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Prototypic Long Pentraxin PTX3 Is Present in Breast Milk, Spreads in Tissues, and Protects Neonate Mice from Pseudomonas aeruginosa Lung Infection

Sébastien Jaillon,*†‡§ Giuseppe Mancuso,§ Yveline Hamon,*†‡§ Céline Beauvillain,*†‡§ Viorica Cotici,§ Angelina Midiri,§ Barbara Bottazzi,‡ Manuela Nebuloni,** Cecilia Garlanda,‡§ Isabelle Frémaux,*†‡§ Jean-François Gauchat,†† Philippe Descamps,§ Concetta Beninati,§ Alberto Mantovani,‡†‡ Pascale Jeannin,*†‡§,† and Yves Delneste*†‡§,†,1

Newborns and infants present a higher susceptibility to infection than adults, a vulnerability associated with deficiencies in both the innate and adaptive immune systems. Innate immune receptors are sensors involved in the recognition and elimination of microbes that play a pivotal role at the interface between innate and adaptive immunity. Pentraxin 3 (PTX3), the prototypic long pentraxin, is a soluble pattern recognition receptor involved in the initiation of protective responses against selected pathogens. Because neonates are generally resistant to these pathogens, we suspected that PTX3 may be provided by a maternal source during the early life times. We observed that human colostrum contains high levels of PTX3, and that mammary epithelial cell and CD11b+ milk cells constitutively produce PTX3. Interestingly, PTX3 given orally to neonate mice was rapidly distributed in different organs, and PTX3 ingested during lactation was detected in neonates. Finally, we observed that orally administered PTX3 provided protection against Pseudomonas aeruginosa lung infection in neonate mice. Therefore, breastfeeding constitutes, during the early life times, an important source of PTX3, which actively participates in the protection of neonates against infections. In addition, these results suggest that PTX3 might represent a therapeutic tool for treating neonatal infections and support the view that breastfeeding has beneficial effects on the neonates’ health. The Journal of Immunology, 2013, 191: 1873–1882.

Newborns have an immature immune system, rendering them more susceptible to microbial infections than adults. Intrinsic and extrinsic cell factors have been involved in both innate and adaptive immunity. Neonatal immune cells exhibit functional defects compared with adult cells (1, 2). They express lower levels of some innate sensors (e.g., CD14) and signaling molecules (e.g., MyD88) than adult cells, resulting in altered cell activation in response to MyD88-dependent TLR agonists (3, 4). Neutrophils from neonates have both quantitative and qualitative insufficiencies, characterized by a defective chemotaxis (5) and a limited stock of some antimicrobial proteins (e.g., lactoferrin and bactericidal/permeability-increasing protein) (6, 7). In addition, the formation of neutrophil extracellular traps, a major component of their antimicrobial arsenal, is also delayed (8, 9).

In response to microbial moieties, neonatal myeloid cells have an increased production of Th2- and Th17-biased cytokines, and a depressed production of Th1 cytokines (2). Dendritic cells (DCs), the most potent APCs involved in the initiation and polarization of the adaptive immune responses, have an impaired production of IL-12 and type I IFN in response to inflammatory signals (10, 11). Moreover, myeloid mononuclear cells, precursors of conventional DCs, have a cytokine profile biased toward a Th2- and Th17-promoting phenotypes (11). However, some studies have reported that neonatal myeloid cells are similar to adult cells in their capacity to produce cytokines (12), and that the deficiency in producing proinflammatory cytokines in neonates is related to immunomodulatory soluble mediators (2). For instance, high levels of adenosine present in neonate blood promote the inhibition of the TLR-induced TNF-α production but preserve the production of IL-6 (1, 13). To date, the nature of the soluble immunomodulatory molecules responsible for the impaired Th1 responses in neonates remains largely undetermined (2, 14).

Breastfeeding provides optimal immune protection to compensate for this immature immune system and is considered the most effective protective means to limit childhood mortality (15–17). In addition to cytokines (e.g., IL-6, IL-10, TGF-β), lactadherin, lactoferrin, and Abs, breast milk contains biologically active and protective innate immune sensors, such as soluble CD14 (sCD14) and soluble TLR2 (16–23).

Innate immune cells sense nonsense through germine-encoded receptors, called pattern recognition receptors (PRRs), which are...
specialized in the recognition of highly conserved motifs expressed by microbes and called pathogen-associated molecular patterns (24). Based on their localization, PRRs can be classified into three groups: 1) endocytic PRRs, such as scavenger receptors and C-type lectin receptors, involved in the recognition and internalization of microbes; 2) signaling PRRs, involved in cell activation; and 3) soluble PRRs, including collectins, ficolins, and pentraxins, which can bind selected microbes and facilitate their disposal by phagocytes (25).

