Complexity and Complementarity of Outer Membrane Protein A Recognition by Cellular and Humoral Innate Immunity Receptors

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Summary

Outer membrane protein A (OmpA) is a conserved major component of the outer membrane of Enterobacteriaceae. Here, we report that OmpA from Klebsiella pneumoniae (KpOmpA) activates macrophages and dendritic cells (DCs) in a TLR2-dependent way. However, TLR2 does not account for binding of KpOmpA to innate immune cells. KpOmpA binds the scavenger receptors (SRs) LOX-1 and SREC-I, but not other members of the same family. LOX-1 colocalizes and cooperates with TLR2 in triggering cellular responses. The TLR2-activated functional program includes production of the long pentraxin PTX3, a soluble pattern recognition receptor involved in resistance against diverse pathogens. PTX3, in turn, binds KpOmpA but does not affect recognition of this microbial moiety by cellular receptors. KpOmpA-elicited in vivo inflammation is abrogated in TLR2−/− mice and significantly reduced in PTX3−/− mice. Thus, SR-mediated KpOmpA recognition and TLR2-dependent cellular activation set in motion a nonredundant PTX3-mediated humoral amplification loop of innate immunity.

Introduction

Innate immunity is the first line of defense against pathogens and plays a key role in the activation and orientation of adaptive immunity. Innate immunity receptors, called pattern-recognition receptors (PRRs), recognize a few highly conserved structures on microorganisms called pathogen-associated molecular patterns (PAMPs). PRRs are either expressed on cell surface or secreted and present in body fluids. There are two functionally different classes of cell surface PRRs: endocytic PRRs (i.e., scavenger receptors and mannose receptors) involved in the binding and uptake of pathogen components and signaling PRRs (members of the Toll-like receptor [TLR] family) involved in cell activation upon contact with pathogens. The humoral arm of the innate immunity includes collectins and pentraxins (Gordon, 2002; Medzhitov, 2001; O’Neill, 2003).

Pentraxins are a superfamily of proteins usually characterized by a pantameric structure and are highly conserved during evolution (Garlanda et al., 2005; Mantovani et al., 2003; Pepys and Hirschfield, 2003; Szalai et al., 1999). The classical short pentraxins C-reactive protein (CRP) and serum amyloid P component (SAP) are acute phase proteins in man and mouse, respectively (Baumann and Gauldie, 1994; Pepys and Baltz, 1983). They are produced in the liver in response to inflammatory mediators, most prominently IL-6. CRP and SAP bind, in a calcium-dependent manner, different ligands, and they are involved in the innate resistance to microbes and the scavenging of cellular debris and extracellular matrix components (Pepys and Hirschfield, 2003; Szalai et al., 1999). Long pentraxins are characterized by an unrelated N-terminal domain coupled to a pentraxin-like C-terminal domain (Garlanda et al., 2005; Mantovani et al., 2003). The prototypic long pentraxin PTX3 is rapidly produced and released by diverse cell types, in particular by mononuclear phagocytes, DCs, and endothelial cells (Breviario et al., 1992; Doni et al., 2003; Lee et al., 1993; Vidal Alles et al., 1994), in response to primary inflammatory signals (e.g., TLR engagement, TNFα, and IL-1). PTX3 binds with high affinity the complement component C1q, the extracellular matrix protein TSG6, and selected microorganisms (Bottazzi et al., 1997; Garlanda et al., 2002; Nauta et al., 2003; Salustri et al., 2004). PTX3 activates the classical pathway of complement activation and facilitates pathogen recognition by macrophages and DCs (Diniz et al., 2004; Garlanda et al., 2002; Nauta et al., 2003). Recent studies in ptx3-deficient (PTX3−/−) mice have shown that this molecule plays complex nonredundant functions in vivo, ranging from the as-
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Figure 1. Binding of KpOmpA to the Scavenger Receptors

(A) Maleylated BSA prevents KpOmpA binding to APCs. Macrophages and myeloid DCs were incubated with 2.5 μM BSA or mBSA. After 10 min, 0.25 μM Alexa 488-labeled KpOmpA was added before FACS analysis. Gray histogram corresponds to cells incubated with 0.25 μM Alexa 488-labeled TT (and is similar to unlabeled cells).

(B) Ligands of SRs prevent KpOmpA binding to APCs. Myeloid DCs, macrophages, and LOX-1- and SREC-I-transfected CHO cells were incubated with the indicated molecules for 10 min. Then, Alexa 488-labeled KpOmpA was added before FACS analysis. Results are expressed as percentage of inhibition of binding (mean ± SD, n = 5).

(C) FACS analysis of Alexa 488-labeled KpOmpA (white histogram) or flagellin (gray histogram; similar to unlabeled cells) binding to CHO cells transfected with expression vectors encoding LOX-1, SREC-I, MARCO, SR-A1, CD36, and CLA-1.

