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Different Domains of *Pseudomonas aeruginosa* Exoenzyme S Activate Distinct TLRs

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Some bacterial products possess multiple immunomodulatory effects and thereby complex mechanisms of action. Exogenous administration of an important *Pseudomonas aeruginosa* virulence factor, exoenzyme S (ExoS) induces potent monocyte activation leading to the production of numerous proinflammatory cytokines and chemokines. However, ExoS is also injected directly into target cells, inducing cell death through its multiple effects on signaling pathways. This study addresses the mechanisms used by ExoS to induce monocyte activation. Exogenous administration resulted in specific internalization of ExoS via an actin-dependent mechanism. However, ExoS-mediated cellular activation was not inhibited if internalization was blocked, suggesting an alternate mechanism of activation. ExoS bound a saturable and specific receptor on the surface of mononuclear cells. ExoS, LPS, and peptidoglycan were all able to induce tolerance and cross-tolerance to each other suggesting the involvement of a TLR in ExoS-recognition. ExoS activated mononuclear cells via a myeloid differentiation Ag-88 pathway, using both TLR2 and the TLR4/MD-2/CD14 complex for cellular activation. Interestingly, the TLR2 activity was localized to the C-terminal domain of ExoS while the TLR4 activity was localized to the N-terminal domain. This study provides the first example of how different domains of the same molecule activate two TLRs, and also highlights the possible overlapping pathophysiological processes possessed by microbial toxins. *The Journal of Immunology*, 2004, 173: 2031–2040.

The complex consequences of activation and exploitation of the innate immune system by single microbial products are poorly understood. It is known that TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) found within diverse microbial products. To date, eleven human homologues have been cloned and published (1–4). TLR4/MD-2 is responsible for transmitting an intracellular signal when a cell is confronted by Gram-negative LPS (5, 6). TLR2 is responsible for recognizing products from Gram-positive organisms such as peptidoglycan (PepG), bacterial lipoproteins, lipoteichoic acid (LTA), and yeast products (7–9). A number of other microbial ligands activate individual TLR (10–12); however, only a few examples of microbial activation of multiple TLR have been described, and the mechanisms by which this occurs are poorly understood. For example, TLR2 governs a response to bacterial lipopeptides and LTA whereas the combination of TLR2/TLR6 mediates responses to PepG and phenol-soluble modulin from *Staphylococcus epidermidis* (8, 13, 14). TLR1 has been shown to recognize the bacterial lipoprotein outer surface protein A (OspA) from *Borrelia burgdorferi*, and may work in combination with TLR2 (15). Heat shock protein (HSP)60 and HSP70 are normally intracellular proteins that become liberated when a cell dies a necrotic death. When extracellular, they are endogenous ligands of TLR; HSP60 activates TLR2 whereas HSP70 activates both TLR2 and the TLR4/MD-2 complex (11, 12). The mechanisms by which microbial products induce activation of multiple receptors are unknown.

Activation of all TLR involves several intracellular adaptors and kinases. Activation of a TLR recruits the adaptor protein MyD88, implicated in the activation of downstream signaling pathways for all TLR (16, 17). MyD88 recruitment is followed by the recruitment of the IL-1-associated kinase (IRAK1) and IRAK2 (18, 19). Upon recruitment to MyD88, IRAK1/2 autophosphorylate, recruit TNFR-associated factor 6 and link TLR stimulation to multiple downstream signaling pathways and proinflammatory cytokine production (20–23). Given that TLR agonists use common signaling pathways, activation by one TLR agonist might influence the signaling by another TLR agonist.

During infection, it is common that cells are exposed to many TLR agonists, both simultaneously and sequentially. Repeated exposure to LPS induces a state of self-regulated unresponsiveness or endotoxin tolerance (24). Tolerance occurs at multiple levels. For example, both LPS stimulation and bacterial lipoprotein stimulation causes a reduction in the expression of TLR4 and TLR2, respectively, thereby preventing subsequent cellular activation to products that used those TLRs (25, 26). The observation that tolerance also acts on the level of IRAK1 (both degradation and sequestration), which is shared by the TLR signaling pathways, led to an understanding of the phenomenon of cross-tolerance between TLR agonists (27–29).

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Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; ExoS, exoenzyme S; HSP, heat shock protein; PepG, peptidoglycan; LTA, lipoteichoic acid; IRAK, IL-1-associated kinase.
In an attempt to understand the complex activation of innate immunity, we have been studying the role of a microbial product that contributes to the LPS-independent inflammatory response induced by the Gram-negative pathogen *Pseudomonas aeruginosa*. It has been disappointing that despite our knowledge of LPS, studies and clinical trials looking at the effects of neutralizing LPS in Gram-negative-induced inflammatory responses have had only limited success, suggesting that other microbial products may be critical elements in the pathophysiology (30). Exoenzyme S (ExoS) is a unique *P. aeruginosa* virulence factor that is produced by many *Pseudomonas* isolates from diverse tissue origins and virtually all pneumonia and cystic fibrosis pulmonary isolates (31). Increased levels of ExoS correlates with increased pulmonary damage in both animal models and cystic fibrosis patients (31–34).

ExoS is a complex bifunctional toxin, possessing two distinct methods of action: extracellular and intracellular. In addition to a type III (contact)-dependent intracellular delivery system that results in cellular cytotoxicity upon delivery of ExoS to the cytosol, ExoS can also interact with target cells by acting as a soluble (extracellular) protein (34–40). Extracellular ExoS stimulates both monocytes and T cells directly (41–43). Extracellular ExoS induces T cell activation resulting in T cell apoptosis, whereas monocyte activation results in the production of a wide variety of proinflammatory cytokines and chemokines (42). Based on these observations, ExoS likely contributes to inflammatory responses during *P. aeruginosa* infection through proinflammatory cytokine induction (44, 45). However, because of the well-characterized ability of ExoS to exploit both extracellular and intracellular pathways, there were a number of possible mechanisms by which cellular activation could occur.