Pentraxins are a superfamily of evolutionarily conserved proteins including the short pentraxins serum amyloid P component and C-reactive protein, and the prototypic long pentraxin PTX3 (24). Several studies have underlined the pleiotropic activities of PTX3 in innate immunity and inflammation (reviewed in Ref. 26). PTX3 is produced by numerous cell types, including myeloid cells, endothelial cells, and epithelial cells, in response to proinflammatory cytokines, microbes, and microbial moieties (24). PTX3 is also stored in specific granules of neutrophils, which serve as a reservoir of preformed PTX3 suitable for a rapid release (27). PTX3 binds specific pathogens (e.g., Aspergillus fumigatus, Pseudomonas aeruginosa) and promotes their phagocytosis and clearance by phagocytes (27–29). PTX3 has also been involved in complement activation (30) and regulation of inflammatory response (31). The pivotal role of PTX3 in innate immunity has been evidenced in Ptx3−/− mice, which are susceptible to selected fungal, bacterial, and viral infections (32, 33). This susceptibility has been related to a deficient activation of the phagocytic cup and an inability to mount a protective Th1 response (28, 29, 32).

Given the central role played by PTX3 in the protection against selected pathogens and the high susceptibility of newborns to microbial infections, we evaluated the expression levels of PTX3 in neonates and assessed whether maternal milk may compensate for a potential defect. Moreover, we assessed whether PTX3 represents a potential therapeutic tool for treating neonatal infections.

Materials and Methods

Blood collection

Blood from healthy adults (Blood Collection Center, Angers, France) was obtained with written informed consent in accordance with the Angers University Hospital ethics committee. Cord blood was obtained after written informed consent of the mother, in accordance with the Angers University Hospital ethics committee.

Breast milk collection and human milk cell isolation

Human breast milk was obtained after written informed consent. As levels of circulating PTX3 are increased during pre-eclampsia or preterm delivery (34, 35), only healthy pregnant women with an uncomplicated term vaginal delivery were selected for this study. After collection, milk samples were immediately placed on ice and centrifuged at 3000 relative centrifugal force (rcf) for 15 min at 4°C. Pelleted human milk cells (HMCs) were washed in PBS (Lonza, Verviers, Belgium) and resuspended in culture medium (see later). Cell-free milk was subjected to a second centrifugation at 16,000 rcf for 30 min at 4°C to remove debris, and stored at −80°C. CD11b+ leukocytes were isolated from HMCs by positive selection using PE-labeled anti-CD11b mAb (BD Biosciences, San Jose, CA) followed by incubation with anti-PE Ab-coated magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany); nonretained cells were considered as epithelial cells and their purity, assessed by FACS analysis using an anti-Epithelial cell adhesion molecule (EpCAM) mAb (Miltenyi Biotech), was >97%.

Mice milk was collected from anesthetized lactating C57BL/6 mice (Charles River, L’Arbresle, France) at day 5 (D5) postdelivery.

Monocyte isolation and DC generation

PBMCs from adults and neonates were separated by Ficoll-Paque (Amersham Biosciences, Upsalla, Sweden) density gradient centrifugation. Monocytes were isolated from PBMCs by positive selection using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotech); purity, assessed by FACS analysis using an anti-CD13 mAb (BD Biosciences), was >98%. Purified CD14+ monocytes were differentiated into DCs by 5-d culture in complete medium (CM) consisting of RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 50 μM penicillin, and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA) at 5 × 10^6 cells/5 ml/well in 6-well tissue culture plates (Costar, Cambridge, MA) with 20 ng/ml IL-4 and 20 ng/ml GM-CSF (both from R&D Systems, Abingdon, U.K.).

Cell activation

DCs from adults and monocytes from neonates (0.5 × 10^6 cells/ml) were cultured in CM and stimulated with LPS (from E. coli serotype O111:B4; Sigma-Aldrich, St. Louis, MO) at the indicated concentrations, without or with human milk (0.125 or 2% final concentration, v:v) or 50 ng/ml recombinant sCD14 (R&D Systems). DCs were also stimulated with PAM3CSK4, polyinosine-polycytidylic acid [poly(I:C)] (both from Invivogen, San Diego, CA) or TNF-α plus IL-1β (R&D Systems), at the indicated concentrations. In some experiments, DCs were cultured with sCD14-depleted milk. In brief, milk was incubated with the anti-human CD14 mAb MY4 (Beckman Coulter, Fullerton, CA) or with an isotype control mAb for 16 h at 4°C. Milk samples were then incubated with protein A–coated microbeads (GE Healthcare, Orsay, France) at room temperature for 2 h. Samples were centrifuged, and sCD14-depleted milks were stored at −80°C until use.