(D) FACS analysis of FITC-labeled wild-type (wt) (gray histogram) and OmpA-deficient Escherichia coli (white histogram, unbroken line) binding to SREC-I- and LOX-1-CHO cells; break line histograms correspond to unlabeled CHO cells.

assembly of a hyaluronic acid-rich extracellular matrix and female fertility to innate immunity against diverse microorganisms (Diniz et al., 2004; Garlanda et al., 2002; Salustri et al., 2004; Varani et al., 2002).

Whether and how the cellular and humoral arms of innate immunity interact in the recognition of the same microbial moieties remains poorly investigated. In addition to LPS, the cell wall of Gram-negative bacteria contains Omp such as OmpA, porins, and lipoproteins. OmpA is a class of proteins highly conserved among the Enterobacteriaceae family and throughout evolution that are essential for bacteria integrity and virulence (Poolman, 1996). Recent data suggest that OmpA is a bona fide PAMP. Recombinant OmpA from KpOmpA binds to and is internalized by antigen-presenting cells (APCs) such as macrophages and DCs via a still unidentified endocytic cell surface receptor(s) and activates them (Jeannin et al., 2000).

Here, we report that innate immunity against KpOmpA involves cellular recognition by the scavenger receptors SREC-I and LOX-1 and TLR2-dependent cellular activation. Activation of cellular innate immunity by KpOmpA is followed by humoral amplification via induction of PTX3, which binds KpOmpA. Analysis of tlr2 and ptx3 gene-targeted mice supports that both the cellular and the humoral arms of innate immunity are essential for a full response to KpOmpA.

Results

Involvement of Scavenger Receptors in KpOmpA Binding to APCs

Previous data, confirmed here, had shown that KpOmpA binds to human macrophages (Jeannin et al., 2000) (Figure 1A) and peripheral blood myeloid DCs (Figure 1A), but not to NK cells, T cells (Jeannin et al., 2000), and plasmacytoid DCs (data not shown), in a manner suggesting the existence of KpOmpA binding...
Figure 2. Role of TLR2 in KpOmpA-Mediated Cellular Activation, but Not KpOmpA Binding
(A) TLR2 transfection does not upregulate KpOmpA binding. CHO cells were transfected (unbroken line) or not (dotted line) with an expression vector encoding TLR2. Top, cells were incubated with a FITC-labeled anti-TLR2 mAb. Gray histogram corresponds to cells incubated with the isotypic control mAb and is similar on both cell types. Bottom, cells were incubated with Alexa488-labeled KpOmpA. Gray histogram corresponds to cells incubated with labeled TT and is similar on both cell types.
(B) KpOmpA binds to DCs from TLR2-deficient mice. DCs were generated from wt (white histogram) or TLR2−/− mice (black). Top, cells were incubated with different concentrations of KpOmpA, and IL-6 was quantified in the 16 h-supernatants by ELISA. Bottom, cells were incubated with Alexa488-labeled KpOmpA before FACS analysis.

In (A) and (B), results are representative of the mean ± SD of one out of three experiments.

(C) LOX-1 or SREC-I expression upregulates KpOmpA-mediated TLR2-dependent IL-8 production. 293 cells were transfected or cotransfected as described. Left column, cells were incubated with Alexa647-labeled KpOmpA before FACS analysis. Right column, cells were incubated with 0.1 μM KpOmpA, and IL-8 was quantified in the 10 h-supernatants by ELISA. Results are expressed in MFI and pg/ml, respectively (mean ± SD, n = 3).

Figure 3. TLR2-Dependent Induction of PTX3 Production by KpOmpA
(A) KpOmpA induces PTX3 mRNA expression. PTX3 mRNA expression was evaluated by Northern blotting in PBMC from two donors after 4 hr stimulation with 100 ng/ml LPS or 0.002–0.5 μM KpOmpA. Expression of β-actin was used as internal transcript control.
(B) KpOmpA induces PTX3 production by APCs. PTX3 protein levels (mean ± SEM) produced by human monocytes (huMo), human DCs (huDC), and murine DCs (muDC) stimulated for 24 hr with different concentrations of KpOmpA were quantified by ELISA.
(C) PTX3 production in response to KpOmpA is TLR2 dependent. PTX3 protein production by murine DCs from wt and TLR2−/− mice stimulated for 16 hr with KpOmpA was quantified by ELISA. Results are expressed as mean ± SEM.

(D) KpOmpA induces TLR2 and LOX-1 colocalization. Immature human DCs layered on gelatin-coated slides were incubated (right) or not (left) with 0.5 μM KpOmpA for 20 min at 37°C and then with FITC-labeled anti-LOX-1 mAb and Cy3-labeled anti-TLR2 mAb. After washing, cells were fixed with 2% PFA and examined by using an LSM510 inverted confocal microscope.
Figure 4. Specific Binding of PTX3 to KpOmpA

(A) The binding of 50–100 nM biotin-labeled PTX3 (bPTX3) to different concentrations of KpOmpA immobilized on plastic wells was analyzed by ELISA.