The goal of this study was to determine the mechanisms by which extracellular ExoS induces proinflammatory cytokine production from monocytes. To determine whether extracellular ExoS was internalized or bound a specific cell surface receptor, flow cytometric analysis, confocal microscopy, and competition studies were used. Tolerization experiments and dominant negative inhibitors were used to identify the class of receptors required for ExoS signaling. Genetic complementation studies were used to identify the specific receptors activated by ExoS and to determine which ExoS domains were involved in cellular activation.

### Materials and Methods

#### Cell lines and reagents

The human promonocytic cell line THP-1 (no. TIB-202; American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640, 10% heat-inactivated FCS, 2 mM l-glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate (all from Invitrogen Life Technologies, Rockville, MD) and grown in 5% CO₂. Human embryonic kidney 293 cells (HEK293) and HEK293 cells stably transfected with TLR2 were obtained from Genentech (South San Francisco, CA) and cultured in a 1/1 mixture of F-12 medium and DMEM, 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin in 7% CO₂. Ba/F3 cells stably transfected with the NF-κB-driven luciferase reporter and Ba/F3 cells stably transfected with NF-κB/TLR4/MD-2 were cultured in RPMI 1640, 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% WEHI-3B conditioned medium as a source of IL-3, and were grown in 7% CO₂ (6). PepG (Sigma-Aldrich, St. Louis, MO), anti-TLR2 (mouse anti-human TLR2, clone TL2.1 IgG2a), anti-TLR4 (mouse anti-human TLR4, clone HTA125 IgG2a), and the control Ab (mouse IgG2a isotype control), were all purchased from eBioscience (San Diego, CA).

#### Preparation of recombinant ExoS

Recombinant ExoS, full-length N-terminal ExoS, N'-ExoS (amino acids 1–234), and C-terminal Exo, C'-ExoS (amino acids 234–435), were all isolated from an *Escherichia coli* strain BL21(DE3) pLysS bearing a plasmid encoding histidine-tagged ExoS cloned from *P. aeruginosa* strain 388 (pETHisExoS, kindly donated by Dr. J. Barbieri, Medical College of Wisconsin, Milwaukee, WI). ExoS, N'-ExoS, and C'-ExoS were purified by Ni²⁺-affinity chromatography (Qiagen, Mississauga, Ontario, Canada) from cellular lysates and migrated at 52-, 32-, and 28-kDa proteins, respectively (data not shown) (43, 46). Proteins were purified to >99% homogeneity. In experiments in which cellular activation was determined, polymyxin B was preincubated with the various ExoS preparations for 5 min on ice before being incubated with cells. Although THP-1 cells were grown in the presence of serum, when they were stimulated with ExoS, the serum-containing medium was removed and cultured in THP-1 medium in the absence of serum. Therefore, there would be no soluble CD14 present.

#### Construction of a GFP-ExoS fusion protein

A flow cytometry-optimized mutant of wild-type GFP was used to construct the GFP-ExoS fusion protein (47). NsiI sites were introduced on both the 5' and 3' end of gfp in the pGy3 vector by PCR using the following PCR primers: NsiI up, GGGATATGTCATATGAGT1AAAGG and NsiI down, GCTTTGCAATGATGCGCTTGTGATATGC. This product was cloned in frame into the NsiI site downstream of the His tag and upstream of exoS gene in pET16bHisExoS vector. The GFP-ExoS fusion protein migrated as an ~80-kDa protein, reacted with anti-ExoS antisera, fluoroscein upon excitation at 488 nm and possessed the ability to induce monocyte cytokine production and T cell activation (data not shown).

#### Labeling of ExoS with the fluorescent dye Alexa 488

ExoS was labeled with Alexa 488 according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Briefly, 0.5 μg of ExoS (in 500 μl) was incubated with the Alexa 488 dye for 1 h at room temperature. The labeled mixture was layered atop a column containing purification resin, and the labeled ExoS-Alexa 488 protein was separated from the unlabeled dye. The progression of the labeled protein was determined by using a UV light. Fractions containing ExoS-Alexa 488 were collected and pooled. The protein concentration was determined and amount of dye labeled per mole of protein was determined by the following equation: moles of Alexa/ moles of ExoS = [A494 (of the ExoS-Alexa 488) × dilution factor]/ (71,000 cm⁻³ M⁻¹ × molar protein concentration). Typically, labeling efficiency was ~1 mole of Alexa 488 to 1 mole of ExoS.

#### Incubation of ExoS-Alexa 488 and GFP-ExoS with THP-1 cells

THP-1 cells were incubated with ExoS-Alexa 488 or GFP-ExoS at either 4°C or 37°C for various times. In some experiments, THP-1 cells were fixed in 1% buffered formalin before incubation with labeled ExoS. Fluorescence of labeled THP-1 cells was analyzed by flow cytometric analysis (FACScan; BD Bioscience, San Jose, CA). For some experiments, cells were preincubated with either cytochalasin D (dissolved in DMSO) or DMPS (alone (Calbiochem, La Jolla, CA) for 30 min before activation with ExoS-Alexa 488. To quench the extracellular ExoS fluorescence, cells were incubated in 0.1% trypan blue (Calbiochem) for 2 min, washed in FACS buffer (below), and then analyzed.