The human mammary epithelial cell lines (MDA-MD-435, MCF-7, MDA-MD-231, MDA-MD-453, MDA-MD-468) were cultured in CM. The cell line MCF-10A was cultured in DMEM/F12 medium (Lonza) supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin (all from Sigma-Aldrich), and antibiotics. In some experiments, MCF10A cells were activated for 24 h with TNF-α plus IL-1β, at the indicated concentrations.

Quantification of PTX3 by ELISA

PTX3 was quantified by ELISA, as previously reported (36). In brief, the anti-PTX3 mAb (MN4B for human or 2C3 for murine PTX3) was coated in ELISA plates (Nunc, Roskilde, Denmark) in 10 mM NaHCO3/NaH2CO3 carbonate buffer, pH 9.6, for 16 h at 4°C. After washings with PBS containing 0.05% Tween 20 (Sigma-Aldrich) (PBS/Tween), wells were saturated with PBS/BSA 1% for 2 h at 37°C. Samples were incubated for 2.5 h at 37°C (or 16 h at 4°C for human milk) before washings with PBS/Tween. Plates were then incubated for 1.5 h at 37°C with affinity-purified polyclonal rabbit anti-PTX3 Ab (100 ng/ml, 100 μg/ml) for human PTX3 or with biotin-labeled anti-PTX3 mAb (clone 6B11; 100 ng/ml, 100 μg/ml, 100 μg/ml) for murine PTX3. After washings, plates were incubated with peroxidase-labeled goat anti-rabbit IgG Ab (1 μg/ml, 100 μg/ml, 100 μg/ml) (Biorec, Camarillo, CA) or peroxidase-labeled streptavidin (Amersham Biosciences) for 1 h at 37°C. Bound Abs were released using the 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich).

Analysis of PTX3 expression by Western blotting

Proteins were electrophoretically separated on a 10% SDS-PAGE in reducing conditions and then transferred to an Immobilon membrane (Millipore, Bedford, MA). After saturation, membranes were incubated for 16 h at 4°C with 1 μg/ml affinity-purified rabbit polyclonal anti-PTX3 Ab. When PTX3 expression was evaluated in cell extracts, protein loading was verified by incubating the membranes with a rabbit anti-actin polyclonal Ab (Sigma-Aldrich). After washing, membranes were incubated with 1 μg/ml peroxidase-labeled anti-rabbit IgG Ab (BioSource). Bound Abs were detected using ECL, according to the manufacturer’s instructions (Amersham Biosciences).

Analysis of PTX3 mRNA expression

PTX3 mRNA expression in human tissues and mammary epithelial cell lines was determined by RT-PCR. In brief, total RNA was extracted using TRIzol reagent (Life Technologies, Saint Aubin, France) following the manufacturer’s recommendations. Single-strand cDNA was synthesized using 1 μg total RNA by reverse transcription using an oligo-dT primer and reverse transcriptase (Amersham Biosciences). Human tissue cDNA and human mammary gland mRNA were purchased from Clontech (Mountain View, CA); mammary gland mRNA was reverse transcribed as mentioned earlier. PCR amplification was performed with an amount of cDNA corresponding to 25 ng starting total RNA using specific oligonucleotides. RNA integrity was assessed by GAPDH cDNA amplification. The PCR products were analyzed on a 1% agarose gel by electrophoresis and visualized with ethidium bromide.
Quantification of TNF-α by ELISA

Human TNF-α was quantified by ELISA using a commercial kit (R&D Systems).

Animals

Wild-type C57BL/6J and CD-1 mice were obtained from Charles River Laboratories (Calco, Italy). Ptx3-deficient mice were generated as described previously (32). Mice were housed in the specific pathogen-free animal facilities of Istituto Clinico Humanitas and University of Messina, in individually ventilated cages. CD-1 mice were time mated and litters used and their suffering.

