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Peroxisiredoxin 1 Stimulates Secretion of Proinflammatory Cytokines by Binding to TLR4

Jonah R. Riddell,* Xiang-Yang Wang,**† Hans Minderman,**† and Sandra O. Gollnick*

Peroxisiredoxin 1 (Prx1) is a member of the typical 2-cysteine peroxiredoxin family, whose major intracellular functions are as a regulator of hydrogen peroxide signaling through its peroxidase activity and as a protein chaperone (1). Prx1 expression is elevated in various cancers, including esophageal, pancreatic, lung, follicular thyroid, and oral cancer (2–9). Elevated Prx1 levels have been linked with poor clinical outcomes and diminished overall patient survival (4, 10, 11). Recent studies have demonstrated that Prx1 can be secreted by non-small cell lung cancer cells, possibly via a nonclassical secretory pathway (12, 13).

The function of extracellular/secreted Prx1 is unknown; however, a number of oxidative stress proteins, including thioredoxin and heat shock proteins, are released from stressed, transformed, and dying cells and act as “endogenous” danger signals by binding danger signal sensors/receptors in the extracellular microenvironment (14–18). Many of these “endogenous” danger signals are recognized by the danger signal receptor TLR4 (17, 19). A recent study by Furuta et al. (20) indicates that the malaria (Plasmodium berghei ANKA) homolog of Prx1/2, PbA, is a TLR4/MD2 ligand that promotes IgE-mediated protection and innate immunity. We hypothesize that mammalian Prx1 acts as an endogenous danger signal by binding to TLR4.

TLR4-induced gene activation is mediated through both MyD88-dependent and -independent pathways (21). MyD88-dependent signaling causes activation of NF-κB and protein kinase cascade-dependent activation of AP-1, which results in the secretion of proinflammatory cytokines such as TNF-α and IL-6 (22, 23). MyD88-independent gene activation occurs via the adaptor protein TRAM and leads to activation of interferon regulatory factor 3 and secretion of type I IFNs (IFN-α/β) (22–24).

Our studies demonstrate that Prx1 stimulates TLR4-dependent cytokine secretion from macrophages and dendritic cells (DCs), that the interaction and subsequent cytokine secretion is peroxidase independent but chaperone/structure dependent, and that TLR4-stimulated cytokine secretion by Prx1 is optimal in the presence of CD14 and MD2 and is MyD88 dependent.

Materials and Methods

Materials

LPS (Escherichia coli serotype 026:B6) polymyxin B sulfate salt, BSA, and ovalbumin (OVA) were obtained from Sigma-Aldrich (St. Louis, MO). 7-Aminoactinomycin D (7-AAD) and thioglycollate (TG) brewer-modified media was purchased from (BD Biosciences, La Jolla, CA). Capture and detection Abs for IL-6 and TNF-α used in Luminox assays, as well as protein standards, were purchased from Invitrogen (Carlsbad, CA). Abs specific for CD11b, Gr-1, F4/80, and all isotypes were purchased from BD Pharmingen (San Diego, CA). Abs specific for Prx1 were obtained from Lab Frontier (Seoul, South Korea); this Ab is specific for Prx1 and detects only a single band in Western analysis of cells that express Prx1 (Supplemental Fig. 1A).

Animals and cell lines

C57BL/6Ncr (TLR4+/+ and TLR2+/+), C57BL/10ScNj (TLR4−/−), B6.129-Tlr2(−/−) (TLR2−/−), C3H/HeNcr (TLR4+/+), and C3H/HeNj (TLR4−/−) pathogen-free mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in microisolation cages in laminar flow units under ambient light. The mice were maintained in a pathogen-free facility at Roswell Park Cancer Institute (Buffalo, NY). The Institutional Animal Care and Use Committee approved both animal care and experiments.

The role of Prx1 in vivo was determined by injecting either C57BL/6Ncr or C57BL/10ScNj mice i.v. with 90 μg Prx1 (~1000 nM). Cardiac punctures were performed 2 h later. Serum was obtained by incubation of blood at 4°C overnight, then samples were centrifuged and supernatants collected.