#### Confocal microscopy

Cells were deposited onto poly-L-lysine coated coverslips and 5% CO₂. Human embryonic kidney 293 cells (HEK293) and HEK293 cells stably transfected with TLR2 were typically taken of cells under either 37 or 63 or 100 magnification. In experiments in which cellular activation was determined, intracellular cytokine levels were determined by the following equation: moles of Alexa/ moles of ExoS = [A494 (of the ExoS-Alexa 488) × dilution factor]/ (71,000 cm⁻³ M⁻¹ × molar protein concentration). Typically, labeling efficiency was ~1 mole of Alexa 488 to 1 mole of ExoS.

#### Intracellular cytokine detection and flow cytometric analysis

To determine intracellular cytokine levels, cells were stimulated for 3 h with ExoS (with 10 μg/ml polymyxin B) in the presence of monensin (0.66 μM/ml of culture), collected and washed twice in FACS buffer (PBS, 1% PBS, 0.1% NaCl). Cells were then fixed in CytoFix/CytoPerm for 20 min at 4°C and washed in PermWash buffer twice according to the manufacturers instruction (BD Pharmingen, Mississauga, Ontario, Canada). Cells were then incubated with either anti-TNF-α PE or anti-IL-8 PE or isotype-matched control Abs (R&D Systems, Minneapolis, MN) for 30 min and washed twice in PermWash buffer. Cells were resuspended in FACS buffer and fluorescence was measured by flow cytometric analysis (FACScan; BD Biosciences). The values obtained from the control Abs were subtracted from the test group in each experiment.

#### Electrophoresis and Western blot

Cells were lysed in Laemmli buffer containing 1 μM DTT, heated for 10 min at 37°C and electrophoresed into a 4–12% Bis-Tris gradient gel for ~1
A total of 2.5 × 10^3 HEK293 or HEK293-TLR2 cells were plated into one-well in a six-well plate (in 3 ml) overnight. pNF-κB-luciferase (1 μg; firefly luciferase, Promega, Madison, WI) was mixed with 6 μl of Superfect (Qiagen) in 100 μl of medium (1/1 mix of DMEM and F12 medium; Invitrogen Life Technologies) for 10 min. The mix was diluted to 700 μl with complete HEK medium (1/1 mix of DMEM and F12 medium, 10% FCS and 1% penicillin/streptomycin). The medium was removed from the HEK cells and 700 μl of the Superfect/plasmid DNA mix was added, and incubated for 2.5 h at 37°C. The cells were washed once in PBS and resuspended in completed medium and plated in 100 μl samples in 96-well plates, and rested for either 3–4 h or overnight. In 100 μl, the HEK293 cells or the Ba/F3 cells were stimulated with various stimuli for 24 h in triplicate. Each sample was collected, washed in PBS and the cell pellet was lysed in 50 μl of passive lysis buffer (Promega). The lysates were centrifuged and 20 μl of the soluble material lysate was assayed by addition of firefly luciferase substrate (100 μl). Samples were read in triplicate in a luminesimeter.

Luciferase measurements

Escherichia coli O111:B4 was purchased from Sigma-Aldrich and repurified by the protocol of Hirschfeld et al. (48). Repurified LPS did not activate TLR2 expressing cells (data not shown). LPS was used at concentrations between 1 ng/ml and 10 μg/ml.

Results

ExoS was specifically internalized via an actin-dependent process

It was hypothesized that extracellular ExoS might contact the cell surface, become internalized and induce cytokine production via its well-characterized ability to modify signaling pathways. Specifically, ExoS possesses N-terminal ADP-ribosyl transferase activity and C-terminal GTPase activating activity for Rho family GTPases (40, 49). If internalization of extracellular ExoS were responsible for cellular activation, it would be through one of two mechanisms. Following internalization, the enzymatic or catalytic activities of intracytoplasmic ExoS could activate cells. Alternatively, the internalization process itself, during which numerous kinases are activated, could trigger cytokine production; this process would be similar to activation of TLR9 by microbial unmethylated CpG DNA or epidermal growth-factor receptor-mediated activation (50, 51).

THP-1 cells, an ExoS-responsive cell line (42) were used to determine the mechanism by which ExoS activates monocyte cells. THP-1 cells were incubated with ExoS-Alexa 488 and using confocal microscopy the cellular distribution of ExoS-Alexa 488 (within a cytoplasmic plane or extracellular plane) was determined. ExoS-Alexa 488 accumulated within THP-1 cells, as was seen in a cytoplasmic/intracellular section, indicating that ExoS was internalized (Fig. 1A). Internalized ExoS-Alexa 488 exhibited a punctate distribution, which was initially located close to the cell surface (15 min incubation), but subsequently ExoS-Alexa 488 was found in greater amounts and dispersed throughout the intracellular space. Internalization was both dose- and time-dependent, but also specific, as the accumulation of ExoS-Alexa 488 was greater than an equifluorescent control protein (goat anti-rabbit IgG-Alexa 488) (Con). The amount of IgG-Alexa 488 was adjusted to be equifluorescent to 1 μM ExoS-Alexa 488. Fluorescence was determined by flow cytometric analysis and is displayed as the mean fluorescence intensity (n = 2–3).

FIGURE 1. ExoS was internalized. A, THP-1 cells were incubated with ExoS-Alexa 488 (5 μM) for 1 h at 4°C, and then for 15 or 60 min at 37°C. The cells were resuspended in anti-fade mounting medium (DAPI 0.1 μg/ml) and cytospun onto poly-L-lysine coated slides. Fluorescence images were captured using a 100× objective and a 400-nm excitation filter. B, THP-1 cells were incubated at 37°C with various concentrations of ExoS-Alexa 488 (5–90 min) or a goat anti-rabbit IgG-Alexa 488 (Con). The amount of IgG-Alexa 488 was adjusted to be equifluorescent to 1 μM ExoS-Alexa 488. Fluorescence was determined by flow cytometric analysis and is displayed as the mean fluorescence intensity (n = 2–3).
with 0.1% trypan blue for 2 min. Cells were analyzed by were stimulated with 1 positive cells (n were washed and extracellular cytochalasin D for 30 min before ExoS-Alexa 488 addition). THP-1 cells were incubated with 1.0 H9262 with 0.333 mM ExoS-Alexa 488 for various times (n tochalasin D (Cyto D) or a vehicle control (DMSO), and then incubated cells were preincubated for 30 min with different concentrations of cytochalasin D (Cyto D) or a vehicle control (DMSO), and then incubated