(B) Binding of 100 nM bPTX3 to exotoxin A, MDP, TSST, KpOmpA, enterotoxin A and B, OMV isolated respectively from K. pneumoniae, E. coli Omp*, and E. coli Omp* immobilized on plastic wells (1 μg/well).

In (A) and (B), the results are presented as O.D. 405 nm (mean ± SD, n = 3).

(C) Fitting analysis of PTX3-KpOmpA interaction. KpOmpA was immobilized on plastic wells and incubated with different amounts of bPTX3. Specific binding was measured in accordance with a standard curve of bPTX3, and nonlinear fitting analysis was performed with GraphPad Prism 3.0a software.

(D) Real-time biomolecular study of KpOmpA-PTX3 interaction. KpOmpA (2.8 μM) was injected over a flow cell of a BIAcore F1 sensor chip containing BSA or PTX3. The overlay of sensograms after blank subtraction showing the binding of KpOmpA to immobilized PTX3 are presented.

(E) PTX3 binding to KpOmpA is Ca2+ dependent. Binding of PTX3 to immobilized KpOmpA was performed in the presence or absence of 10 mM EGTA. Results are presented as mean O.D. 405 nm ± SD.

(F) Binding of PTX3 to Klebsiella pneumoniae and Burkholderia cepacia. 10^8 bacteria were incubated with 100 nM bPTX3 and then with FITC-labeled streptavidin before FACS analysis. Gray histograms correspond to bacteria incubated with FITC-streptavidin alone.

SD, n = 5) (Figure 1A). SRs are cell surface glycoproteins that bind modified lipopolysaccharides (such as oxidized [Ox-] and acetylated [Ac-]LDL) and a broad spectrum of structurally unrelated ligands (Dhaliwal and Steinbrecher, 1999; Peiser and Gordon, 2001). Ox-LDL, Ac-LDL, and fucoidan, but not BSA (used as a negative control), also significantly prevented KpOmpA binding to APCs (Figure 1B). Collectively, these data suggest that SRs could be cell surface-capturing receptors for KpOmpA.

LOX-1 and SREC-I Are Receptors for KpOmpA Expressed on APCs

In order to identify the SRs that bind KpOmpA, Chinese hamster ovary (CHO) cells were transfected with cDNA encoding different SRs. KpOmpA bound to LOX-1- and SREC-I-transfected CHO cells (MFI = 1835 ± 168 and 820 ± 86, respectively; mean ± SD, n = 5), but not to the other SR tested (MARCO, SR-A1, CD36, and CLA-1) (Figure 1C). MARCO, SR-A1, CD36, and CLA-1 expression by CHO cells is shown in Figure S1 available with this article online. KpOmpA and fluorescent Ac-LDL did not bind to mock-transfected CHO cells (data not shown). As controls, other bacterial proteins, labeled flagellin (Figure 1C) and tetanus toxoid (TT) (data not shown), did not bind to SR-transfected CHO cells. Ac-LDL and fucoidan significantly inhibited KpOmpA binding to LOX-1- and SREC-I-CHO cells (Figure 1B). In agreement with these results, human macrophages and peripheral blood myeloid DCs constitutively express LOX-1 (Delneste et al., 2002) and SREC-I (P.J., unpublished data), in contrast to T cells, NK cells, and plasmacytoid DCs.

The scavenger receptors LOX-1 (Shimaoka et al., 2001) and SREC-I (P.J., unpublished data) support adhesion of Gram-negative bacteria. We therefore evaluated the role of OmpA in the binding of Gram-negative bacteria to LOX-1 and SREC-I. As recombinant OmpA from Escherichia coli binds to SREC-I and LOX-1 in a manner similar to KpOmpA (data not shown) (OmpA from E. coli and Klebsiella pneumoniae share 88% identity), we used wild-type (wt) and OmpA-deficient E. coli (Ec OmpA* and Ec OmpA) to evaluate this hypothesis. LOX-1- and SREC-I-transfected cells retain the ability to bind FITC-labeled OmpA* E. coli but to a lower extent than wt bacteria (Figure 1D), thereby suggesting that in addition to OmpA, other structures expressed on bacteria are recognized together with OmpA by LOX-1 and SREC-I.