In vivo experiments

C57BL/6J neonate (5 d old) or adult mice (8–12 wk old) were injected i.p. with 4 mg/kg LPS. Sera were collected 5 h later and levels of PTX3 quantified by ELISA. Recombinant murine PTX3 was biotinylated, according to the manufacturer’s instruction (Pierce, Rockford, IL). C57BL/6J neonate mice (5 d old) were given orally 5 μg biotin-labeled PTX3. Organs were collected 15 and 45 min after gavage and were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.6, 0.25% SDS, 1% Triton X-100) containing protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany). After sonication and 1-h incubation at 4˚C, lysates were centrifuged at 16,000 rcf for 15 min at 4˚C to remove debris. Proteins (60 μg/organ) were electrophoretically separated on a 10% SDS-PAGE in reducing conditions and then transferred to an Immobilon membrane. After saturation, membranes were incubated for 16 h at 4˚C with streptavidin-poly-HRP (Sanquin, Amsterdam, The Netherlands). After washing, bound streptavidin-poly-HRP was detected using ECL (Amersham Biosciences). The reference clinical P. aeruginosa strain RP73, kindly provided by A. Bragonzi (San Raffaele Scientific Institute, Milan, Italy) (37), was used in a neonatal mouse model of P. aeruginosa lung infection. Bacteria were grown at 37˚C in tryptic soy broth, as previously indicated (29). Anesthetized neonate mice (5 d old) were orally administered with recombinant PTX3 (5 μg/mouse in 5 μl PBS) or PBS (volume equivalent) 10 min before intranasal challenge with 1.5 × 109 P. aeruginosa (5 μl/mouse; placed directly into the nares). In addition, a group of neonate mice were intranasally challenged with 1.5 × 109 P. aeruginosa (5 μl/mouse) preopsonized with recombinant PTX3. Lungs were removed at 4, 24, or 48 h postinfection and homogenized in 1 ml PBS containing protease inhibitors. Samples were serially diluted and plated on tryptic soy agar plates for CFU counts.

Immunohistochemistry analysis

Frozen sections of lung tissue were cut and mounted on SuperFrost slides. After fixation with acetone/chloroform for 3 min, slides were washed in PBS and incubated for 2 h with affinity-purified polyclonal rabbit anti-PTX3 Ab. Bound Abs were revealed using nonbiotin HRP rabbit EnVision Detection System (Dako, Glostrup, Denmark) and the chromogen 3,3′-diaminobenzidine.

Statistical analysis

Expression levels of PTX3 were shown as means ± SD or means ± SEM. Median values were shown for the CFU levels. Mann-Whitney U test or Student t test were used as specified; p ≤ 0.05 was considered significant. Statistics were calculated with GraphPad Prism version 4 (GraphPad Software).

Results

Neonates have a defective expression of PTX3

Serum PTX3 levels were measured in healthy human adults and neonates. Results showed lower levels of circulating PTX3 in cord blood (0.45 ± 0.05 ng/ml; mean ± SEM, n = 11) compared with adult blood (1.143 ± 0.18 ng/ml; n = 27; Fig. 1A). In an effort to assess the capacity of neonates to produce PTX3 in vivo, circulating PTX3 was quantified in sera of adult and neonate mice, 5 h after LPS injection (i.p.). Levels of circulating PTX3 were lower in neonate mice (175.86 ± 29.94 ng/ml; mean ± SEM, n = 4) compared with adult mice (378.95 ± 15.64 ng/ml; Fig. 1B), suggesting that neonates have a reduced capacity to produce PTX3 in response to a microbial component.

PTX3 is present in human milk

Breast milk contains immune mediators that may compensate for defective production in neonates (16). Thus, we hypothesized that breast milk contains PTX3. In comparison with the levels found in the sera of healthy subjects (<2 ng/ml; Figs. 1A, 2C) (38), human colostrum contains high levels of PTX3 at D1 and D2 postdelivery (47.62 ± 4.29 and 35.24 ± 5.87 ng/ml at D1 and D2, respectively; mean ± SEM, n = 10). Then the levels of PTX3 in human milk decreased over time, as reported for sCD14 (20), and similar to the ones found in sera of healthy subjects at D5–6 (Fig. 2A and data not shown). Western blotting analysis of colostrum collected at D1, D2, and D3 revealed two major immunoreactive bands with an apparent molecular mass of ~86 and ~45 kDa (Fig. 2B), corresponding to the dimeric and monomeric forms of the protein, respectively (39). A similar profile was observed for the human recombinant PTX3 (Fig. 2B). In contrast, no PTX3 was evidenced in milk formula of bovine origin (Fig. 2B), even if the polyclonal anti-PTX3 Ab used in Western blotting recognizes the human, mouse, and bovine PTX3 (data not shown), and in agreement with the high degree of sequence homology observed in PTX3 among these three species (~80% of identity; http://www.blast.ncbi.nlm.gov).