FIGURE 2. ExoS was internalized by an actin-dependent process, although internalization was not required for cytokine production. A, THP-1 cells were preincubated for 30 min with different concentrations of cytochalasin D (Cyto D) or a vehicle control (DMSO), and then incubated with 0.333 nM ExoS-Alexa 488 for various times (n = 3). B, THP-1 cells were incubated with 1.0 μM ExoS-Alexa 488 for 3 h at either 4°C or 37°C (cells incubated at 37°C were first pretreated with either DMSO or 10 μM cytochalasin D for 30 min before ExoS-Alexa 488 addition). THP-1 cells were washed and extracellular fluorescence was quenched by incubation with 0.1% trypan blue for 2 min. Cells were analyzed by flow cytometric analysis and the data are expressed as the percentage of ExoS-Alexa 488-positive cells (n = 3). C, THP-1 cells were preincubated for 30 min with different concentrations of cytochalasin D or DMSO, and then cells were stimulated with 1 μg/ml ExoS for 3 h in the presence of monensin. Intracellular TNF-α production was detected by permeabilization and incubation with a fluorescently conjugated anti-TNF-α Ab. The data were expressed as the percentage of TNF-α− cells above unstimulated cells based on a 100% value for ExoS alone (n = 3).

Given that the process of internalization of ExoS or the enzymatic activity of ExoS could modify intracellular signaling pathways, it was necessary to determine whether internalization was required for proinflammatory cytokine production. Surprisingly, at concentrations that inhibited internalization, cytochalasin D was not able to inhibit ExoS-induced TNF-α production, suggesting that internalization or intracellular delivery of ExoS was not required to induce cytokine production (Fig. 2C).

ExoS binding to monocytic cells was saturable and specific

Because internalization was not required for ExoS-induced activation, an alternate mechanism of cellular activation was investigated. Experiments were performed to determine whether ExoS activated cells through a cell surface receptor. THP-1 cells were incubated with ExoS-Alexa 488 or an equifluorescent amount of a control protein (goat anti-rabbit-Alexa 488) and binding was assessed by flow cytometric analysis. ExoS-Alexa 488 bound to the THP-1 cells as indicated by an increase in fluorescence, whereas there was no increase in fluorescence when the control protein was used. There was a shift in the fluorescence of the entire population, suggesting that ExoS bound to the entire THP-1 cell population (Fig. 3A).

The two hallmark characteristics of receptor binding kinetics are saturability and specificity. To test the first characteristic, THP-1 cells were incubated with various amounts of ExoS-Alexa 488. At lower ExoS-Alexa 488 concentrations a rapid rise in binding occurred that was saturated at greater concentrations were used (Fig. 3B). The curve fit an exponential equation that was consistent with saturable binding, with half-maximal binding occurring at ~100 nM of ExoS-Alexa 488. Unlabeled ExoS competed with the binding of labeled ExoS-Alexa 488 (to a maximum of ~40%) (Fig. 3C). The inability of unlabeled ExoS to completely block binding of ExoS-Alexa 488 may be due to the propensity of ExoS to self-aggregate (46). To ensure that the Alexa 488 labeling procedure did not affect the binding of ExoS, an additional approach was taken. An N-terminal GFP-ExoS fusion protein was constructed. GFP-ExoS binding could also be similarly outcompeted by unlabeled ExoS (Fig. 3D). Together, these observations suggested the presence of a saturable and specific receptor on the surface of THP-1 cells.

ExoS demonstrated cross-tolerization with LPS and PepG

Having demonstrated binding characteristics consistent with a receptor, it became of real interest to determine the receptor identity. Based on the ability of ExoS to directly activate monocytes, it was hypothesized that TLR were involved in ExoS recognition (42, 43). ExoS demonstrated cross-tolerization with LPS and PepG.

FIGURE 3. ExoS binds a specific and saturable receptor on mononuclear cells. A, THP-1 cells were fixed, and either incubated with PBS (shaded area), ExoS-Alexa 488 (0.96 μM, solid line) or equifluorescent goat anti-rabbit-Alexa 488 (dashed line) for 20 min at 4°C (n = 3). Fluorescence was measured by flow cytometric analysis. B, Saturable binding of ExoS to THP-1 cells was demonstrated by incubating different concentrations of ExoS-Alexa 488 with fixed THP-1 (20 min, 4°C) followed by thorough washing. The R2 value was generated in Microsoft Excel, as was the curve that describes the distribution of data. The data points are the average of at least two independent experiments. Specific binding was demonstrated by preincubating THP-1 cells with unlabeled ExoS for 20 min at 4°C before incubation with either ExoS-Alexa 488 (300 nM, n = 2) (C) or GFP-ExoS (900 nM, n = 3) (D) for an additional 20 min. The cells were thoroughly washed and fluorescence determined as above.
To explore this possibility, experiments exploited the observation that stimulation of monocytes by LPS, followed by a secondary stimulation with LPS induces endotoxin tolerance, or an inability of a cell type to be activated by a secondary exposure to LPS (24). The phenomenon also holds for other TLR ligands, as activation with one TLR ligand leads to tolerization of other family members (52). Tolerance is not a result of general cellular activation, as LPS pretreatment does not inhibit cellular activation in response to phorbol ester stimulation (53). Because the hypothesis was that ExoS was operating through a TLR, the possibility that there could be some cross-tolerance induced between ExoS and TLR agonists was explored.