Role of TLR2 in Signaling, but Not in Binding

The signaling molecule TLR2 has been suggested to be involved in KpOmpA-mediated APC activation (Jeannin et al., 2000). We therefore tested whether TLR2 could
also act as a KpOmpA binding element on the cell surface. TLR2 transfection does not upregulate KpOmpA binding to CHO cells (Figure 2A). Although they failed to be stimulated by KpOmpA (Figure 2B, top), DCs from TLR2−/− mice bind KpOmpA in a way similar to wt cells (Figure 2B, bottom). Moreover, KpOmpA binding to LOX-1- or SREC-I-transfected cells is not upregulated by cotransfection with TLR2 (Figure 2C). However, cotransfection of TLR2 with LOX-1 or SREC-I renders 293 cells more responsive to a stimulation with KpOmpA (as assessed by measuring IL-8 production) than TLR2 alone or TLR2 plus CD36 (Figure 2C). TLR4 expression does not render LOX-1-transfected 293 cells responsive to KpOmpA (Figure 2C). In resting DCs, TLR2 is associated to the cell membrane, whereas LOX-1 is mainly localized to the membrane and, to a larger extent, in intracellular vesicles (Figure 2D, left). Upon contact with KpOmpA, LOX-1 and TLR2 colocalize at the cell membrane, with the appearance of double-stained intracellular vesicles (Figure 2D, right). We therefore evaluated whether SRs may also participate in KpOmpA-induced cell activation. First, the SR ligand mBSA (1) is internalized by APCs but does not stimulate them, and (2) it does not activate 293 cells transfected with LOX-1 (without or with TLR2) (data not shown). Second, the crosslinking of LOX-1 or SREC-I with specific mAbs does not stimulate DCs or macrophages (data not shown). Third, we observed that OmpX, another Omp that binds to, is internalized by, but does not activate DCs and macrophages (Maisnier-Patin et al., 2003), also binds SREC-I- and LOX-1-transfected cells (but not the other scavenger receptor tested: CD36, CLA-1, MARCO, and SR-A1) (Figure S2, left). However, in contrast to KpOmpA, OmpX does not activate 293 cells transfected with TLR2 (Figure S2, right), TLR4, or TLR2 plus LOX-1 (data not shown). Together these data show that the SR-mediated binding and internalization can occur independently of TLR-mediated activation.

Collectively, these results suggest that the activation of an innate cellular response by KpOmpA involves the cooperation of SR and TLR2 for recognition and signaling, respectively.

KpOmpA Induces PTX3 Production via TLR2

We then examined whether KpOmpA interaction with APCs may modulate soluble PRR production. KpOmpA, at concentrations ranging from 0.002 to 0.5 μM, induces PTX3 production by human monocytes and DCs, with a maximal production of 7.3 ± 2.4 (mean ± SEM, n = 4) and 26.8 ± 8.4 ng/ml (n = 7), respectively (Figures 3A and 3B), by acting at the transcriptional level (Figure 3A). A similar induction of PTX3 production is observed with murine DCs (Figure 3B). The activity of KpOmpA as a PTX3 inducer was comparable to that of LPS, used as reference agent (Doni et al., 2003), and is abrogated when boiled KpOmpA is used (data not shown). As expected, DCs are better producers of PTX3 than monocytes (Doni et al., 2003). In parallel, the production of other soluble PRRs such as mannose binding protein is unaffected (data not shown). Finally, by using DCs from wt and TLR2−/− mice, we demonstrate that KpOmpA-induced PTX3 production is TLR2 dependent (Figure 3C).

PTX3 Binds KpOmpA with High Affinity

As PTX3 is a soluble PRR, we tested whether it may interact with KpOmpA. PTX3 binds KpOmpA (Figure 4A), but not other microbial moieties tested, some of which are shown in Figure 4B, with an apparent dissociation constant (Kd) equal to 36 × 10−9 M (Figure 4C). By using the BIAcore technology, an association rate constant (ka) equal to 1.77 × 104 M−1 s−1 and a dissociation rate constant (kd) equal to 8.57 × 10−4 s−1, with an estimated Kd equal to 50 × 10−9 M, comparable to that estimated by conventional binding, were obtained (Figure 4D). Although the interaction of PTX3 with C1q is Ca2+-independent, PTX3 binding to KpOmpA was Ca2+ dependent (Figure 4E). PTX3 binds Klebsiella pneumoniae (Figure 4F) and a protein extract obtained from outer membrane vesicles (OMVs) of Klebsiella pneumoniae (Figure 4B), but not Burkholderia cepacia used as irrelevant control (Figure 4F). In addition, it binds more efficiently OMVs of OmpA+ E. coli than OMVs of OmpA− E. coli (Figure 4B), as well as intact OmpA+ versus OmpA− E. coli (data not shown).