We next verified that PTX3 present in human colostrum did not result from a systemic increase of circulating PTX3 during pregnancy and/or labor. Circulating PTX3 levels were quantified in pregnant women during labor (D-1) and in the sera and milk of the same mothers at D2. In accordance with previous reports (34, 40), a slight increase in circulating PTX3 levels was observed at the time of delivery, compared with nonpregnant healthy women (1.54 ± 0.14 and 0.83 ± 0.17 ng/ml, respectively; mean ± SEM, n = 4; Fig. 2C). The levels of circulating PTX3 at D2 were similar to the ones in nonpregnant women (0.96 ± 0.33 and 0.83 ± 0.17 ng/ml, respectively; Fig. 2C), whereas elevated concentrations of PTX3 were found in the colostrums collected at the time of blood sampling (30.94 ± 2.96 ng/ml). Therefore, human colostrum contains high levels of PTX3 that does not result from elevated levels of circulating PTX3.

FIGURE 1. Analysis of PTX3 expression levels in neonates. (A) Levels of circulating PTX3 in serum of human cord blood (n = 11) and blood from healthy adults (n = 27). (B) Neonate (5-d-old C57BL/6J mice) or adult mice (8-wk-old C57BL/6J mice) received one administration of LPS (4 mg/kg i. p.). Sera were harvested 5 h after injection, and PTX3 levels were analyzed by ELISA. (A and B) The mean value ± SEM is shown. *p ≤ 0.05, ***p ≤ 0.001, two-tailed Student t test.
Identification of PTX3-producing cells

The high concentrations of PTX3 in colostrum compared with the levels found in blood suggested a local production of this molecule in mammary gland. Therefore, we assessed whether mammary tissue and mammary epithelial cells produce PTX3. As previously reported (41–43), the PTX3 transcript is constitutively expressed in lung, ovary, and skeletal muscle (Fig. 3A, left panel). In an unexpected way, mammary gland constitutively expressed PTX3 mRNA (Fig. 3A, left panel). In addition, a constitutive expression of the PTX3 mRNA associated with a constitutive secretion of the protein and its accumulation in the culture supernatants were evidenced in the MDA-MD-231 and MDA-MD-453 mammary epithelial tumor cell lines and in the normal mammary epithelial cell line MCF-10A (Fig. 3A, right panel, 3B). In contrast, the MDA-MD-435, MDA-MD-468, and MCF-7 mammary epithelial tumor cell lines expressed low or no PTX3 mRNA, and the protein was not detected in their supernatants (Fig. 3A, right panel, 3B, and data not shown). Moreover, the production of PTX3 by the normal mammary epithelial cell line MCF-10A increased, in a dose-dependent manner, after stimulation with TNF-α and IL-1β (Fig. 3C), two PTX3-inducing cytokines present in the colostrum (16).

Human milk contains cells (referred to as HMCs), mainly epithelial cells and leukocytes. Western blotting analysis showed a constitutive expression of PTX3 in HMCs (Fig. 3D) with an electrophoretic profile similar to the one observed in human neutrophils (Fig. 3D) (27). To identify the cellular source for PTX3 production, we separated HMCs into CD11b+ EpCAM2 leukocytes and CD11b2 EpCAM+ epithelial cells. Results showed that CD11b+ EpCAM− cells, containing mainly neutrophils (16, 44, and data not shown), expressed high levels of PTX3 compared with CD11b− EpCAM+ epithelial cells (Fig. 3E).

Collectively, these data showed that mammary epithelial cells and CD11b+ HMCs constitutively produced PTX3, and that the expression of PTX3 by mammary epithelial cells can be regulated by proinflammatory cytokines.

PTX3 expression is potentiated by human milk

Previous studies have reported that molecules present in human milk may exhibit immunoregulatory properties (20, 21, 45). We thus evaluated whether human colostrum and maternal milk may modulate the LPS-induced expression of PTX3 by myeloid cells. Colostrum or milk alone did not induce significant levels of PTX3 by DC (Fig. 4A, 4B). In contrast, the production of PTX3 induced by LPS was highly potentiated in the presence of human colostrum (Fig. 4A). Similar potentiating activity was observed using colostrum collected at early time points (D1/D4) or maternal milk collected at later time points (D30/D60), underlining a long-lasting potentiating effect of milk on PTX3 production (Fig. 4B). Interestingly, human colostrum also amplified the production of PTX3 by neonate monocytes (Fig. 4C).