When THP-1 cells were pretreated with ExoS, PepG or LPS, a secondary stimulation by ExoS was inhibited (60–90% inhibition) (Fig. 4A). Additionally, pretreatment with ExoS inhibited secondary stimulation by both PepG and LPS (Fig. 4B). In fact, all three ligands induced tolerance and cross-tolerance to themselves and to each other. These results suggested that a TLR was involved in the recognition of ExoS or that there were common elements in the activation pathways between PepG, LPS, and ExoS.

**FIGURE 4.** ExoS and other microbial products induced cross-toleration to each other. A, THP-1 cells were pretreated with either medium (−), 1μg/ml ExoS (E), 0.5μg/ml peptidoglycan (P), or 10μg/ml LPS (L) for 24 h in the absence of FCS. THP-1 cells were washed and the stimulated with 1μg/ml ExoS (+) for an additional 3 h in the presence of monensin. B, THP-1 cells were pretreated as above and stimulated for an additional 3 h with either 0.5μg/ml peptidoglycan (P) or 1μg/ml LPS (L) in the presence of monensin. Intracellular TNF-α was assessed by flow cytometric analysis (n = 3–4). *p < 0.05: paired, one-tailed Student’s t test.

**Activation by ExoS was inhibited by a dominant negative of MyD88.**

All TLR have been demonstrated to use the intracellular adaptor molecule MyD88 as one of the most proximal intracellular signaling molecules, linking TLR to proinflammatory cytokine production (17, 19, 23, 54). To determine whether a TLR was involved in ExoS induced cellular activation, or some other receptor that did not use MyD88, THP-1 cells were transfected with a dominant negative version of MyD88 (MyD88 152–296, MyD88DN) (18). THP-1 cells were cotransfected with pGFP and either pCDNA3 (control vector) or pMyD88DN. The cells were rested overnight, stimulated with ExoS and TNF-α production assessed in GFP+ cells by flow cytometric analysis. MyD88DN inhibited ExoS-induced TNF-α production in a dose-dependent manner (Fig. 5). This result suggested that an MyD88-dependent pathway was involved in the activation of monocytes by ExoS and is consistent with a TLR being involved in ExoS recognition.

**ExoS activated cells expressing either TLR2 or TLR4/MD-2/CD14.**

Because TLR2 and TLR4/MD-2 agonists (PepG and LPS) induced a state of tolerance to further stimulation by ExoS, experiments were performed to investigate whether TLR2 and/or TLR4/MD-2 were involved in the recognition of ExoS. To determine whether the TLR4/MD-2 complex was involved in ExoS signaling, a human IL-3-dependent B cell line Ba/F3 that expressed TLR4, MD-2, and an NF-κB-luciferase reporter was compared with a cell line that expressed only the reporter (6). ExoS induced dose-dependent induction in the cell line expressing TLR4/MD-2, but did not activate cells lacking TLR4/MD-2 (Fig. 6A). Similarly, LPS induced activation of Ba/F3 cells only if they expressed the TLR4/MD-2/CD14 complex.

**FIGURE 5.** ExoS induced TNF-α production via a MyD88-dependent pathway. THP-1 cells were electroporated with pGFP (1μg/1×10⁶ cells) and either pCDNA3 or pMyD88DN at various concentrations. THP-1 cells were washed and rested. The following day, THP-1 cells were stimulated with either 10μg/ml polymyxin B alone, or 1μg/ml ExoS in the presence of 10μg/ml polymyxin B for 3 h and intracellular TNF-α levels determined by FACS analysis. For each group, unstimulated (polymyxin B alone) TNF-α mean fluorescence intensity was subtracted from that of the stimulated group to determine the net TNF-α induction. The data are presented as a percentage of net TNF-α production based on the control plasmid (pCDNA3) for each of the plasmid concentrations. TNF-α production was assessed by first gating on GFP+ cells and determining TNF-α production within that subset. This data presented are the average of two independent experiments ± SEM.
least 1 μg of LPS, so that LPS contamination was not responsible for the TLR4-mediated cellular activation by ExoS. To further assess whether any other contaminating molecules were present in the ExoS preparation we performed the following experiment. E. coli BL21 (DE3) cells containing the plasmid encoding ExoS were grown for 2 h. The cells were split into two groups—ExoS production was induced with isopropyl β-D-thiogalactoside (IPTG) in one group, whereas the other group received only PBS as a control for an additional 2 h. Both groups were then subject to the identical ExoS purification protocol. The eluted fractions from each group were used to stimulate THP-1 cells and intracellular TNF-α production was assessed. Only the eluted fractions from the IPTG induced group were capable of inducing TNF-α from THP-1 cells. The IPTG-induced eluent plus polymyxin B induced TNF-α on 8.2 ± 1.2% THP-1 cells vs 0.1 ± 0.3% of THP-1 cells when stimulated by the uninduced eluent, n = 3, p = 0.015. The data indicated that ExoS was responsible for the cytokine induction, and not any other molecules that were copurified with ExoS. Experiments were also performed to determine whether ExoS activated cells expressing TLR2. We used HEK293 cells that expressed TLR2 (11). These cells were stimulated with ExoS and the phosphorylation of Erk1/2, a critical factor in the induction of TNF-α was determined (21, 55). Both ExoS, and the previously characterized TLR2 agonist PepG, induced Erk1/2 phosphorylation only in cells that expressed TLR2 (Fig. 7A). HEK-TLR2 cells were also transfected with an NF-κB reporter plasmid, and they

**Figure 6.** ExoS activated cells that express TLR4/CD14/MD-2. A. Ba/F3 cells stably transfected with either NF-κB or Ba/F3 cells stably expressing NF-κB-Luciferase/TLR4/MD-2 were stimulated with different concentrations of ExoS (in micrograms per milliliter) in the presence of 10 μg/ml polymyxin B for 24 h. Triplicate samples were lysed and luciferase levels were determined. Data are presented as the mean of the triplicate samples (n = 2–4) for all groups. B. Ba/F3 cells stably transfected with either NF-κB-Luciferase/TLR4/MD-2 or NF-κB-Luciferase/TLR4/MD-2/CD14 were stimulated with different concentrations of ExoS (μg/ml) in the presence of 10 μg/ml polymyxin B or LPS for 24 h. The samples were analyzed in quadruplicate. One of four representative experiments. *, p < 0.05 when compared with Ba/F3 cells stably transfected NF-κB/TLR4/MD-2.