The cultured mouse macrophage cell line (RAW264.7) was maintained in DMEM containing 10% defined FBS and 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5.0% CO2. RAW264.7 cells were transfected with the pcDNA3.1 plasmid containing either control or MyD88 dominant-negative (DN) encoding oligonucleotides using FuGENE 6.
DCs were identified by the expression of CD11c. Culture of BM-derived cells in GM-CSF as described previously (30, 31). Determined by ELISA. TNF-α, IL-6, and IL-10 cytokine-specific ELISA or the Luminex multiplex assay system. Serum blocking or control Abs. In the indicated experiments, Prx1 proteins or concentrations were added in the presence or absence of Prx1-, MD2-, and CD14- adherent TG-elicited macrophage cells were washed 5–10 times with PBS, a Superdex 200 (16/60; GE Healthcare) and equilibrated with 50 mM containing 0.1 M NaCl. The unbound proteins from the DEAE column were dialyzed with 50 mM sodium phosphate buffer (pH 6.5) normalized by FITC labeling per nanomole proteins. Immunofluorescence staining was performed using the ImageStream multispectral imaging flow cytometer (34). At least 5000 events were thus acquired for each sample. FITC-labeled Prx1 and PE-conjugated anti-TLR4 to the media of TG-elicited macrophages. Colocalization experiments were performed by the addition of 200 nM FITC-labeled Prx1 and PE-conjugated anti-TLR4 to the media of TG-elicited macrophages. Colocalization of Prx1/TLR4 and NF-κB translocation

Colocalization of Prx1 and TLR4 formed in the following manner. TG-elicited macrophages obtained from C3H/HeNCr (TLR4+/+) and C3H/HeNJ (TLR4−/−) mice were obtained by adherence selection for 1 h in complete media (DMEM supplemented with 10% defined FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin) (28) and were characterized through FACS analysis for expression of CD11b, Gr1, and F4/80 as described previously (29); cells that were CD11b−Gr1−F4/80− were identified as macrophages. Immature bone marrow-derived DCs (iBMDCs) were generated by culture of BM-derived cells in GM-CSF as described previously (30, 31). DCs were identified by the expression of CD11c.

Protein purification

Recombinant human Prx1, Prx1C52S, and Prx1C83S proteins were purified as described previously (32, 33). Briefly, bacterial cell extracts containing recombinant proteins were loaded onto DEAE-Septarose (GE Healthcare Piscataway, NJ) and equilibrated with 20 mM Tris-Cl (pH 7.5). The proteins were dialyzed with 50 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The unbound proteins from the DEAE column containing Prx1, Prx1C52S, or Prx1C83S were pooled and loaded onto a Superdex 200 (16/60; GE Healthcare) and equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The fractions containing Prx1, Prx1 C52S, or Prx1C83S were pooled and stored at −80°C. Endotoxin levels of purified proteins were quantified with a Limulus amebocyte lysate assay (Lonza, Walkersville, MD), according to the manufacturer’s directions. Prx1, Prx1C52S, and Prx1C83S were found to contain 14.14 ± 0.050, 14.07 ± 0.67, and 14.17 ± 0.025 endotoxin units (EU)/ml, respectively.

Cytokine analysis

Adherent TG-elicited macrophage cells were washed 5–10 times with PBS, to remove any nonadherent cells. Once washed, complete media containing purified Prx1, Prx1C52S, Prx1C83S, or LPS at the specified concentrations were added in the presence or absence of Prx1-, MD2-, and CD14-blocking or control Abs. In the indicated experiments, Prx1 proteins or LPS were incubated with polymyxin B or were boiled for 20 min prior to addition. After 24 h, the supernatant was collected and analyzed by cytokine-specific ELISA or the Luminox multiplex assay system. Serum samples were collected as indicated above, and IL-6 levels were determined by ELISA. TNFα and IL-6 ELISA kits were purchased from BD Biosciences (Franklin Lakes, NJ), and assays were completed according to the manufacturer’s instructions. Luminex analyses were performed by the Institute Flow Cytometry Facility in 96-well microtiter plates (Multiscreen HV plates; Millipore, Billerica, MA) with polystyrene diisocoulophane membranes using a TECAN Genesis liquid handling robot (Tecan, Research Triangle Park, NC) for all dilutions, reagent additions, and manipulations of the microtiter plate. Bead sets, coated with capture Ab were diluted in assay diluents and pooled, and ~1000 beads from each set were added per well. Reconstituent protein standards were titrated from 9000 to 1.4 μg/ml using 3-fold dilutions in diluent. Samples and standards were added to wells containing beads. The plates were incubated at ambient temperature for 120 min on a rocker and then washed twice with diluent using a vacuum manifold to aspirate. Biotinylated detection Abs to each cytokine were next added, and the plates were incubated 60 min and washed as before. Finally, PE-conjugated streptavidin was added to each well, and the plates were incubated 30 min and washed. The beads were resuspended in 100 μl wash buffer and analyzed on a Luminex 100 (Luminex, Austin, TX). Each sample was measured in duplicate, and blank values were subtracted from all readings. Using BeadView Software (Millipore), a log-regression curve was calculated using the bead mean fluorescence intensity (MFI) values versus concentration of recombinant protein standard. Points deviating from the best-fit line, i.e., beyond detection limits or above saturation, were excluded from the curve. Sample cytokine concentrations were calculated from their bead’s MFIs by interpolating the resulting best-fit line. Samples with values above detection limits were diluted and reanalyzed.