PTX3 Does Not Modulate KpOmpA Recognition by APCs

Soluble cytokines (e.g., IL-6 receptor α chain) (Romano et al., 1997) or PRRs (e.g., CD14) can present ligands to membrane bound receptor moieties and activate signaling. We therefore tested whether KpOmpA recognition by PTX3 may activate a trans-signaling cascade or affect KpOmpA interaction with APCs. Cells were ex-

Figure 5. Effect of PTX3 on KpOmpA-Induced CCL2 Production

(A) CCL2 production was measured in culture supernatants of murine peritoneal cells stimulated for 24 hr with 0.002–0.25 μM KpOmpA in the presence or absence of 0.2 μM PTX3. (B) Levels of CCL2 produced by murine peritoneal macrophages stimulated with 0.25 μM KpOmpA in the presence or absence of different concentrations of PTX3 (0.044–1.1 μM). In (A) and (B), CCL2 production was determined by ELISA, and results representative of one out of four experiments performed are from wt and TLR2−/− mice, we demonstrate that KpOmpA-induced PTX3 production is TLR2 dependent (Figure 3C).
Figure 6. In Vivo Role of TLR2 and PTX3 in KpOmpA-Induced Inflammation

(A and B) Role of TLR2 in KpOmpA-induced inflammation. KpOmpA or BSA were injected into the footpad of wt and TLR2−/− mice. At different time points, footpad swelling was measured (A) and inguinal LNs were excised and LN cells counted (B). Results are mean ± SD of three separate experiments.

(C) Inflammatory response to KpOmpA in PTX3−/− mice. PTX3−/− and wt mice were injected into the footpad with KpOmpA (0.625 nmol), and footpad swelling was measured. Results are mean ± SD of three separate experiments.

(D) Reconstitution of the inflammatory response in PTX3−/− mice. PTX3−/− mice were injected with KpOmpA (0.5 nmol) and treated with 0.55 nmol PTX3 coadministered in the footpad. Results are mean ± SEM (n = 8; one experiment performed). *p = 0.01, **p = 0.03 (Student’s t test).

(E) PTX3 amplifies the inflammatory response to KpOmpA. Mice were injected in the air pouch with PBS, KpOmpA (0.625 nmol), PTX3 (0.55 nmol), or KpOmpA + PTX3 and sacrificed after 4 or 24 hr. The pouch fluid (1 ml) was collected, and total cells were counted. Results are mean ± SEM. *p = 0.003, **p = 0.04 (Student’s t test).

In Vivo Relevance

The above results indicate that KpOmpA is recognized by the cellular SRs SREC-I and LOX-1 and activates cells via TLR2. In an effort to examine the actual in vivo relevance of TLR2, we measured the inflammatory activity of KpOmpA in TLR2−/− mice. In response to KpOmpA, footpad swelling and lymph node (LN) cell counts are dramatically reduced in TLR2−/− mice (Figures 6A and 6B). KpOmpA induces the production of PTX3, which in turn binds to KpOmpA. We therefore tested the in vivo relevance of this interaction by measuring the inflammatory response to KpOmpA in PTX3−/− mice. After KpOmpA injection in the footpad, PTX3−/− mice showed a significant reduction of footpad swelling at early time points (44% reduction at 2 hr, p = 0.01, two experiments performed) (Figure 6C). As shown in Figure 6D, in a third experiment, exogenous injection of PTX3 together with KpOmpA abrogated the 56% reduction in inflammation observed in PTX3-deficient mice at the time of maximal reduction relative to wt mice (2 hr) and reduced the difference at later time points. Injection of PTX3 subcutaneously per se does not elicit an inflammatory response (data not shown). Finally, in an effort to further explore the capacity of PTX3 to amplify the response to KpOmpA, an air pouch model was used. As shown in Figure 6E, KpOmpA injection caused a leukocyte recruitment in the pouch, whereas PTX3 alone was inactive. When KpOmpA and PTX3 are cojected in the pouch, a 145% and 85% increase in leukocyte counts is observed at 4 and 24 hr, respectively (p = 0.003 and p = 0.04, respectively). Thus, PTX3 inoculation represents a nonredundant humoral amplification loop of the local inflammatory response elicited by KpOmpA.
Recent studies showed that β-glucan, a yeast cell wall component, is recognized by Dectin-1 but that cellular activation requires TLR2 (Brown et al., 2003; Gantner et al., 2003). Recognition of and activation by KpOmpA involves a similar cooperative interaction between members of the SR family and TLR2.

The genetic program activated by KpOmpA in macrophages or DCs includes induction of the long pentraxin PTX3. PTX3 is a soluble PRR that interacts with fungi and bacteria (Diniz et al., 2004; Garlanda et al., 2005; Garlanda et al., 2002). Moreover, it binds the extracellular matrix protein TSG6 and has a key role in the assembly of a hyaluronic acid-rich extracellular matrix (Salustri et al., 2004). PTX3 plays a nonredundant role against selected pathogens, including Aspergillus fumigatus and Pseudomonas aeruginosa (Garlanda et al., 2002). The results reported here show that PTX3, whose production is induced by KpOmpA and by inflammatory signals (e.g., LPS, IL-1, and TNFα), binds KpOmpA with high affinity.