**Figure 7.** ExoS activated cells that express TLR2. A. HEK293 cells or HEK293-TLR2 cells were stimulated for various times with either 5 μg/ml peptidoglycan (P) or 5 μg/ml ExoS (E) for 30 min in the presence of 10 μg/ml polymyxin B. Cells were lysed and a Western blot was performed for phospho-Erk1/2 and actin. One of two representative experiments is shown. B. HEK293 cells or HEK293-TLR2 cells were transiently transfected with an NK-κB-Luciferase reporter plasmid and then stimulated with ExoS or peptidoglycan (PepG) for 6 h, in the presence of 10 μg/ml polymyxin B. The cells were then lysed, luciferase production determined, and the data are presented as fold induction over unstimulated cells in triplicate (n = 3). *, p < 0.05 when compared with HEK293 cells alone.

MD-2 complex (data not shown). To determine whether CD14 enhanced cellular activation, Ba/F3 cells that expressed TLR4/MD-2/NF-κB-Luciferase were compared with cells expressing TLR4/CD14/NF-κB-Luciferase. Both cells lines were stimulated with LPS and ExoS using conditions that maximized the difference between the cell lines. The presence of CD14 enhanced the responsiveness of Ba/F3 cells to both LPS and ExoS (Fig. 6B).

Previous studies had demonstrated that LPS contamination was not responsible for ExoS-induced proinflammatory cytokine production (42, 44). In addition, the amount of LPS activity in the ExoS preparation was calculated using titration experiments (4.3 ± 1.3 ng of LPS activity per 1.0 μg of ExoS). The amount of polymyxin B (10 μg/ml) present was sufficient to neutralize at
too were responsive to stimulation by ExoS, the 19-kDa lipoprotein from Mycobacterium tuberculosis, but not highly purified LPS, although the magnitude of activation was not as great as seen with Erk1/2 phosphorylation (Fig. 7B and data not shown).

Additional experiments were performed to ensure that TLR2 and TLR4 activity was indeed specific. For this purpose, neutralizing Abs to TLR2 and TLR4 were used to block ExoS-induced TNF-α production from THP-1 monocytes. Blocking TLR2 resulted in a modest but statistically significant reduction (19.0 ± 8.6%) in the expression of TNF-α (Fig. 8). Blocking TLR4 resulted in a substantial reduction (57.2 ± 7.8%) in the expression of TNF-α. Together, the blocking Abs resulted in an additive effect (71.4 ± 9.9% inhibition). The specificity of the Abs was demonstrated by blocking the response to PepG and LPS. The anti-TLR2 Ab inhibited PepG induced TNF-α production (70.4 ± 4.9% inhibition, n = 5, p < 0.05) and the anti-TLR4 Ab inhibited highly purified LPS induced TNF-α production (83.3 ± 4.6% inhibition, n = 3, p < 0.05).

**TLR2 and TLR4 activity was dependent on different domains of ExoS**

Given that ExoS activated both TLR2 and the TLR4/MD-2 complex, we hypothesized that the same domain of ExoS was responsible for both activities. Recombinant protein preparations of ExoS were produced that contained full-length ExoS (amino acid 1–435), N terminus (N’-ExoS amino acid 1–234), or C terminus (C’-ExoS amino acid 232–435). It has previously been shown that these domains are biologically active when they are expressed in the truncated form (46, 56). Full-length ExoS, N’-ExoS, and C’-ExoS were used to stimulate both TLR4/MD-2 and TLR2-expressing cell lines. N’-ExoS induced activation of Ba/F3 cells that expressed TLR2 and TLR4 mediated activation whereas the C-terminal domain was responsible for TLR2-mediated activation. A, Ba/F3 stably transfected with either an ELAM-1 promoter driven luciferase vector (NF-κB) or Ba/F3 cells stably transfected with NF-κB-luciferase/TLR4/MD-2 were stimulated with different concentrations of either the N-terminal ExoS (N’) or C-terminal ExoS (C’) in the presence of 10 μg/ml polymyxin B. Triplicate samples were lysed and luciferase levels were determined. Data are presented as the mean of the triplicate samples (n = 3). B, HEK293 cells or HEK293-TLR2 cells were stimulated for 5 min with various concentrations of either N’-ExoS or C’-ExoS. Cells were lysed and a Western blot analysis was performed for phospho-Erk1/2 and actin. One of two representative experiments is shown.

**FIGURE 8.** Neutralization of both TLR2 and TLR4 blocks ExoS-induced TNF-α production. THP-1 cells were pretreated with medium (ExoS), 10 μg/ml control Ab (ExoS + Con), 10 μg/ml anti-TLR2 (Exo + anti-2), 10 μg/ml anti-TLR4 (Exo + anti-4), 20 μg/ml control Ab (ExoS + Con*), or 10 μg/ml anti-TLR2 + 10 μg/ml anti-TLR4 (Exo + anti-2/4) for 45 min at 37°C. Cells were then stimulated with either 10 μg/ml polymyxin B alone, or 500 ng/ml ExoS in the presence of 10 μg/ml polymyxin B for 4 h at 37°C and intracellular TNF-α levels were determined by FACS analysis. The percentage of control +/– SE are of control +/– SEM for each group. *, p < 0.05 as compared with the average ± SEM (n = 4–5 for all groups). #, p < 0.05 as compared with 10 μg/ml control Ab. 

