa* lung infections, such as those seen in CF patients. The *Journal of Immunology*, 2011, 186: 000–000.

Cystic fibrosis (CF) is an autosomal recessive disease caused by lack of function of a CAMP-regulated chloride channel, the cystic fibrosis transmembrane conductance regulator, expressed in many epithelial and blood cells, such as neutrophils and macrophages (1, 2). The lung is the most important site of disease, which accounts for much of the morbidity and mortality in CF. CF is not considered a primary immunodeficiency; however, several innate immune responses, such as mucociliary clearance, activity of β-defensins and cathelicidins, production of ceramide, leukocyte migration, and bacterial encounter and killing, are severely impaired by the viscous mucus layer and high salt concentration present in the respiratory tract (3). The accumulation of airway-secretory products, including proteoglycans, cytokines, chemokines, neutrophil proteases, and growth factors, causes airway inflammation and poor bacterial clearance (4). A limited number of microbial pathogens adapted to this specific environmental niche infect CF patients, including *P. aeruginosa*, the most common isolate, *Staphylococcus aureus Stenotrophomonas maltophilia* and *Burkholderia cepacia* complex (5). Early acquisition of *P. aeruginosa* is a predictor for a worse prognosis in CF patients, because pathogen-induced inflammation, which is meant to contain infection, fails in the airway and is responsible for much of the pathology in the lung. Actually, persistent chronic infections lead to bronchiectasis, self-sustaining cycles of airway obstruction, and infection and exacerbate neutrophilic airway inflammation, which lead to tissue damage, impairment of immune responses, and, ultimately, to respiratory failure and death (6, 7). In particular, neutrophil-derived elastase cleaves IgGs, complement components, surfactant proteins, and chemokine receptors (8).

Early *P. aeruginosa* isolates in CF patients are usually acquired from the environment and are nonmucoid, motile, and highly susceptible to antibiotics. A number of *P. aeruginosa* virulence factors, including flagella, pili, LPS, quorum-sensing molecules, proteases, and toxins, are critical in the establishment of acute infections and the progression to chronic infections (9, 10). This repertoire of virulence factors promotes adherence to host cells,
PTX3 IN CHRONIC LUNG INFECTION BY P. AERUGINOSA

damages host tissues, elicits inflammation, and possibly disrupts host defenses by altering gene expression in host cells (11, 12). However, a prominent feature of P. aeruginosa strains infecting CF patients is the conversion to a mucoid phenotype characterized by overproduction of a exopolysaccharide, known as alginate, and the loss of synthesis of the LPS-O side chains (13, 14). This phenotypic conversion, which is associated with an increased rate of decline in CF lung function, confers increased resistance to the innate and acquired immune defenses by impeding phagocytosis, as well as providing protection against reactive oxygen species and antibiotics (15, 16). New treatment options for patients with CF are critically needed because the median age for survival is still in the mid-30s (16).

Pentraxins are a superfamily of phylogenetically conserved proteins characterized by a multimeric structure, some of which are humoral pattern recognition receptors of innate immune responses (17). Pentraxins are divided into short (C-reactive protein [CRP] and serum amyloid P component) and long pentraxins. PTX3 is the prototype of the long pentraxin subfamily and is produced and released by a variety of cell types, including phagocytes, dendritic cells, fibroblasts, and endothelial cells, in response to primary inflammatory signals and TLR engagement. In neutrophils, 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 (18). PTX3 is essential and has therapeutic potentials in innate resistance to a variety of fungi, bacteria and viruses, including P. aeruginosa, by playing opsonic activities, facilitating phagocytosis, innate immune cell activation and orienting the development of adaptive immune response (18–25). PTX3 was also observed to have a regulatory role in inflammation by acting as a feedback mechanism of inhibition of leukocyte recruitment (26).

PTX3 is involved in complement activation through the classical, lectin, and alternative pathways. PTX3 binds the first component of the classical complement cascade, C1q, modulating the activation of the classical pathway (27–29). In addition, PTX3 interacts with factor H, the main soluble regulator of the alternative pathway of complement activation, protecting factor H deposition on PTX3-coated surfaces and preventing an exaggerated complement activation (30). Finally, PTX3 synergizes with ficolin-2 in recognizing pathogens and triggering complement deposition (31). Recognition of the bacterial component OMP-A from Klebsiella pneumoniae by PTX3 triggers a complement-mediated inflammatory response (32, 33).

It was reported that pentraxins, including PTX3, interact with FcyRs and mediate part of their biological activity through the activation of these receptors (34, 35). Actually, we recently demonstrated that PTX3 facilitates phagocytosis of Aspergillus fumigatus conidia through a complex interplay among complement, CR3 and FcyRs (36). All of these properties suggest that PTX3 mediates resistance to pathogens, facilitating complement activation and opsonization.