The potentiating effect of colostrum on the production of PTX3 was not observed in response to Pam3CSK4, poly(I:C), or TNF-α plus IL-1β (Fig. 4D), suggesting that milk contains soluble molecule(s) that selectively modulate(s) the TLR4-mediated activation of myeloid cells. A previous study reported that sCD14 contained in human milk had the capacity to potentiate the LPS-
induced cell activation (20). Accordingly, the increased secretion of PTX3 induced by colostrum was abrogated after sCD14 depletion and restored by addition of exogenous sCD14 (Fig. 4E). Collectively, these data demonstrate that human milk potentiates the LPS-induced production of PTX3 via sCD14.

PTX3 contained in maternal milk diffuses in neonate tissues

The neonatal gastrointestinal system is permeable for biomolecules present in milk, which may rapidly accumulate in the neonate tissues (22, 46, 47). To evaluate whether PTX3 contained in milk diffuses in neonatal tissues, we analyzed whether orally administered PTX3 in neonate mice can be detected in the tissues. Biotin-labeled recombinant PTX3 (biot-PTX3) was detected in the lung, liver, kidney, and, at a lower level, in the heart as soon as 15 min after oral administration (Fig. 5A). An accumulation of PTX3 was observed 45 min after gavage. According to previous works (48), Western blotting analysis (SDS-PAGE under denaturing conditions) of biotin-labeled recombinant PTX3 revealed an apparent molecular mass for the monomer of ~50 kDa (Fig. 5A). PTX3 is a multimeric protein composed of identical subunits linked by a network of disulfide bonds and high molecular masses (>50 kDa) correspond to not fully reduced PTX3 (39). In addition, we recently demonstrated that PTX3 is degraded by endogenous proteases (e.g., elastase), creating low molecular mass breakdown products (48). Therefore, low molecular mass bands (<50 kDa) observed in the kidney and in the liver of neonate mice are likely to correspond to degradation products, as reported for TGF-β (22) (Fig. 5A).

Western blotting analysis showed that PTX3 is expressed in murine colostrum, exhibiting an electrophoretic profile similar to the one observed in human colostrum (Fig. 5B). Therefore, we evaluated whether PTX3 ingested during lactation may be detected in neonates. In Ptx3−/− neonate mice breastfed by heterozygote Ptx3+/− mother, PTX3 was detected in the sera of 1 in 9 neonates at D4 and in 8 in 14 neonates at D8 after birth (Fig 5C). Moreover, immunohistochemistry analysis showed interstitial staining for
PTX3 in lung interalveolar septa of Ptx3−/− neonate mice (8 d old) born and breastfed by Ptx3−/− female mice (Fig. 5D). In contrast, PTX3 was not detected in the sera and lung sections of Ptx3−/− neonate mice born and breastfed by Ptx3+/− female mice (Fig. 5C, 5D). As positive control, high levels of PTX3 were found in the sera and lung sections of Ptx3+/+ and Ptx3−/+ neonates born and breastfed by Ptx3+/+ female mice (Fig. 5C, 5D). Collectively, these data showed that PTX3 present in maternal milk can rapidly diffuse in neonatal tissues.