Soluble receptors can present agonists to membrane-anchored signaling receptors and be part of signaling receptor complexes, as exemplified by CD14 and MD2 for TLR4 and the soluble IL-6 receptor α chain (Romano et al., 1997). It was therefore important to assess whether soluble PTX3 could act as a component of cellular signaling receptor complexes, which recognize KpOmpA. PTX3 did not amplify or block the responsiveness of macrophages or DCs to KpOmpA, and cells from PTX3−/− mice responded normally to KpOmpA. Thus, PTX3 represents a bona fide humoral KpOmpA-recognizing molecule and it does not interact with cellular receptors, which bind this bacterial component.

When given subcutaneously, i.v., or i.p., PTX3 had essentially no inflammatory activity per se (Garlanda et al., 2005 and data not shown). However, when administered with KpOmpA, PTX3 significantly amplified the inflammatory response elicited by this microbial moiety (Figure 6E). Moreover, PTX3+/− showed a significant reduction (20%–50% at early time points) of local inflammation elicited by KpOmpA. Thus, PTX3 acts as a nonredundant humoral amplification system of the response elicited by KpOmpA. PTX3 is the only element of the cascade of mediators (cytokines, chemokines, etc..) set in motion by TLR2-dependent activation of innate immunity by KpOmpA. The finding that PTX3 accounts for 40%–50% of the inflammatory response at early time points is fully consistent with it being a nonredundant amplification loop in the cascade, in contrast to TLR2, which sets in motion the whole cascade and whose deficiency essentially abrogates the response.

PTX3 binds C1q and activates the complement cascade (Nauta et al., 2003). Moreover, a cellular PTX3 binding site expressed on macrophages and DCs has been identified. PTX3 binding facilitates recognition and ingestion of conidia and zymosan (Diniz et al., 2004; Garlanda et al., 2002). The relative importance of these two pathways in the role played by PTX3 in the inflammatory response to KpOmpA remains to be determined.
The innate immune system consists of a cellular and a humoral arm, a general organization reminiscent of adaptive immunity. The studies reported here highlight the complexity and complementarity of cellular and humoral recognition of KpOmpA (Figure 7). KpOmpA is recognized by cellular receptors, which include SRs (LOX-1 and SREC-I) for binding and TLR2 for signaling. Cellular recognition of KpOmpA activates a proinflammatory program that includes the humoral PRR PTX3, which in turns binds this microbial moiety. Cellular and humoral (PTX3-mediated) recognition of KpOmpA are complementary in mediating the innate response to this conserved key component of *enterobacteria*.

**Experimental Procedures**

**Cell Culture Media and Reagents**

The following reagents were used for tissue culture: pyrogen-free saline (SALF, Bergamo, Italy); phosphate-buffered saline (PBS) with calcium and magnesium (Bio Whittaker, Walkersville, MD); RPMI 1640, L-glutamine, and Ficoll Hypaque (Biochrom, Berlin, Germany); and aseptically collected fetal calf serum (FCS; HyClone, Logan, UT). All reagents contained <0.125 endotoxin U/ml, as determined by the Limulus amoebocyte lysate assay (Bio Whittaker). Lipopolysaccharide (LPS) from *Escherichia coli* strain 055:BSb was obtained from Difco Laboratories (Detroit, MI). Bovine serum albumin (BSA), with low endotoxin characteristics, exotoxin A from *Pseudomonas aeruginosa*, toxic shock syndrome toxin (TSST), and enterotoxin A and B from *Staphylococcus aureus* and *N*-acetylglucosamino-1,3-2,4-di-aminoglutamine (MDP) were from Sigma-Aldrich (St. Louis, MO). GM-CSF and murine GM-CSF were from R&D Systems (Abingdon, UK).

Recombinant human PTX3 was purified from CHO cells constitutively expressing the protein as described (Bottazzi et al., 1997). Biotinylated PTX3 (bPTX3) was obtained following standard protocols. Recombinant KpOmpA expression and purification were performed as described (Jennin et al., 2000) with the following additional steps. Briefly, after protein precipitation and solubilization in 7 M urea, KpOmpA was submitted to size exclusion chromatography in water. Purification was carried out in apyrogenic conditions. KpOmpA batches used were produced according to pharmaceutical quality standards intended for clinical trials in human. Endotoxin contamination was <0.25 EU/mg of KpOmpA (Limulus amoebocyte lysate assay) and was also excluded by using cells from *tlr4*-deficient mice (Jennin et al., 2000). Lipoprotein contamination was excluded by the extensive characterization of contaminating traces of proteins present in KpOmpA batches (Haeuw and Beck, 2004). OmpX expression and purification was performed as described (Jennin et al., 2000). TT was purchased from SBL vaccine (Stockholm, Sweden); endotoxin contamination checked by the limulus assay test was <0.25 EU/mg of proteins. KpOmpA and TT were labeled with Alexa 647 as described by the manufacturer (Molecular Probes, Eugene, OR).