Previous studies showed that ptx3-deficient mice have an increased susceptibility to infection with a laboratory strain of P. aeruginosa (19). Therefore, it was important to assess the actual therapeutic potential of PTX3 in a system representative of unmet medical needs, such as chronic infection in patients with CF (10). Moreover, we recently observed that PTX3 gene polymorphisms affect CF-associated P. aeruginosa lung infections (37), in line with previous findings on the links between PTX3 polymorphisms and tuberculosis infection (38). In the current study, we addressed the potential therapeutic role of recombinant PTX3 in a murine model of chronic lung infection with a clinical P. aeruginosa strain, which mimics the persistent and progressive colonization occurring in CF patients. Our results suggested that PTX3 plays a potential therapeutic role against infection by P. aeruginosa, promoting the reduction of the bacterial load and limiting the inflammatory response. The therapeutic effect of PTX3 was mediated by facilitated recognition and phagocytosis of this pathogen through the interplay between complement and FcyR. These data suggested that PTX3 is a potential therapeutic tool in chronic P. aeruginosa lung infections, with particular relevance for CF patients.

Materials and Methods

Bacterial strains

The reference laboratory P. aeruginosa strain PA01 (39) and the clinical strain RP73, isolated at the late stage of chronic infection from a patient with CF (40), were used for in vitro experiments and mouse-infection studies. P. aeruginosa clinical strain RP73 was kindly provided by Prof. Burkhard Tümmler (Klinische Forscherguppe, Medizinische Hochschule Hannover, Hannover, Germany). Strains were cultured in trypticase soy broth and plated on Pseudomonas isolation agar or trypticase soy agar plates at 37°C.

Recombinant PTX3

Recombinant human clinical grade and murine PTX3 were purified from CHO cells constitutively expressing the proteins, as described previously (27, 41). Purity of recombinant proteins was assessed by SDS-PAGE, followed by silver staining. Recombinant PTX3 contained <0.125 endotoxin units/ml, as checked by the Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD).

Binding of PTX3 to bacteria

A total of 109 bacteria was incubated in 50 μl HBSS, BSA 0.5% with biotinylated PTX3 (50 μg/ml) or biotinylated BSA (70 μg/ml). After 1 h at room temperature, samples were extensively washed with HBSS. Bound proteins were eluted with 150 μl 0.5 M ammonium formate (pH 6.4) and detected by ELISA. ELISA plates (96 well; Nunc Immuno Plate, Maxisorp; Nunc) were coated with 100 μl/well supernatant diluted in coating buffer (15 mM carbonate buffer [pH 9.6]) and were incubated overnight at 4°C. After washing (Dubelco’s PBS, 0.05% Tween 20), plates were incubated with 5% dry milk to block nonspecific binding sites and then with streptavidin-peroxidase conjugated to dextran backbone (AmDex, Copenhagen, Denmark) (1:4000), followed by 3 mM 3,3’,5,5’-tetramethyl-benzidine dihydrochloride (Sigma Aldrich). Absorbance values were read at 405 nm in an automatic ELISA reader.

Animals

C57BL/6 male mice were obtained from Charles River Laboratories (Calco, Italy). C1q-deficient mice were generously provided by M. Botto (Imperial College, London, U.K.). FcR common γ-chain (FcγR)-deficient mice were provided by Takashi Saito (Osaka University, Osaka, Japan), and C3-deficient mice were from Jackson Laboratories.

Procedures involving animals and their care conformed with protocols approved by the Istituto Clinico Humanitas and San Raffaele Scientific Institute in compliance with national (4D.L. N.116, G.U., Suppl. 40, 18-2-1992) and international law and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, 12-1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals). All efforts were made to minimize the number of animals used and their suffering.

Chronic lung infection

The P. aeruginosa agar-beads mouse model was used (42). Briefly, a starting amount of 5 × 107 clinical strain RP73 was used for inclusion in the agar beads, which were prepared as described previously (14, 43). Mice (20–22 g) were anesthetized with 375 mg/kg Avertin (2,2,2-tribromoethanol, 97%; Aldrich), intubated with a 22-gauge venous catheter and inoculated with 109 clinical strain RP73 in agar-beads mouse model was used (42). Briefly, a starting amount of 5 × 107 clinical strain RP73 was used for inclusion in the agar beads, which were prepared as described previously (14, 43). Mice (20–22 g) were anesthetized with 375 mg/kg Avertin (2,2,2-tribromoethanol, 97%; Aldrich), intubated with a 22-gauge venous catheter and inoculated with 2 × 107/50 μl P. aeruginosa. After the intratracheal (i.t.) injection, mice were treated once a day for 3, 7, or 14 d with recombinant PTX3 (10 μg/mouse i.p.) or with sterile saline. When specified, the treatment started 7 d postinfection. Bronchoalveolar lavage (BAL) was performed 3, 7, 14, or 21 d after the infection, and murine lungs were excised, homogenized, and plated onto trypticase soy agar.