**Oral administration of PTX3 protects neonate mice from P. aeruginosa lung infection**

Previous studies showed that Ptx3−/− mice had an increased susceptibility to P. aeruginosa infection, and that i.p. administration of recombinant PTX3 had a potential therapeutic effect (29, 32). Therefore, we evaluated the protective role of orally administered PTX3 in neonatal host defense against P. aeruginosa lung infection. At 4 h postinfection, similar levels of lung bacterial burden were observed in PTX3-treated (oral PTX3 + P. aeruginosa) and nontreated (P. aeruginosa) mice, showing that mice were equivalently infected (Fig. 6). In contrast, oral administration of PTX3 significantly reduced the lung bacterial burden found at 24 h postinfection (0.50 × 10^5 CFU/lungs; 6.5 × 10^5 – 1 versus 7.25 × 10^4 CFU/lungs; 2 × 10^6 – 250 in PTX3-treated and nontreated mice, respectively; median; interquartile range [IQR], p = 0.03) and 48 h postinfection (1 CFU/lungs; 6 × 10^4 – 1 versus 9.5 × 10^3 CFU/lungs; 2.5 × 10^3 – 1 in PTX3-treated and nontreated mice, respectively; median; IQR, p = 0.03), suggesting a long-lasting protective effect of orally administered PTX3 (Fig. 6). In addition, we assessed whether bacterial preopsonization with recombinant PTX3 (PTX3-opsonized P. aeruginosa) has a protective effect, as shown in adult mice (29). At 24 h postinfection, lung bacterial burden was significantly reduced in mice infected with PTX3-preopsonized P. aeruginosa (1.25 × 10^5 CFU/lungs; 7 × 10^5 – 1; median; IQR, p = 0.03) compared with mice infected with nonopsonized bacteria (Fig. 6). However, only a nonstatistically significant trend toward a protective effect was observed at 48 h postinfection (1.1 × 10^5 CFU/lung; 7.5 × 10^4 – 1; median; IQR, p = 0.15), suggesting the lack of long-lasting protective effect in the case of bacterial preopsonization alone (Fig. 6). Collectively, these data showed that PTX3 contained in colostrum/milk may prevent the colonization of the lung by P. aeruginosa, and that oral administration of PTX3 represents a potential therapeutic tool for neonatal infections.

**Discussion**

Newborns and infants present a higher susceptibility to infection than adults, a vulnerability associated with deficiencies in both the innate and adaptive immune systems. In this study, we report the constitutive expression of PTX3 in human and murine colostrum both in soluble and cell-associated forms that could compensate for defective production in neonates. PTX3 spreads rapidly in neonatal tissues, and one oral administration of PTX3 protects neonate mice against P. aeruginosa lung infection.
In the early months of life, breastfeeding represents an important source of protective immune molecules and is believed to be the main preventive means to reduce the death rate of children younger than 5 years (16). Breast milk contains immunomodulatory mediators, such as cytokines (e.g., IL-10, TGF-β, IL-1β), Abs, and soluble PRR (e.g., sCD14, soluble TLR2), which can compensate and/or instruct the immature immune system of neonates (16–23).

In this article, we show that colostrum contains high levels of soluble PTX3 compared with the ones found in the sera of healthy adults and mothers (before or after delivery). As reported for sCD14 (20), the concentration levels of PTX3 in colostrum decreased in a time-dependent manner, and at D5/6 postdelivery, these levels were equivalent to the ones found in the sera of healthy adults. We assume that this decrease is related to the increasing volume of colostrum/milk ingested by the neonates over time. Therefore, the quantity of PTX3 incorporated by the neonates remains high over time.

Except in neutrophils (27), which store the molecule, PTX3 is produced by diverse cell types on demand, in response to proinflammatory signals (24). In this study, we extended this observation to mammary epithelial cells. Interestingly, this production is potentiated by TNF-α, which plays an important role in the mammary gland development and in the regulation of milk protein expression (49). Because adipocytes also produce PTX3 (50), we cannot...
were counted at 4, 24, and 48 h after data as pooled uncensored values of individual mice. Total lung CFUs repeated the experiment three times with similar results and present the value is shown. *

mice. Therefore, breastfeeding induces the accumulation of PTX3 from adipocytes surrounding the mammary ducts and alveoli.

PTX3, accumulated in tissues, conserved a multimeric structure (46, 47). In accordance with this property, orally administered molecules and milk cells. Consequently, ingested molecules and especially neutrophils, represent an alternate source of PTX3 for neonates.

In addition to containing PTX3, human colostrum (collected at D1 and D4 postdelivery) and also maternal milk (collected at D30 and D60 postdelivery) increased the production of PTX3 by immune cells in response to LPS, a property related to the presence of sCD14 in milk and in line with the high concentration of sCD14 in maternal milk until 6 mo postdelivery (>10 μg/ml) (20). A previous study reported that milk can prevent the Pam3CSK4- and poly(I:C)-induced cell activation (45). In our experimental conditions, the expression of PTX3 induced by these TLR agonists was not or was poorly modulated by maternal milk. These differences may result from the use of different cell isolation protocols and/or origin of the TLR agonists.