Wt and OmpA-deficient *Escherichia coli* (kindly provided by Dr. A. Robert, Centre d’Immunologie Pierre Fabre, F-Saint-Julien en Genevois) were labeled with FITC as described (Van den Berg et al., 1999) and UV irradiated. *Burkholderia cepacia* and *Klebsiella pneumoniae* were from clinical isolates kindly provided by Dr. M. Conese (H.S. Raffaele, Milan) and by Dr. E. De Vecchi (L. Sacco Teaching Hospital, Milan), respectively. The method for preparation of membrane fractions from outer membrane vesicles extracted from *K. pneumoniae*, *E. coli* (OmpA*−*), and *E. coli* OmpA*+* was adapted from Davies et al. (1990). Mice

129/sv mice were from Charles River (Calco, Italy), *px3*-deficient mice were generated as described (Garlanda et al., 2002). C57BL/6 (H-2b) mice were from Harlan (Gannat, France). C57BL/6 *tr2* knock-out (TLR2−/−) have been previously described (Fukeuchi et al., 1999). Procedures involving animals and their care conformed to institutional guidelines in compliance with national (D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,11,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council 1996). All efforts were made to minimize the number of animals used and their suffering.

**Human Cells**

Peripheral blood mononuclear cells (PBMC) were purified under endotoxin-free conditions from fresh buffy coats of healthy donors (courtesy of the Centro Trasfusionale, Ospedale Niguarda, Milan, Italy) by Ficoll gradients. Monocytes were purified by Percoll gradients (Amersham Biosciences, Uppsala, Sweden), and DCs were generated from monocytes as described (D’Amico et al., 1998; Pie- mantelli et al., 1995). Human BDCa-1~, CD1c~high~ and CD123~low~ blood myeloid DCs were isolated by using magnetic beads per the manufacturer’s instructions (Miltenyi Biotech, Bergish Gladbach, Germany). Macrophages were generated by culturing monocytes for 5 days in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FCS, and antibiotics (cRPML) containing 2 ng/ml GM-CSF and 20 ng/ml M-CSF.

**Murine Cells**

To obtain peritoneal exudate cells (PECs), mice were injected i.p. with 1 ml of 3% thioglycolate medium (Difco Laboratories), and PECs were harvested after 4 days by washing the peritoneal cavity with 5 ml saline. PECs collected in that way contain more than 80% of macrophages, as determined by morphological evaluation on cytospin. Murine DCs were generated by culturing bone marrow cells in cRPMI supplemented with 50 μM β-mercaptoethanol and 3 ng/ml murine GM-CSF. At day 5, nonadherent immature DCs expressing intermediate levels of I-A were used.

**Transfectants**

CHO cells transfected with LOX-1, CD36, MARCO, SRA-1, and CLA-1 were generated as described (Delneste et al., 2002). The cDNA-encoding SREC-I was subcloned in the expression vector pCDNA3.1 containing a sequence encoding the protein C tag (EDQVPRDIQKGD) in frame with the inserted sequence. CHO cells (ATCC, Manassas, VA) were transfected by using Fugene-6 (Roche Diagnostic, Meylan, France) and cultured in ISCOVE medium supplemented with 10% FCS and selected with hygromycin (all from Life Technologies) before clones were picked. SREC-I, SRA-1, CD36, and CLA-1 expressing cells were isolated by FACS sorting using 5 μg/ml fluorescent Dil-Ac-LDL (Harbor Bioproducts, Strou-thon, MA); LOX-1 and MARCO-expressing cells were sorted by using anti-LOX-1 (clone 22G11) (Delneste et al., 2002) and anti-tag protein C mAb, respectively, revealed with FITC-labeled anti-mouse Ig (Silenus, Melbourne, Australia).

**PTX3 Protein and Transcript Levels**

Monocytes and DCs were cultured for 24 hr 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, Franklin Park, NJ) in the presence of 100 ng/ml LPS or displaying negative results, the limulus assay test was <0.25 EU/mg of proteins. KpOmpA and TT were labeled with Alexa 647 as described by the manufacturer (Molecular Probes, Eugene, OR).

**PtX3 Protein and Transcript Levels**

Monocytes and DCs were cultured for 24 hr 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, Franklin Park, NJ) in the presence of 100 ng/ml LPS or different concentrations of KpOmpA. PTX3 levels in culture supernatants were quantified by ELISA as described (Peri et al., 2000). To evaluate PTX3 mRNA induction, cells were stimulated as described for 4–6 hr in 50 ml tubes (Falcon; BD Biosciences). Total RNA for Northern analysis was extracted by the guanidine isothiocyanate method, blotted, and hybridized as previously described (Breviario et al., 1992). Human full-length cDNA for PTX3 was used as probe and labeled by using the Megaprime DNA labeling system with [32P]dCTP (3000 Ci/mmol; Amersham Biosciences). β-actin was used as internal transcript control.