BAL fluid collection and analysis

BAL was performed three times with 1 ml RPMI 1640 with protease inhibitors (Complete tablets, Roche Diagnostic; PMSF, Sigma) with a 22-

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P. aeruginosa strain RP73 (10^8/ml) in the logarithmic phase was incubated at 37°C in PBS, with 1, 5, or 10% normal human serum (NHS) or heat-inactivated human serum, in the presence of 20 mM in 100 μl HBSS, washed three times, and resuspended in 50 μl HBSS. The percentage of CFSE+ bacteria was evaluated by FACS analysis. A total of 10^7 CFSE-labeled RP73, eventually opsonized with PTX3 (20 μg/ml, 1 h at room temperature), was added to WT alveolar neutrophils in the presence of 5% NHS, C1q-, or C3-deficient serum (C1qDHS and C3DHS; Calbiochem) or to FcR-deficient alveolar neutrophils in the presence of 5% NHS and incubated for 30 min at 37°C in an orbital shaker. Samples were placed on ice to block phagocytosis, and red cells were lysed by adding ACK. CFSE MFI was analyzed by FACS analysis.

### Statistical analysis

CFU, cells, MPO, total proteins, cytokines, and histological results are expressed as mean ± SEM. Two-tailed Student t test was used; p < 0.05 was considered significant. Experiments were repeated two or three times, as specified.

### Results

#### Treatment with recombinant PTX3 protects mice from chronic P. aeruginosa lung infection

Previous studies showed that PTX3 interacts with a laboratory strain of *P. aeruginosa* and plays a nonredundant role in resistance to lung infection caused by this strain (19). It was important to extend these studies on PTX3 interaction with *P. aeruginosa* by testing recognition of a clinical isolate. As shown in Fig. 1, the RP73 clinical isolate was recognized more efficiently than was the laboratory strain PAO1 and *A. fumigatus* conidia. We then assessed the therapeutic potential of recombinant PTX3 in chronic *P. aeruginosa* lung infection. We used the agar-beads model, which mimics the microaerobic growth conditions of *P. aeruginosa* in the CF lung (43). As previously demonstrated (14), i.t. inoculation of *P. aeruginosa* clinical strains embedded in agar beads induces chronic pneumonia, and RP73 was ranked as the most efficient in establishing chronic infection at 14 d after challenge. Within the first 3 d postinfection, *P. aeruginosa* growth reaches its peak, and mice can eventually die from sepsis; however, after 7 d, a chronic infection is established, and the bacterial load is maintained at a constant level. Based on previously performed pharmacokinetic analyses and therapeutic approaches in a murine model of lung aspergillosis (25), we chose a treatment schedule of daily i.p. injections with recombinant human PTX3 (10 μg/mouse) or vehicle, starting from the day of i.t. inoculum injection.
with $2 \times 10^6$ 

$P. aeruginosa$ RP73. During the first 3 d, we observed a mortality of 2 and 8.3% in PTX3-treated and untreated mice, respectively (1/48 versus 4/48; $p = 0.168$, $\chi^2$ test). Given the low mortality in mice infected with the RP73 

$P. aeruginosa$ strain, a survival benefit to PTX3 treatment was not appreciable. When surviving mice were sacrificed 14 d after RP73 injection, lungs appeared swollen and inflamed. As shown in Fig. 2A, PTX3 treatment reduced the total CFU recovered from lungs and BALF compared with sterile saline treatment ($2 \times 10^5 \pm 3 \times 10^5$ in control mice ($p = 0.05$) after 7 d of treatment (Fig. 2B) and $5.6 \times 10^5 \pm 1.3 \times 10^5$ in treated mice versus $1.7 \times 10^5 \pm 5.4 \times 10^4$ in control mice ($p = 0.04$) after 14 d of treatment (Fig. 2C). This result suggested that PTX3 treatment is effective, even if the chronic infection is already established. In contrast, treatments for 3 or 7 d after infection did not modify the bacterial load (data not shown), possibly because of the high variability in bacterial load in early time points in this model (44) or because long-term treatment is necessary to fight against this chronic infection, as demonstrated in CF patients.