**FIGURE 9.** The N-terminal domain of ExoS was responsible for TLR4-mediated activation whereas the C-terminal domain was responsible for TLR2-mediated activation. A, Ba/F3 stably transfected with either an ELAM-1 promoter driven luciferase vector (NF-κB) or Ba/F3 cells stably transfected with NF-κB-luciferase/TLR4/MD-2 were stimulated with different concentrations of either the N-terminal ExoS (N’) or C-terminal ExoS (C’) in the presence of 10 μg/ml polymyxin B. Triplicate samples were lysed and luciferase levels were determined. Data are presented as the mean of the triplicate samples (n = 3). B, HEK293 cells or HEK293-TLR2 cells were stimulated for 5 min with various concentrations of either N’-ExoS or C’-ExoS. Cells were lysed and a Western blot analysis was performed for phospho-Erk1/2 and actin. One of two representative experiments is shown.

**Discussion**

In this report we have made six observations: 1) ExoS was internalized by monocytic cells, although internalization was not required for cellular activation; 2) ExoS bound a specific and saturable receptor; 3) primary stimulation with either ExoS, LPS or PepG induced a state of cross-tolerance to one another; 4) ExoS-induced NF-κB production was MyD88-dependent; 5) ExoS activated cells by both TLR2 and TLR4/MD-2/CD14-dependent pathways; and 6) the ability to activate cells expressing TLR2 was...
attributed to the C terminus of ExoS and the ability to activate the TLR4/MD-2 complex was attributed to the N terminus of ExoS.

It is known that ExoS is secreted by a bacterial type III secretion system through both contact-dependent (intracellular delivery) and contact-independent pathways (extracellular action). In addition to the contact-dependent activity (attributed to ExoS injection into target cells) (35), contact-independent secretion results in the release of soluble extracellular ExoS, which has previously been shown to possess potent immunoinflammatory activity. Extracellular ExoS activates both monocytes and T cells directly, resulting in monocyte proinflammatory cytokine production, T cell proliferation and T cell apoptosis (41, 42, 44, 45, 57, 58). However, the mechanism by which extracellular ExoS activated these immune cells was not known, nor was it known whether activation was dependent on the enzymatic/catalytic domains possessed by ExoS. Extracellular ExoS was internalized into monocytes, whereas a control protein was not internalized, suggesting that the process was specific. Internalization was dependent on actin polymerization and led to the accumulation of ExoS within monocytes. Internalized ExoS labeled with a punctate pattern within the cell, which suggested that ExoS remained in vesicles rather than being diffusely localized through the cytosol. Additionally, when THP-1 cells were allowed to internalize ExoS-Alexa 488 (incubation at 37°C), the fluorescent signal was protected from quenching by trypan blue, confirming that ExoS was in fact intracellular.

We felt that there were a number of possible mechanisms by which internalized ExoS may have been involved in cellular activation. Internalization may have been a prerequisite step before the entry of ExoS into the cytosol, a compartment in which the enzymatic activities of ExoS could play a role. Alternatively, the internalization process, during which numerous kinases are activated, could trigger cytokine production; this process would be similar to the internalization of membrane-bound vesicles containing TLR9 during CpG DNA-induce activation, or could follow from internalization of the epidermal growth-factor ligand/receptor complex (50, 51, 59). However, blockade of internalization did not inhibit ExoS-induced cytokine production indicating that although ExoS was specifically internalized, neither internalization nor the process of internalization was required for ExoS-induced proinflammatory cytokine production.

This lead to an alternate hypothesis whereby ExoS might bind a specific cell surface receptor that initiated a signal transduction cascade leading to proinflammatory cytokine production. ExoS-Alexa 488 bound THP-1 cells in specific and saturable manner. However, only 40% of the binding could be outcompeted by unlabeled ExoS, which may be due to either ExoS binding nonspecifically to the cell membrane and/or may be related to the ability of ExoS to self-aggregate (46). It was clear that ExoS bound a saturable and specific receptor and the preferential internalization of ExoS may be due to receptor ligation, rather than simply part of the natural endocytic process of mononuclear cells.

Because microbial products can activate cells via pattern recognition receptors, and TLR are a major class of these molecules, the possibility that ExoS activated a TLR was considered. TLR bear the responsibility for recognizing a wide variety of stimuli including LPS, LTA, lipoproteins, and PepG (60, 61). There are a number of similarities in the inflammatory response to these molecules and ExoS. LTA, PepG, and lipoproteins are best known for inducing proinflammatory cytokine production from monocytes, and the cytokine profile is similar to that induced by ExoS (44, 62–64). In addition, LTA, PepG, and lipoproteins have been shown to have direct effects on T cells. LTA has been shown to bind directly to T cells and act as a T cell mitogen, similarly to ExoS (65). PepG was also shown to be mitogenic for human T cells (66) and B. burgdorferi OspA can act directly on T cells and provide costimulatory signals for T cell activation (67). Taken together, these observations suggest that ExoS may use a TLR to activate monocytes.

