Production of the soluble pattern recognition receptor PTX3 by myeloid, but not plasmacytoid, dendritic cells

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PTX3 is a prototypic of long pentraxin consisting of an N-terminal portion coupled to a C-terminal pentraxin domain, the latter related to short pentraxins (C-reactive protein and serum amyloid P component). PTX3 is a soluble pattern recognition receptor, which plays a non-redundant role in resistance against selected pathogens and in female fertility. The present study was designed to analyze the production of PTX3 by human dendritic cells (DC) and to define the role of different innate immunity receptors in its induction. Human monocyte-derived DC produced copious amounts of PTX3 in response to microbial ligands engaging different members of the Toll-like receptor (TLR) family (TLR1 through TLR6), whereas engagement of the mannose receptor had no substantial effect. DC were better producers of PTX3 than monocytes and macrophages. Freshly isolated peripheral blood myeloid DC produced PTX3 in response to diverse microbial stimuli. In contrast, plasmacytoid DC exposed to influenza virus or to CpG oligodeoxynucleotides engaging TLR9, did not produce PTX3. PTX3-expressing DC were present in inflammatory lymph nodes from HIV-infected patients. These results suggest that DC of myelomonocytic origin are a major source of PTX3, a molecule which facilitates pathogen recognition and subsequent activation of innate and adaptive immunity.

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1 Introduction

Pentraxins are a superfamily of proteins conserved during evolution from *Limulus polyphemus* to man, usually characterized by a pentameric structure [1–4]. The classical short pentraxins CRP and SAP are acute phase proteins in man and mouse, respectively, and are made in the liver in response to inflammatory mediators, most prominently IL-6 [3, 4]. A number of ligands, recognized in a calcium-dependent manner, have been identified for CRP and SAP, including phosphoethanolamine (PE), phosphocholine (PC), DNA, immunecomplexes, sugars. Due to these properties short pentraxins regulate innate resistance to microbes and scavenging of cellular debris and extracellular matrix components [1–4].

Pentraxin 3 (PTX3) is the first long pentraxin identified. PTX3 has similarities with the classical short pentraxins, however, has an unrelated long N-terminal domain coupled to the C-terminal domain, and differs in gene organization, chromosomal localization, cellular source and ligands recognized [5, 6]. PTX3 was cloned as an IL-1β inducible gene in endothelial cells [7] and as a TNF-α inducible gene (TSG14) in fibroblasts [8]. PTX3 is rapidly produced and released by diverse cell types, in particular by mononuclear phagocytes and endothelial cells, in response to primary inflammatory cytokines, *in vitro* and *in vivo* [7–13]. Increased circulating levels of this protein have been detected in different infectious and inflammatory conditions [11, 14–16].

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Abbreviations: CpG ODN: Bacterial oligodeoxynucleotide with CpG motifs **MoDC:** Monocyte-derived DC **MyDC:** Myeloid DC **Omp A:** Outer membrane protein A **pDC:** Plasmacytoid DC **PGN:** Peptidoglycan **poly (I): (C):** Polyionisic-polycytidylic acid **PTX3:** Pentraxin 3 **TLR:** Toll like receptors

PTX3 does not bind classical pentraxin ligands [5]. In contrast PTX3 binds with high affinity C1q [5, 17] and selected microorganisms like *Aspergillus fumigatus* (*A. fumigatus*), *Salmonella typhimurium* and *Pseudomonas aeruginosa* (*P. aeruginosa*) [18]. Recent studies in PTX3–/– mice have shown that this molecule plays complex non-redundant functions *in vivo* [13, 18, 19], ranging from female fertility to innate immunity. In particular PTX3 binds to selected pathogens, including conidia of *A. fumigatus* and facilitates their recognition by macrophages and dendritic cells (DC). PTX3–/– mice show defective resistance against *A. fumigatus*, and infected mice have Th2 polarization of lung adaptive immunity [18].

DC play a key role in activation and orientation of adaptive immunity [20–22]. It was therefore important to investigate the capacity of different DC populations to produce PTX3. Here we report that DC belonging to the myelomonocytic differentiation pathway produce copious amounts of PTX3 upon engagement of different Toll-like receptors (TLR) [23, 24]. In contrast, plasmacytoid DC (pDC) exposed to appropriate stimuli are unable to produce this molecule. Therefore, myeloid DC are a major source of the prototypic long pentraxin PTX3, a soluble pattern recognition receptor which facilitates interaction with pathogens and amplifies innate immunity.