**Treatment with recombinant PTX3 reduces airway and parenchymal inflammatory response associated with chronic $P. aeruginosa$ lung infection**

We next analyzed the inflammatory response associated with RP73-induced chronic pneumonia in terms of leukocyte recruitment in the airways, vascular permeability, and local cytokine production. As shown in Fig. 3A, 14 d postinoculation, PTX3-treated mice had significantly fewer leukocytes in BALF than did control mice ($1 \times 10^6 \pm 2 \times 10^5$ versus $3 \times 10^6 \pm 4 \times 10^5$ total cells, respectively; $n = 18$; $p = 0.001$). In particular, we observed a significant reduction in neutrophils in PTX3-treated mice, but macrophages and lymphocytes were not modified ($5.1 \times 10^5 \pm 1.4 \times 10^5$ versus $2.1 \times 10^6 \pm 3.5 \times 10^5$, $p < 0.0003$ for neutrophils; $4.9 \times 10^5 \pm 7.6 \times 10^5$ versus $6.1 \times 10^5 \pm 7.6 \times 10^5$, for macrophages; $1.3 \times 10^5 \pm 3 \times 10^5$ versus $1.2 \times 10^5 \pm 2.2 \times 10^5$ for lymphocytes, in PTX3-treated and untreated mice, respectively; $n = 18$) (Fig. 3A). The percentage of neutrophils was significantly reduced in PTX3-treated mice (43 ± 5% versus 72 ± 3%, in PTX3-treated and untreated mice, respectively; $p < 0.001$), whereas the percentages of macrophages and lymphocytes were increased compared with untreated mice (47 ± 4% versus 23 ± 3%, $p < 0.0001$ for macrophages; 11 ± 1% versus 5 ± 1%, $p < 0.0004$ for lymphocytes, in PTX3-treated and untreated mice, respectively) (Fig. 3B). Accordingly, the MPO activity in BALF (Fig. 3C) was significantly lower in treated mice. Moreover, BALF protein content, which is a parameter of vascular leakage, was significantly reduced in PTX3-treated mice compared with untreated mice (1354 ± 72 µg/ml versus 1546 ± 71 µg/ml, respectively; $n = 10$; $p = 0.04$, one-tailed Student $t$ test).

**Table 1. Cytokines and chemokines in lung homogenates of PTX3-treated mice 14 d after RP73 injection**

<table>
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<tr>
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<th>Saline (pg/ml, mean ± SEM)</th>
<th>PTX3 (pg/ml, mean ± SEM)</th>
<th>$p^*$</th>
<th>$n$</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>6323 ± 1288</td>
<td>2438 ± 529</td>
<td>0.009</td>
<td>24</td>
</tr>
<tr>
<td>CCL2</td>
<td>116 ± 10</td>
<td>90 ± 8</td>
<td>0.04</td>
<td>28</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1266 ± 86</td>
<td>1630 ± 114</td>
<td>0.01</td>
<td>18</td>
</tr>
<tr>
<td>IL-17</td>
<td>25 ± 6</td>
<td>9 ± 5</td>
<td>0.04</td>
<td>8</td>
</tr>
<tr>
<td>CXCL1</td>
<td>978 ± 148</td>
<td>624 ± 86</td>
<td>0.04</td>
<td>14</td>
</tr>
<tr>
<td>CXCL2</td>
<td>858 ± 178</td>
<td>441 ± 71</td>
<td>0.04</td>
<td>20</td>
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<tr>
<td>IFN-γ</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
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<tr>
<td>IL-13</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>IL-6</td>
<td>nd</td>
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<td>IL-4</td>
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<tr>
<td>IL-10</td>
<td>nd</td>
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</table>

*Two-tailed Student $t$ test.
nd, Not detectable
Similar results were observed in mice sacrificed at day 21 of infection (14 d of treatment starting on day 7 postinfection), with regard to MPO activity (0.04 ± 0.009 OD in treated mice versus 0.1 ± 0.02 OD in control mice, \( p = 0.01 \)).

Finally, we measured the concentration of cytokines and chemokines (IL-1β, IL-6, IFN-γ, CCL2/MCP-1, CXCL2/MIP-2, CXCL1/KC, TGFβ, IL-13, IL-4, IL-10) in lung homogenates of chronically infected mice. As shown in Table I, mice treated with PTX3 had significantly lower levels of proinflammatory cytokines (IL-1β, IL-17) and chemokines (CCL2/MCP-1, CXCL2/MIP-2, CXCL1/KC) compared with control mice. In contrast, the level of the anti-inflammatory cytokine TGFβ-1 was significantly increased in PTX3-treated mice compared with control mice. A significant decrease in IL-1β levels was also observed in mice at day 21 after infection (234 ± 61 pg/ml in treated mice versus 316 ± 119 pg/ml in control mice, \( p = 0.05 \)), whereas a 7-d treatment upon infection did not modify IL-1β levels (data not shown).