The susceptibility of neonates to infections has been related to the functional immaturity of their immune cells (1, 16). In vivo studies have underlined the pivotal role played by PTX3 in the protection against selected bacteria, fungi, and viruses (28, 32, 33). Therefore, we evaluated the ability of orally administered PTX3 to protect neonate mice against *P. aeruginosa*, which is responsible for a wide variety of clinical symptoms from mild infection (e.g., pneumonia) to sepsis or death among infants in neonatal intensive care (57). Moreover, the high rate of resistance to antibiotics underlines the current need to develop alternative therapies against *P. aeruginosa* infection (58). Given that Ptx3 deficiency has been associated with severe female subfertility (42), it was technically impossible to get a sufficient number of Ptx3-deficient neonate mice breastfed by Ptx3+/− female mice and to compare them with Ptx3-deficient neonate mice breastfed by Ptx3+/− female mice. Therefore, we evaluated the protective role of orally administered PTX3 in wild-type neonate mice infected intranasally with *P. aeruginosa*. Moreover, we assume that a treatment of PTX3 in Ptx3-competent mice better reflects physiological conditions. Our results showed that a single administration of PTX3 is sufficient to significantly reduce the number of *Ptx3*-deficient neonate mice breastfed by *Ptx3*−/+ female mice and to compare them with *Ptx3*-deficient neonate mice breastfed by *Ptx3*−/+ female mice. Therefore, we evaluated the protective role of orally administered PTX3 in wild-type neonate mice infected intranasally with *P. aeruginosa*. Moreover, we assume that a treatment of PTX3 in Ptx3-competent mice better reflects physiological conditions. Our results showed that a single administration of PTX3 is sufficient to significantly reduce the number of *P. aeruginosa* in the lung of infected neonate mice. Interestingly, the protective effect, observed at 24 h postinfection, was maintained 48 h after the bacterial challenge, suggesting a long-lasting protective effect of orally administered PTX3. In contrast, the protective effect observed at 24 h postinfection by a preopsonization of bacteria with recombinant PTX3 was partially lost 48 h postinfection, suggesting the lack of long-lasting protective effect in the case of bacterial preopsonization.

In conclusion, this study shows that breastfeeding constitutes an important source of the humoral PRR PTX3, which may protect the neonates against infections. In addition, these results suggest that PTX3 might represent a therapeutic tool for treating neonatal infections and support the view that breastfeeding has beneficial effects on the neonates’ health.

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**FIGURE 6.** Oral administration of PTX3 protects neonate mice from *Pseudomonas aeruginosa* lung infection. Neonate mice (5 d old) were intranasally inoculated with 1.5 × 10⁶ *P. aeruginosa* 10 min after one oral administration of PTX3 (5 μg/mouse in 5 μl PBS) or PBS (5 μl/mouse). In addition, a group of neonate mice was intranasally challenged with 1.5 × 10⁶ *P. aeruginosa* (5 μl/mouse) preopsonized with recombinant PTX3. We repeated the experiment three times with similar results and present the data as pooled uncensored values of individual mice. Total lung CFUs were counted at 4, 24, and 48 h after *P. aeruginosa* infection. The median value is shown. *p ≤ 0.05, two-tailed Mann–Whitney U test.

exclude that soluble PTX3 found in colostrum may also derive from adipocytes surrounding the mammary ducts and alveoli.

In addition to epithelial cells, milk also contains leukocytes, notably neutrophils (40–65%), macrophages (35–55%), and lymphocytes (5–10%). These leukocytes colonize the tissues of the neonate and can participate in the immune response (16, 44, 51, 52). Our results showed that leukocytes from HMC express PTX3, and that HMC-associated PTX3 exhibits an electrophoresis migration profile similar to the one observed in neutrophil-associated PTX3 (27), suggesting that neutrophils may represent the main cellular source of cell-associated PTX3 in HMC. The absence or low expression of PTX3 by CD11b⁺ EpCAM⁺ epithelial cells present in milk, compared with mammary epithelial cell lines, may be related to the fact that these cells are dying at the time of sampling.

The gastrointestinal system of neonates is permeable for macromolecules and milk cells. Consequently, ingested molecules and cells are rapidly found in plasma and organs after feeding (22, 44, 46, 47). In accordance with this property, orally administered recombinant PTX3 was rapidly accumulated in neonatal tissues. In addition, Western blotting analysis revealed that exogenous PTX3, accumulated in tissues, conserved a multimeric structure required for its biological activity (39, 53). Endogenous PTX3 was detected in the sera and in the interalveolar septum of lung sections from *Ptx3*−/− neonates born and breastfed by *Ptx3*−/+ female mice. Therefore, breastfeeding induces the accumulation of PTX3 in neonatal tissues. We cannot exclude that a part of PTX3 ob-
References