2 Results

2.1 Production of PTX3 by MoDC

Previous studies had shown that mononuclear phagocytes are major producers of the prototypic long pentraxin PTX3 [25–27]. Here we wanted to examine how differentiation to DC affected their capacity to produce PTX3. Fig. 1 shows experiments in which monocytes, MoDC and monocyte-derived macrophages from the same individual were assessed for their capacity to produce PTX3 in response to LPS (100 ng/ml). There was considerable donor-to-donor variability, but in all individuals tested MoDC were by far better producers of PTX3 than monocytes and monocyte-derived macrophages. As summarized in Fig. 1, in this series of experiments conducted over a period of 6 months MoDC produced 53±8.1 ng/ml/10^6 cells (n =33) of PTX3, compared to 11±4.3 ng/ml/10^6 cells (n =20) and 12±6 ng/ml/10^6 cells (n =15), for monocytes and monocyte-derived macrophages, respectively. Similar results were obtained when different LPS concentrations and different incubation times were tested (Fig. 2).

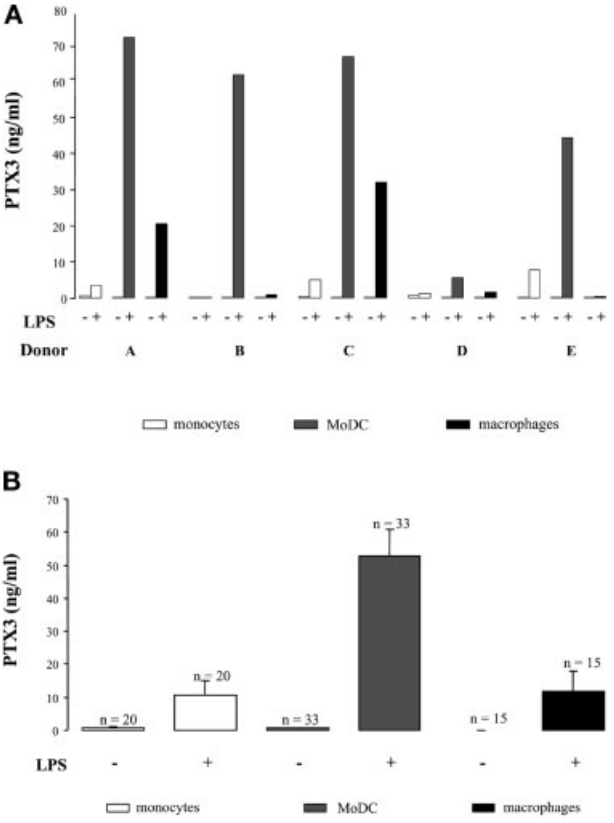

Fig. 1. Production of PTX3 by monocytes, MoDC and *in vitro*-generated macrophages. Cells were stimulated for 24 h in the presence of LPS (100 ng/ml). PTX3 was measured by ELISA. (A) Cells generated from each donor and tested in parallel. (B) Values of PTX3. Results are mean ± SE of several experiments; *n*, number of experiments performed.

2.2 Induction of PTX3 production by different TLR agonists

MoDC express a wide spectrum of TLR [28]. TLR activate a similar signaling pathway [29, 30], but different members of the TLR family differ i their capacity to activate collateral signaling molecules (*e.g*. MAL/TIRAP) and to induce production of downstream effectors [24, 31, 32]. It was therefore important to assess whether different TLR agonists induce production of PTX3 in MoDC. As shown in Fig. 3, in addition to LPS (TLR4), Omp A (TLR2), PGN (TLR2), poly (I):(C) (TLR3), *Candida* (TLR4), and flagellin (TLR5) induced PTX3 production, with LPS being the most effective stimulus. As discussed below, CpG ODN 2006 did not induce PTX3 production in MoDC (not shown), consistently with the low expression of TLR9 in myeloid DC. PTX3 protein production was associated with induction of PTX3 transcripts, already evident after 1 h of stimulation (Fig. 4).