**Treatment with PTX3 reduces histopathological lesions of chronic pneumonia**

The histopathological analysis of *P. aeruginosa* RP73-induced chronic pneumonia indicated that, in this model (14 d of infection and treatment), the lung was not totally compromised: the infection was pluri-focal and generally involved one or more lung lobes, whereas the others were unaffected or marginally involved. As shown in Fig. 4A–D, the bronchial involvement was weak.

FIGURE 4. Effect of PTX3 on histological lesions in *P. aeruginosa* chronic lung infection. A, Representative photomicrograph of lung involvement in untreated mice. Bronchi are characterized by granulocytic (arrows) and macrophagic lesions (*), the pulmonary parenchyma is infiltrated by macrophages and lymphocytes. B, Representative photomicrograph of lung involvement in PTX3-treated mice: alveolar spaces and most bronchi (*) are not involved. Few bronchi are characterized by the presence of inflammatory cells and bacteria (arrows). C, Representative photomicrograph of granulocytic lesions characterized by massive granulocyte infiltration of bronchia containing bacteria and agar beads (**). D, Representative photomicrograph of macrophagic lesions characterized by disruption of the bronchial wall (arrow) and infiltration of the bronchial lumen by foamy macrophages. E, Hyperplasia of BAL is observed in peribronchial spaces. GC, germinal center. ** represents bronchial epithelium. F, BALt is identified by the presence of a CD79a+ germinal center (GC). ** represents bronchial epithelium. Immunofluorescence staining showing fluorescent RP73 bacteria localized in beads in untreated (G) and PTX3-treated (H) mice. DAPI was used to stain nuclei. I, Percentage of parenchyma involved by inflammation and number of infected bronchi with granulocytic or macrophagic lesions in untreated and PTX3-treated mice. \( * p = 0.05; ** p < 0.01 \); two-tailed Student t test. (A, B, original magnification \( \times 4; C–F, \) original magnification \( \times 40; G, H, \) original magnification \( \times 20. \))
characterized by two kinds of lesions: one characterized by granulocytic infiltration and the second characterized by macrophagic infiltration. In granulocytic lesions of the involved area, bronchi were filled by agar beads surrounded by a massive neutrophilic infiltration. In macrophagic lesions, the bronchial wall was disrupted, the neutrophilic infiltration was replaced by foamy macrophages, and bacteria invaded the parenchyma. The surrounding parenchyma was principally infiltrated by macrophages and lymphocytes, as well as a few neutrophils. Finally, hyperplasia of the BALT, characterized by CD79⁺ germinal centers (Fig. 4E, 4F), was observed in the peribronchial area of most bronchi of involved lobes. Immunofluorescence staining showed that persisting bacteria were localized in the beads located in the bronchial lumen (Fig. 4G, 4H). As shown in Fig. 4I, we analyzed the percentage of inflamed parenchyma and the number of infected bronchi, distinguishing those with granulocytic or macrophagic lesions, in untreated and PTX3-treated mice. Histopathological lesions were less severe in mice treated with PTX3 (Fig. 4B) compared with untreated mice (Fig. 4A). In particular, the percentages of inflamed parenchyma and BALT hyperplasia and the number of infected bronchi with granulocytic or macrophagic lesions were significantly lower (Fig. 4F). Moreover, in PTX3-treated mice, the infected bronchi contained fewer and smaller agar beads compared with untreated mice, which were partially degraded by surrounding neutrophils (Fig. 4H, 4G, for treated and untreated mice, respectively).

The PTX3-mediated effect is complement and FcγR dependent

PTX3 is involved in complement activation through the classical, lectin, and alternative pathways. Moreover, pentraxins, including PTX3, interact with FcγRs and mediate part of their biological activity through the activation of these receptors (34, 35). We recently demonstrated that a complex interplay between complement activation and FcγRs underlies the opsonic activity of PTX3.