**Binding Assay**

Binding of PTX3 to KpOmpA was performed essentially as previously described (Bottazzi et al., 1997). Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 1–5 μg of purified KpOmpA and blocked with 0.5% dry milk in PBS (2 hr at room temperature) before incubation (30 min, 37°C) with 100 μl of bPTX3 (50–100 nM) considering a molecular weight of 45 kDa for
the PTX3 monomer) in PBS containing 0.05% Tween 20 (PBST). After washing, plates were incubated with HRP-labeled avidin (Bio-spa, Milan, Italy), and absorbance values were read at 405 nm after addition of the chromogen substrate ABTS (Kirkegaard and Perry, Gaithersburg, MD). Binding of βPTX3 to exotoxin A, MDP, TTSt, enterotoxin A and B, OMV from *K. pneumoniae*, Omp*E* col, Omp*F* col (1 μg/ml) was analyzed with the same protocol.

To characterize the specific binding, βPTX3 was added to triplicate wells coated with 125 pmol/well of KpOmpA, and bound PTX3 was detected as described above. The results were converted to picomolar concentrations by using a standard curve of bPTX3. Kd and Bmax were obtained by nonlinear fitting of the saturation curves by means of GraphPad Prism 3.0a software.

Cytokine Production

Murine CCL2 levels were measured by ELISA (Peri et al., 1994) in 24 hr cell-free supernatants from PEC (1 × 10⁶/ml in 96-well plates) cultured in cRPMI in the presence of 0.002–0.25 μM KpOmpA without or with different concentrations of PTX3. Murine IL-6 was quantified by ELISA (R&D Systems) in the 16 hr cell-free supernatants of DCs from wt versus TLR2−/− mice cultured in cRPMI at 10⁵ cells/ml in 96-well tissue culture plates and exposed or not to 0.06–0.5 μM KpOmpA. Human IL-8 was quantified by ELISA (R&D Systems) in the 10 hr supernatants of 293 cells in RPMI (without FCS) exposed to 0.1 μM KpOmpA.

Flow Cytometry

Flow cytometry analysis was performed by using a FACSvantage cytofluorometer (Becton Dickinson, San Jose, CA). 2 × 10⁵ cells/well in 96-V-bottom-well plates were incubated for 20 min either at 4°C in FACS buffer (0.1% BSA in RPMI 1640 medium) with 0.5 μM Alexa488-labeled KpOmpA or TT or at RT with 5 μM BL-FITC-labeled bacteria. In neutralization experiments, cells were or were not preincubated with BSA, maleylated BSA, Ox- or Ac-LDL (both from Biogenesis, Poole, UK), or fucoidan (Sigma-Aldrich) for 10 min at 4°C before incubation with Alexa488-labeled KpOmpA. Results are in MFI or as a percentage of inhibition as follows: A-B/A × 100 where A and B are the MFI values in the absence or presence of the unlabeled molecule, respectively.

Mice Inoculation

WT and TLR2−/− mice were injected subcutaneously with 0.625 nmol BSA or KpOmpA. At different time points, inguinal lymph nodes were excised and LN cells were counted as previously described (Jeannin et al., 2003). For footpad swelling, PTX3−/−, TLR2−/−, and control mice were injected subcutaneously in the footpads with PBS or 0.625 nmol KpOmpA in PBS. In the reconstitution experiments, wt and the specific binding mice were injected with 0.5 μM KpOmpA alone or preincubated with 0.44 nmol PTX3. At different time points, foot swelling was measured with a calliper. The air pouch model was used as described (Romano et al., 1997). Briefly, mice were injected with PBS, 0.625 nmol KpOmpA, 0.55 nmol PTX3, or 0.625 nmol KpOmpA preincubated with 0.55 nmol PTX3. At 4 or 24 hr after treatment, animals were anesthetized, the pouches were washed with 1 ml of saline, and total cells were counted.

Confocal Microscopy

Anti-LOX-1 (Delneste et al., 2002) and anti-TLR2 mAbs (eBioScience, San Diego, CA) were labeled with FITC and Cy3, respectively, by using commercial kits (Sigma-Aldrich and Amersham Biosciences, respectively). Myeloid DCs were cultured on gelatin-coated slides for 2 hr at 3°C, washed with PBS and incubated with 0.5 μM KpOmpA or not for 20 min at 37°C, washed in PBS and incubated for 30 min with 5 μg/ml FITC-labeled anti-LOX1-1 mAb and 5 μg/ml Cy3-labeled anti-TLR2 mAb in PBS-BSA for 30 min. After washing, cells were fixed with 4% PFA, mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark) and examined by using a LSM510 inverted confocal microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

Statistical analysis was performed by using the Student’s t-test. To estimate the Kd (i.e., the equilibrium dissociation constant) binding of PTX3 to plastic immobilized KpOmpA was analyzed by nonlinear fitting using the equation of the “one site binding curve” (GraphPad Prism 3.0a software, GraphPad, San Diego, CA).

Supplemental Data

Supplemental Data include two figures and are available with this article online at http://www.immunology.com/cgi/content/full/22/5/551/DC1/.

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