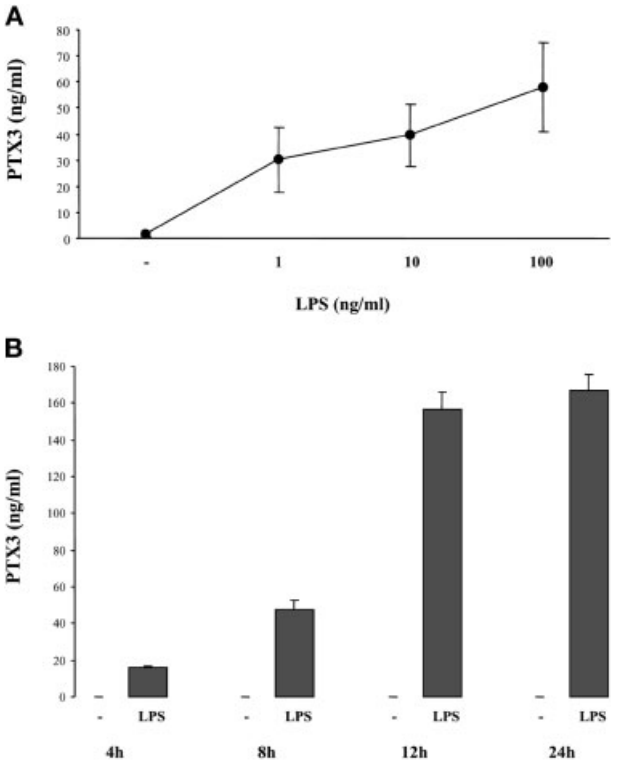

Fig. 2. Dose/response (A) and kinetics (B) of PTX3 production in MoDC treated with LPS. Results are mean ± SE of four different donors.

Similar to human cells, murine bone marrow-derived myeloid DC produced copious amounts of PTX3 after challenge with different TLR ligands (unpublished observations).

2.3 Production of PTX3 by myeloid, but not plasmacytoid, DC

The results discussed so far were obtained using DC generated from blood monocytes in appropriate culture conditions. DC are heterogeneous and pDC represent a well defined cell population present in blood and lymphoid tissues with distinct surface phenotype and functional properties [20, 22, 33–36]. It was therefore important to ascertain whether circulating MyDC (HLA-DR$^+$, Lin$^-$, CD11c$^+$, CD19$^-$, CD1a$^+$ and BDCA-1$^+$) have the capacity to produce PTX3 and whether this property is shared by pDC (CD4$^+$, CD11c$^-$, Lin$^-$, CD123$^+$ and BDCA-4$^+$). As shown in Fig. 5 and 6, exposure to LPS, PGN, poly (I):(C) or whole *A. fumigatus* conidia or bacteria (*P. aeruginosa*) induced PTX3 production in freshly isolated MyDC, consistently with their ample repertoire of functional TLR. pDC did not produce PTX3 in response to the same signals, as expected on the basis of defective expression of TLR2, TLR3, TLR4, TLR5 and TLR8 [37, 38]. Unlike MyDC, pDC have high levels of TLR7 and TLR9 in humans. However, CpG ODN 2006 (TLR9) did not induce PTX3 production in pDC. Similarly, exposure to influenza virus failed to cause PTX3 production in pDC. It should be emphasized that under the same conditions CpG ODN and influenza virus induced copious amounts of selected chemokines [39].

2.4 PTX3 in DC *in vivo*

In an effort to assess whether DC actually produce PTX3 *in vivo* under inflammatory conditions, lymph nodes from HIV-infected patients were examined by immunohistology. As shown in Fig. 7, PTX3$^+$ cells were identified in inflammatory lymph nodes from HIV-infected patients. They were localized in the lymph node paracortex and expressed CD1a. These results are consistent with the

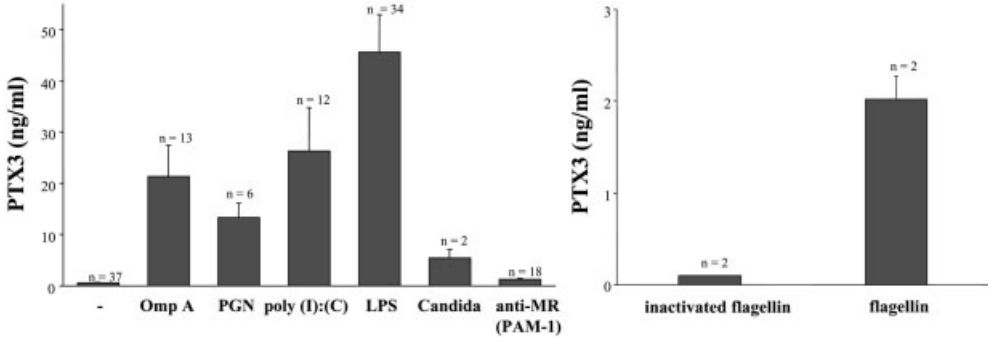

Fig. 3. Production of PTX3 in MoDC in response to various ligands recognized by pattern recognition receptors. The left and right panel refer to two different series of experiments (*n*, number of experiments performed).