**FIGURE 5.** PTX3 facilitates the phagocytosis of P. aeruginosa through complement and FcγR. Peritoneal (A) and lung (B) CFU were counted 4 h after i.p. or i.t. injection of P. aeruginosa RP73 in WT or C1q−, C3−, or FcRγ-deficient mice. P. aeruginosa was preincubated with PTX3 (20 μg/ml) (TREATED) or PBS (CTR). C. In vitro phagocytosis of CFSE-labeled P. aeruginosa by neutrophils (CD45high, Ly6Ghigh, CD11bhigh cells) was analyzed by FACS and reported as CFSE MFI. Whole blood from C57BL/6 (WT) or C1q−, C3−, or FcRγ-deficient mice (n = 5–7) was used. P. aeruginosa was preincubated with PTX3 (20 μg/ml) or PBS (CTR). D. In vitro phagocytosis of CFSE-labeled P. aeruginosa by alveolar neutrophils from C57BL/6 (WT) and FcRγ-deficient mice was analyzed by FACS and reported as CFSE MFI (n = 5–6 replicates from two experiments). Phagocytosis was performed in the presence of 5% NHS, C1qDHS, or C3DHS. P. aeruginosa was preincubated with PTX3 (20 μg/ml) or PBS (CTR). Data were normalized and expressed as the percentage of the mean of control neutrophil MFI. *p = 0.05, **p < 0.01, ***p < 0.001; two-tailed Student t test.
against *A. fumigatus* (36). To address the molecular mechanisms underlying the protective role of PTX3 against *P. aeruginosa* RP73, we analyzed the role of complement and FcγR using two acute models of in vivo *P. aeruginosa* phagocytosis and killing in mice deficient for C1q, C3, or FcγR. In the first model, killing was analyzed by counting peritoneal and blood CFU 4 h after i.p. injection of RP73 bacteria. In the second model, killing was analyzed by counting BALF and lung CFU 4 h after i.t. injection of RP73. Treatment with PTX3 reduced the peritoneal bacterial load (*p* = 0.003) (Fig. 5A) and bacteremia (71 ± 17 CFU/ml versus 526 ± 152 CFU/ml, in treated and control mice, respectively, *p* = 0.007) (data not shown). We also observed a significant reduction in recruited cells and MPO activity in the peritoneal lavage fluid (*p* = 0.0003 and *p* = 0.004, respectively) (Table II). We first addressed the role of C1q and the classical pathway of complement activation in PTX3-mediated antibacterial activity. As shown in Fig. 5A and Table II, PTX3 retained its therapeutic potential in C1q-deficient mice, reducing the peritoneal bacterial load (*p* = 0.006), the number of recruited cells (*p* = 0.008), and MPO activity (*p* = 0.0003). In contrast, in C3-deficient mice, we did not observe a reduction in the peritoneal bacterial load upon treatment with PTX3 or the bacteremia and MPO activity (Fig. 5A, Table II, data not shown), which suggests the involvement of complement in the antibacterial activity of PTX3. Similarly, treatment with PTX3 reduced the lung bacterial load (*p* = 0.04) (Fig. 5B) and BALF cells (*p* = 0.07) (Table II). Therapeutic activity was also observed when we used recombinant murine ptx3 (4.5 × 10⁷ ± 3.6 × 10⁶ CFU in treated mice versus 9.3 × 10⁷ ± 1.5 × 10⁷ CFU in control mice, *p* = 0.01). PTX3 retained its therapeutic potential in C1q-deficient mice, reducing the lung bacterial load (*p* = 0.009) (Fig. 5B) and the number of recruited cells (*p* = 0.007) (Table II). In contrast, we did not observe a reduction in the lung bacterial load in C3-deficient mice upon treatment with PTX3 (Fig. 5B, Table II). We next analyzed the role of PTX3 in modulating the complement activity against *P. aeruginosa*. In an in vitro cytolytic assay, the complement activity of 1, 5, or 10% serum was not modified by the addition of recombinant PTX3 (20 µg/ml) to the culture (data not shown), thus excluding a role for PTX3 in amplifying a complement-dependent cytolytic effect. Finally, PTX3 lost its therapeutic potential in FcγR-deficient mice, and no differences were observed in peritoneal or lung bacterial load or in leukocyte recruitment (Fig. 5A, 5B, Table II).

Because PTX3 did not increase the complement activity against *P. aeruginosa*, we next addressed the effects of PTX3 on phagocytosis. As shown in Fig. 5C, in vivo phagocytic assays performed with CFSE-labeled RP73 in whole blood, PTX3 amplified CFSE MFI in CD45<sup>high</sup>, Ly6<sup>G</sup>high, Ly6<sup>C</sup>low neutrophils (3.3 × 10⁵ ± 5 × 10⁴ MFI versus 1.6 × 10⁶ ± 7 × 10⁵ MFI, in the presence and absence of PTX3, respectively, *p* = 0.03). Moreover, and consistent with the data obtained with the i.p. and i.t. infection models, PTX3 amplified RP73 phagocytosis independently of C1q deficiency (2.1 × 10⁴ ± 2 ± 10⁴ MFI versus 1.5 × 10⁵ ± 2 × 10⁴ MFI, in the presence and absence of PTX3, respectively, *p* = 0.03). In contrast, PTX3 phagocytic activity was abrogated when the blood of C3-deficient (5.4 × 10⁴ ± 5 × 10⁵ MFI versus 6.9 × 10⁵ ± 7 × 10⁵ MFI, in the presence and absence of PTX3, respectively, *p* = 0.12) and FcγR-deficient mice (1.5 × 10⁵ ± 5 × 10⁴ MFI versus 1.9 × 10⁵ ± 2 × 10⁵ MFI, in the presence and absence of PTX3, respectively, *p* = 0.21) was used.