One of the important characteristics of TLR is that they display cross-tolerization. That is, ligands that bind and activate cells via one TLR cause decreased sensitivity to different ligands that bind the same, but also different TLR (52). Experiments were performed to determine whether stimulation with ExoS would decrease the sensitivity to ligands known to activate cells via TLR2 or TLR4 and vice versa. Pretreatment of THP-1 cells with ExoS resulted in tolerance to subsequent stimulation with ExoS, and both PepG and LPS. Conversely, pretreatment with PepG or LPS resulted in tolerance to ExoS. In fact, cross-tolerance was demonstrated between all the products, suggesting use of similar signaling molecules involved in the TLR pathway. This process is similar to that observed with LPS or LTA, which render monocytes tolerant to further stimulation with either one of the two stimuli (52). Tolerization is known to occur at multiple levels, including at the level of receptor down-regulation and degradation IRAK1, a common signaling intermediate. However, an unlikely possibility existed that the tolerance observed was not due to TLR activation, but a secondary effect due to secretion of cytokines during the primary stimulation period, such as TNF-α and IL-1. ExoS induces both TNF-α and IL-1 (42, 44) and both cytokines have been shown to tolerate monocyctic cells to secondary stimulation by LPS (68). Although this possibility was unlikely (52), additional experiments were required to definitely demonstrate that TLR were the class of receptors involved in ExoS recognition.

ExoS induced MyD88-dependent TNF-α production. MyD88 is required for NF-κB activation and subsequent proinflammatory cytokine production downstream of all TLR, which provided strong evidence that a TLR receptor was involved in ExoS recognition (10, 17, 20, 54, 69). Because TNF-α production was assessed in the presence of monensin, a secretion inhibitor, the effects of the MyD88DN was a primary event, and not due to the release of a soluble factor. Together with the ability to tolerate the cells, the data implicated a TLR in the recognition of ExoS. As PepG and LPS induced a state of tolerance to further ExoS stimulation, we tested ability of TLR4/MD-2 and/or TLR2 to transform ExoS nonresponsive cell lines into ExoS-responsive lines.

Studies with stably transfected cell lines and Ab neutralization experiments indicated that ExoS activated cells through both TLR2 and the TLR4/MD-2/CD14 complexes. This response is similar to the endogenous TLR agonist HSP70 (11), which has also been shown to work through these two receptors, and indicates that a single microbial protein can also activate multiple TLR (although the active domains of HSP70 have not been established). In either the case of HSP70 or ExoS, it is not known whether these products directly bind a TLR, or whether they act similarly to LPS and bind a high affinity receptor (CD14), in which a complex of molecules then interacts with TLR4 (70, 71). The presence of CD14 enhanced the responsiveness of cells to ExoS, but was not required indicating that CD14 was not essential in ExoS-mediated cellular activation. Both ExoS and HSP70 do however bind specific receptors. In the case of ExoS, the receptor binding studies indicated that ExoS did bind specifically to a receptor, although whether this was to a TLR, CD14, or to a molecule analogous to CD14 has yet to be determined. HSP70 binds specifically to CD91, and it is possible that a complex between CD91 and a TLR forms (72). Additionally, it was possible that ExoS bound to two different high affinity receptors, one that interacted with TLR2 and one that interacted with TLR4/MD-2. However, when examining the results of ExoS binding, it did not appear that it followed second order
kinetics, which would have suggested a second receptor. Other TLR agonists interact with several different cell surface proteins. For example, PepG binds directly to TLR2, although PepG also binds to a host of transmembrane PepG binding proteins and also CD14 (73, 74). In addition to the well-characterized interactions between LPS and CD14, LPS responsiveness is enhanced by the CD11/CD18 complex, acting in an analogous fashion to CD14 (75). Therefore, it is possible that multiple proteins/receptors may interact with ExoS to facilitate TLR-dependent activation (76).

Interestingly, the TLR2 and TLR4 activity of ExoS could be localized to different ExoS domains. ExoS possesses a C-terminal ADP-riboseyl transferase domain and an N-terminal GAP domain for Rho family GTPases. When translocated intracellularly by a bacterial type III secretion system, intracellular ExoS inhibits DNA synthesis and actin polymerization via these two domains, respectively (35, 40, 56, 77). Extracellularly, it also appears that these two domains play different roles, although this is not likely to be due to their enzymatic activity. N’-ExoS possessed the ability to activate cells expressing TLR4, whereas C’-ExoS activated cells expressing TLR2. Amino acids 36–68 of the N’-ExoS, when expressed intracellularly, target ExoS to the cytoplasmic leaflet of the plasma membrane (56). Whether this intracellular targeting domain is involved during extracellularly delivery is not yet known.

To our knowledge, this is first report indicating that two different domains of the same molecule activate two different TLR. Simultaneous stimulation of TLR2 and TLR4/MD-2/CD14 induces additive activation, which may explain why such an extensive array of proinflammatory cytokines and chemokines is induced by ExoS (76). However, this process may be different from coligation/coaggregation of TLR2 and TLR4/MD-2/CD14 together (42). Unlike simultaneous activation of TLR2 and TLR4/MD-2/CD14, in which both signal transduction pathways would be activated in parallel, but spatially could be segregated; coligation would bring the signaling pathways into close proximity with one another, which could qualitatively change the outcome. For example, inhibition of FceRI signaling occurs only when the FcyRII receptor is cross-linked to the FceRI by the same multivalent Ag, and if both FcyRII and FceRI are ligated, but not cross-linked, FceRI signaling remains normal (78). The idea of coligation is an interesting, and as of yet totally unexamined concept in the field of TLR biology. This is particularly relevant given the likelihood of TLR being simultaneously stimulated and/or coligated during infection with organisms that produces numerous TLR agonists, or as in the case of ExoS, different TLR being coligated by the same molecule.

The importance of such PAMP is an important link between innate and adaptive immunity. The current work has identified an important ligand from a Gram-negative bacteria that has both intracellular, and specific TLR-mediated extracellular activity. There is much to be learned about PAMP and the current study brings us one step closer by identifying a mechanism by which a single ligand can stimulate multiple TLR.

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References