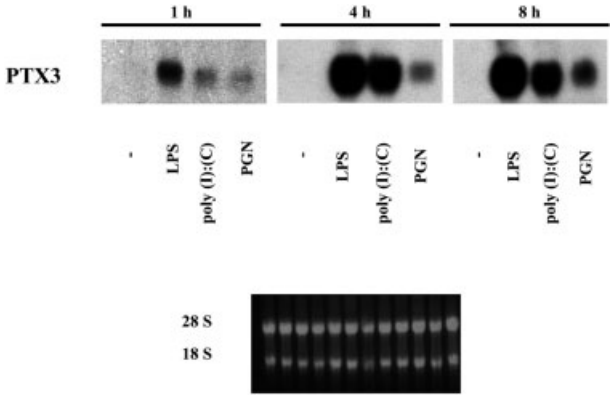

Fig. 4. mRNA expression of PTX3 in MoDC stimulated by LPS (100 ng/ml), poly (I):(C) (10 μg/ml) and PGN (10 μg/ml). The lower part is the ethidium bromide-stained membrane.

notion that DC exposed to maturation signals produce PTX3 *in vitro* and *in vivo*.

3 Discussion

The long pentraxin PTX3 is a soluble pattern recognition receptor produced by diverse cell types in response to primary inflammatory cytokines and microbial products [7–13]. Previous studies have shown that mononuclear phagocytes produce copious amounts of PTX3 in response to these signals [25–27]. The results presented here demonstrate that DC of myelomonocytic origin are the most efficient producers of PTX3 among the cell types considered in the present study.

DC express a wide repertoire of TLR [28, 37, 38]. Molecules recognized by different members of the TLR family (TLR1, TLR2, TLR3, TLR4, TLR5 and TLR6) are able to induce PTX3 production in DC and monocytes. Members of the TLR family activate a signaling cascade initiated by the adapter protein MyD88, leading to NFkB activation [29, 30]. However, different TLR differ in the capacity to recruit alternative adapter proteins (*e.g.* TIRAP/MAL) [24, 32], and to induce the production of selected downstream effector molecules, such as the interferon (IFN)-inducible chemokine CXCL10. The finding that agonists of different TLR induce production of PTX3, a NF-κB-regulated gene [40, 41], suggests that PTX3 is downstream of the common core signaling MyD88 pathway.

DC are heterogeneous in terms of morphology, membrane phenotype and function [20–22]. pDC are a specialized subset characterized by high production of IFN. In humans, pDC express high levels of TLR9, unlike DC