We obtained similar results in vitro phagocytosis assays performed with CFSE-labeled RP73 and neutrophils recruited from the airways of WT or FcγR-deficient mice in the presence of normal serum or sera deficient of C1q or C3. As shown in Fig. 5D, PTX3 amplified phagocytosis in WT neutrophils in the presence of NHS (*p* = 0.03) or C1qDHS (*p* = 0.01) but not C3DHS, and its activity was abrogated in FcγR-deficient mice. Altogether, these data showed that PTX3 increases innate resistance to *P. aeruginosa* RP73 in a complement-dependent manner and, in particular, in a C3-dependent, but C1q-independent, manner and by facilitating recognition and phagocytosis of pathogens through the interplay between complement and FcγRs, as recently described for *A. fumigatus* (36).

**Table II.** Effects of treatment with PTX3 on cell recruitment after i.p. or i.t. injection of *P. aeruginosa* RP73

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<th>Peritoneal Cells</th>
<th>BALF Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPO (OD)</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (n = 25)</td>
<td>8 × 10⁶ ± 10⁶</td>
<td>3 ± 10⁵</td>
</tr>
<tr>
<td>PTX3 (n = 23)</td>
<td>5 × 10⁶ ± 7 × 10⁵</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>C1q knockout</td>
<td>0.0003</td>
<td>0.004</td>
</tr>
<tr>
<td>C3 knockout</td>
<td>7 × 10⁶ ± 7 × 10⁵</td>
<td>0.8 ± 0.08</td>
</tr>
<tr>
<td>PTX3 (n = 17)</td>
<td>5 × 10⁶ ± 3 × 10⁵</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>p</td>
<td>0.008</td>
<td>0.0003</td>
</tr>
<tr>
<td>FcR common γ-chain knockout</td>
<td>3 × 10⁶ ± 4 × 10⁵</td>
<td>0.4 ± 0.08</td>
</tr>
<tr>
<td>Saline (n = 15)</td>
<td>3 × 10⁶ ± 4 × 10⁵</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>PTX3 (n = 15)</td>
<td>4 × 10⁵ ± 4 × 10⁵</td>
<td>0.5 ± 0.07</td>
</tr>
<tr>
<td>p</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

**Discussion**

Chronic lung infections by *P. aeruginosa* strains are a major cause of morbidity and mortality in CF patients. Successful strategies to prevent the initial colonization, to assist in the clearance of infections, to prevent the adaptation of *P. aeruginosa* to the CF lung environment, or even to limit the excessive inflammatory response have therapeutic potential in patients with CF.

Prompted by previous studies showing increased susceptibility of ptx3-deficient mice to lung infection caused by a laboratory *P. aeruginosa* strain, as well as by genetic evidence that PTX3 gene polymorphisms affect CF-associated *P. aeruginosa* lung infections (37), we addressed the potential therapeutic effect of the long pentraxin PTX3 in chronic pneumonia induced by the *P. aeruginosa* clinical strain RP73, using an animal model of chronic bronchopulmonary infection, which closely resembles the lung pathology seen in patients with CF. Treatments, including antibiotics and anti-inflammatory agents against biofilm in murine models of chronic infection, including that reported in this article,
and in humans, are rarely completely effective, raising concerns about the eradication of chronic infections (44–46). Actually, biofilm bacteria are a major concern for clinicians in the treatment of infectious diseases because of their resistance to a wide range of therapies. In this study, we demonstrated that treatment with PTX3 significantly reduced the bacterial load and inflammation in murine lungs, facilitating pathogen recognition, phagocytosis, and killing, through the interplay with complement and FcγRs, suggesting that PTX3 is a potential therapeutic tool in chronic *P. aeruginosa* lung infections.

PTX3 activates different effector pathways that could be involved in innate resistance to this pathogen, including the classic, lectin, and alternative pathways of complement activation by binding C1q, ficolin-2, and factor H, respectively (27, 28, 30). Moreover, PTX3 could promote phagocytosis by interacting with FcγRs (35, 36).