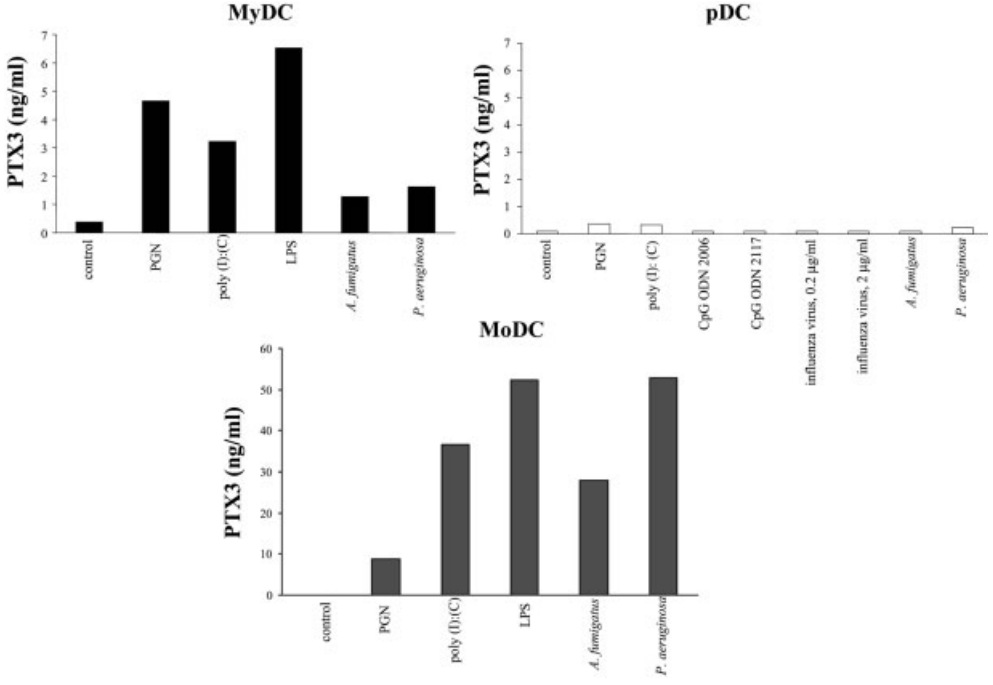

Fig. 5. Production of PTX3 by different DC populations. MyDC, pDC and monocyte-derived DC were obtained from the same donor. Shown is a representative experiment of three performed.

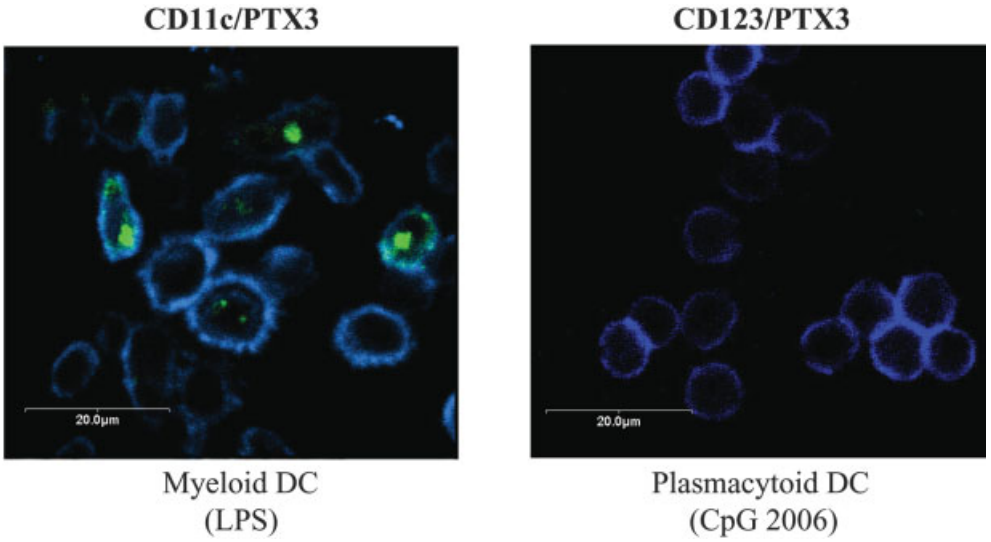

Fig. 6. Single-cell analysis of PTX3 expression in myeloid and plasmacytoid DC. Confocal analysis after 4 h of stimulation with LPS (MyDC) or CpG (pDC). PTX3$^+$, green, CD11c$^+$ (MyDC) and CD123$^+$ (pDC), blue. Control unstimulated cells were negative for PTX3 (not shown).

of myelomonocytic origin [37, 38]. The results presented here demonstrate that pDC are unable to produce PTX3 in response to a wide range of stimuli, including CpG ODN, which activate TLR9. Therefore PTX3 production is restricted to DC of myelomonocytic origin.

Analysis of gene-targeted mice has revealed that PTX3 is a unique pattern recognition receptor, which plays a non-redundant role in female fertility and in resistance against selected pathogens (*A. fumigatus* and *P. aeruginosa*) [18, 19]. The susceptibility of PTX3 deficient mice to *A. fumigatus* is associated with failure to mount an adaptive

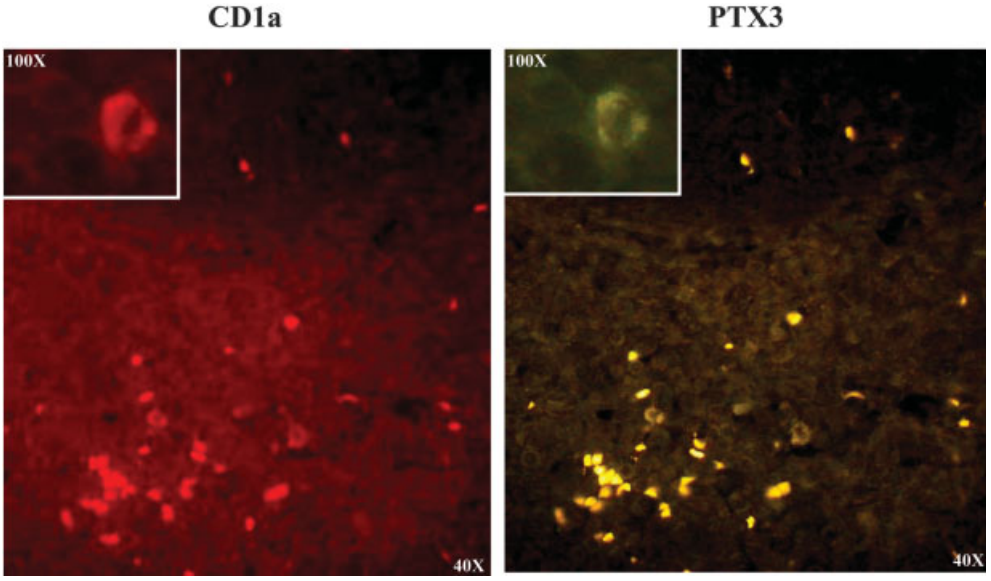

Fig. 7. Expression of PTX3 in DC *in vivo*. Lymph node section from an HIV-infected subject. Double immunofluorescent staining using anti CD1a and anti PTX3 mAb is shown. A few cells in the paracortex are positive for both antibodies (CD1a$^+$, red; PTX3$^+$, green).

type I immune response [18]. It binds selected microbial agents (*e.g*. conidia of *A. fumigatus*; *P. aeruginosa*) and activates at least two effector pathways: by binding C1q, it activates the classic pathway of complement activation [5, 17]; by interacting with unidentified cellular receptor(s) it facilitates pathogen recognition and disposal by macrophages [18]. Therefore, rapid triggering of copious amounts of PTX3 production in DC via members of the TLR family may result in the amplification of innate resistance in tissues. Moreover, PTX3 production is likely to facilitate pathogen recognition by DC themselves and hence the activation and orientation of adaptive immunity.

4 Materials and methods

4.1 Cell culture media and reagents

The following reagents were used for tissue culture: pyrogen-free saline (S. A. L. F., Bergamo, Italy), RPMI 1640 (Biochrom, Berlin, Germany), 200 mM L-glutamine (Biochrom, Berlin, Germany), aseptically collected fetal calf serum, FCS, (HyClone Laboratories, Logan, UT), and bovine serum albumin (BSA), with low endotoxin characteristics, (SIGMA-Aldrich). Human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) was a gift from Novartis (Milan, Italy) and human IL-13 was a gift from Dr. A. Minty (Sanofi Elf Bio Recherches, Labège, France). Human recombinant macrophage (M)-CSF was obtained from Peprotech (London, GB). All reagents contained $<$0.125 endotoxin units/ml, as checked by the *Limulus* amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD).

Lipopolysaccharide (LPS), from *Escherichia coli*, strain 055:B5 (LPS) was obtained from Difco (Detroit, MI). Omp A, from *Klebsiella pneumoniae*, was kindly provided by Dr. Pascale Jeannin (Institute de Recerche P. Fabbre, St. Julien en Genevois, France). Peptidoglycan (PGN), from *Staphilococcus aureus*, was purchased from Fluka Chemie (Switzerland). Poly (I):(C) was obtained from Amersham Pahrmacia Biotech (Amersham, GB). Conidia of *A. fumigatus* and *Candida albicans* were provided by Dr. L. Romani, University of Perugia (Italy). *Candida albicans* was prepared as previously described [42]. *Pseudomonas aeruginosa* was obtained from ATCC (10145). Flagellin, from *Salmonella typhimurium*, and trypsin inactivated flagellin were a kind gift of Dr. Jean-Claude Sirard (ISREC, Epalinges, Switzerland) [43]. CpG ODN motif GTCGTT (2006) and GACGTT (2117) were provided by Invitrogen Life Technologies (Rockville, MD). Inactivated influenza virus strain A/Moscow/10/99 was a gift from Dr. Adorini (BioXell, Milan, Italy) and Dr. T. Magistris (Istituto Superiore di Sanità, Rome, Italy). The properties of an anti-mannose receptor monoclonal antibody (mAb) PAM-1 have been described [44, 45].