Complement plays a key role in resistance to *P. aeruginosa* infection. The bactericidal activity of serum against specific *P. aeruginosa* strains is mostly complement mediated. Moreover, C3 opsonizes the organism via the alternative and mannose-binding lectin pathways, C5 convertase assembly occurs on the bacterial surface (47, 48), and CR3 mediates phagocytosis (49). Accordingly, complement deficiency in mice caused increased susceptibility and mortality to *P. aeruginosa* (47, 48), and mannose-binding lectin deficiency is associated with poor outcome in CF patients (50). Recently, it was shown that CRP and L-ficolin interact, leading to amplification of the classical and lectin pathways and complement-mediated anti-*P. aeruginosa* activity, such as direct lysis and phagocytosis (51). Moreover, C5a receptor plays a non-redundant role in *P. aeruginosa*-induced pneumonia, suggesting that C5a is involved in functionally activating myeloid cells (52). In particular, among its actions, C5a induces an increased expression of FcyRIII, which plays protective roles in the infection (53). Recently, it was shown that one mechanism of immune evasion of *P. aeruginosa* consists of the production of factor H and factor H-related protein-1 binding proteins, which favor factor H-dependent degradation of C3b on the pathogen surface (54). In our study, we demonstrated that the antimicrobial activity of PTX3 in acute models of infection or in vitro assays is complement dependent, because it was abolished in C3-deficient mice. We did not observe amplification of the direct lytic effect of plasma, in contrast to what was suggested for CRP (51). However, we observed amplification of phagocytosis, as observed in the case of *A. fumigatus* (18, 36). We also observed that PTX3 activity was C1q independent, thus suggesting that the interaction of PTX3–L-ficolin might synergize in complement deposition on this pathogen (31). Alternatively, we speculate that through interaction with factor H (30), PTX3 could play a role in counterbalancing the factor H-dependent immune-evasion system of *P. aeruginosa* (54).

Pentraxins, including PTX3, interact with FcγRs and mediate part of their biological activity through the activation of this pathway (34, 35). We recently demonstrated that PTX3 facilitates phagocytosis of *A. fumigatus* conidia through a complex interplay among complement, CR3, and FcγR (36). The results of this study obtained with FcRγ-deficient mice, which lack signaling from any functional activating FcRs, also demonstrated their involvement in PTX3 therapeutic activity against *P. aeruginosa*. PTX3-mediated amplification of phagocytosis and in vivo resistance to acute infection were lost in FcRγ-deficient mice.

The neutrophil burden and the concentration of proinflammatory cytokines, such as IL-1β and IL-8, in the airways correlate with the severity of pulmonary dysfunction in children and adults with CF (55, 56). IL-23 and the downstream cytokines IL-17A and IL-17F are critical molecules for proinflammatory gene expression in bronchial epithelial cells, as well as for in vivo inflammatory responses to *P. aeruginosa* (57, 58). Thus, they are likely involved in the proinflammatory cytokine network related to CF pathogenesis. Actually, levels of IL-17A and IL-17F, which regulate lung neutrophil recruitment, and of the proximal regulator IL-23p19 are elevated in the sputum of *P. aeruginosa*-colonized patients with CF undergoing pulmonary exacerbation, and they decline with therapy directed against *P. aeruginosa* (58). We found a reduction in proinflammatory cytokine (IL-1β, IL-6, IL-17) and chemokine (MCP-1/CCL2, CXCL1/KC, CXCL2/MIP-2) levels in the lung parenchyma of mice treated with PTX3 that paralleled the reduced neutrophil infiltration of the airways. We recently demonstrated that PTX3 binds to P-selecint and negatively modulates neutrophil infiltration in diverse in vivo inflammatory models, including acute lung injury (26). Moreover, PTX3 released from activated leukocytes acts locally, dampening neutrophil recruitment and regulating inflammation (26). The relative importance of antimicrobial activity versus direct inhibition of recruitment in the decreased levels of neutrophils and inflammatory cytokines associated with PTX3 therapy remains to be elucidated.

PTX3-dependent protection was also described in a LPS-dependent acute lung injury model (59, 60). However, under certain experimental conditions, such as in a very severe model of intestinal ischemia and perfusion or in severe bacterial infection, increased expression of PTX3 in transgenic mice was associated with more severe lung injury and amplification of the inflammatory response (21, 24), suggesting that, depending on the model used, PTX3 may play a dual role. Our results suggested that in the pathological context presented in this study, PTX3’s roles as pattern recognition receptor or modulator of inflammatory responses through the interaction with complement and P-selectin prevail.

The reduction of proinflammatory cytokine levels in the lungs of PTX3-treated mice was associated with increased levels of TGF-β, a powerful suppressor of inflammation. In CF patients, TGF-β expression was greater in those with mild lung disease and infrequent exacerbations, whereas low or no expression was detected in patients during an acute exacerbation (56).

In summary, we found that treatment of chronic pneumonia caused by *P. aeruginosa* with PTX3 resulted in increased bacterial clearance, reduced influx of neutrophils in the airways, and decreased levels of proinflammatory cytokines and chemokines in the lungs. Phagocytosis data suggested that FcγR and complement are involved in PTX3-mediated anti-*Pseudomonas* activity. These data suggested that PTX3 might represent a potential therapeutic tool for CF patients with chronic lung infections.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Corrections


The first institution in the author affiliations was published incorrectly. The corrected list is shown below.

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