Endotoxin contamination was monitored by *Limulus* amebocyte lysate assay (0.125 EU/ml sensibility endpoint, BioWhittaker).

4.2 Mononuclear phagocytes and DC

Blood monocytes were purified under endotoxin-free conditions from fresh buffy coats of healthy donors (courtesy of the Centro Trasfusionale, Ospedale Niguarda, Milan, Italy) by Ficoll (Biochrom) and Percoll (Amersham, Uppsala, Sweden) gradients as described [46, 47]. Monocytes were cultured in RPMI 1640 medium with 2 mM L-glutamine and 0.2% of BSA at 1×10^6 cells/well/ml in 24-well tissue culture plates (Falcon; BD Biosciences, Franklin Park, NJ) in the presence of LPS (100 ng/ml). To generate macrophages, monocytes were cultured for 5 days at 1×10^6 cells/ml in 6-well tissue culture plates (Falcon; BD Biosciences) in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FCS, 20 ng/ml M-CSF, in endotoxin-free conditions. MoDC were generated as previously described [46, 47]. Briefly, blood monocytes were cultured for 6 days at 1×10^6 cells/ml in 6-well tissue culture plates (Falcon; BD Biosciences) in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FCS, 50 ng/ml GM-CSF and 20 ng/ml IL-13. MoDC were then cultured for 24 h in RPMI 1640 medium supplemented with 2 mM L-glutamine and 0.2% BSA at 1×10^6 cells/well/ml in 24-well tissue culture plates (Falcon; BD Biosciences) in the presence of different stimuli: LPS, 100 ng/ml; Omp A, 10 μg/ml; PGN, 10 μg/ml; poly (I):(C), 10 μg/ml; flagellin, 1 μg/ml; *Candida albicans*, 1 μg/ml; conidia of *A. fumigatus* at cells to fungi ratio of 1:2; *P. aeruginosa*, at cells to bacteria ratio of 1:2; anti-mannose receptor mAb PAM-1, used at 2 μg/ml.

Peripheral blood MyDC and pDC were magnetically sorted with BDCA-1 and BDCA-4 cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, as described [36] to a purity of 95–98%. MyDC and pDC were cultured at 2×10^5 cells/well/200 μl in 96-well plates (Falcon; BD Biosciences) in culture medium supplemented with 0.2% of BSA and 2 mM L-glutamine, with the above-described stimuli. Moreover CpG oligodeoxynucleotides 2006 and 2117, 2 μg/ml, and inactivated influenza virus strain A/Moscow/10/99, 0.2 μg/ml and 2 μg/ml, were used. After 24 h of culture, supernatants were collected, and PTX3 concentrations were measured by ELISA.

4.3 PTX3 protein and transcripts levels

PTX3 concentrations were detected using an ELISA assay previously described [11, 16]. For Northern analysis total RNA was extracted by the guanidinium thiocyanate or by TRIzol method in according to manufacturer's instructions (Invitrogen, Life Technologies), blotted, and hybridized as described [7]. Briefly, RNA (10 μg/lane) was fractionated on 1% agarose-formaldehyde gels and transferred to a nylon

membrane (NEN™ Life Science Products, Boston, MA). Probes were labeled using the Megaprime DNA labeling system (Amersham International, Little Chalfont, GB) with [^{32}P]dCTP (3,000 Ci/mmol; Amersham International). Membranes were prehybridized at 42°C in Hybrisol (Oncor, Gaithersburg, MD) and hybridized overnight with 1×10^6 cpm/ml ^{32}P-labeled probe. Membrane were then washed three times with 2× SSC (1× SSC =0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at room temperature for 10 min, twice with 2× SSC/1% SDS at 60°C for 20 min, and then with 0.1× SSC for 5 min before being autoradiographed using Kodak XAR-5 films (Eastman Kodak, Rochester, NY) and intensifier screen at –80°C. For Northern analysis, a human full-length cDNA probe for PTX3 were used.

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