FITC labeling of proteins

BSA, Prx1, Prx1C52S, and Prx1C83S proteins were conjugated to FITC using an FITC conjugation kit (Sigma-Aldrich). A 20-fold excess of FITC and individual proteins were dissolved into a 0.1 M sodium bicarbonate/ carbonate buffer (pH adjusted to 9.0); the mix was incubated for 2 h at room temperature with gentle rocking. The excess free FITC was removed with a Sephadex G-25 column (Pharmacia, Piscataway, NJ). Protein amounts were quantified using a standard Lowry assay. The fluorescence:protein ratio was calculated according to the manufacturer’s instructions using the optical density at 495 nm (FITC absorbance) and 280 nm (protein absorbance). FITC per nanomole protein for BSA, Prx1, Prx1 C52S, and Prx1 C83S were 31.00 ± 1.92, 38.52 ± 2.39, 74.49 ± 2.64, and 44.44 ± 2.64, respectively.

Saturation assay

FITC-conjugated BSA, Prx1, Prx1C52S, and Prx1C83S were diluted in 1% BSA in PBS to the specified concentrations and a total reaction volume of 100 μl. These mixtures were incubated with 1.0 × 106 cells/ml for 20 min on ice to prevent internalization. Cells were washed twice with 1% BSA in PBS, and cells were incubated to demonstrate viable from nonviable cells with 7-AAD, <30 min before FACS Calibur analysis. Data were acquired from a minimum of 20,000 cells, stored in collatral list mode, analyzed using the WinList processing program (Verity Software House, Topsham, ME). Cells positive for 7-AAD (nonviable) were gated out of the events. FITC-conjugated BSA was used as a negative binding control, and for mutant studies, variations in FITC labeling were normalized by FITC labeling per nanomole proteins.

Competition assay

Unlabeled OVA, Prx1, Prx1C52S, and Prx1C83S were briefly mixed with FITC-conjugated Prx1 at the specified concentrations in 100 μl 1% BSA in PBS. The mixture was incubated for 20 min on ice, before washing twice with 1% BSA in PBS. Cells were then incubated with 7-AAD and analyzed within 30 min by flow cytometry. OVA was used as a negative competition control in all competition assays. Data were acquired from a minimum of 20,000 cells, stored in collatral list mode, and analyzed using the WinList processing program (Verity Software House). When using WinList to analyze results, 7-AAD-positive cells were gated out of the events.

Immunoprecipitation

Immunoprecipitation was carried out with 500 μg cell lysates and 4 μg anti-TLR4 or anti-TLR2 overnight at 4°C. After the addition of 25 μl protein G-agarose (Santa Cruz Biotechnology), the lysates were incubated for an additional 4 h. To validate specific protein interactions, goat IgG (Santa Cruz Biotechnology) or mouse IgG (Santa Cruz Biotechnology) was used as a negative control. The beads were washed thrice with the lysis buffer, separated by SDS-PAGE, and immunoblotted with Abs specific for Prx1. The proteins were detected with the ECL system (Bio-Rad, Hercules, CA).

Colocalization of Prx1/TLR4 and NF-κB translocation

Colocalization experiments were performed by the addition of 200 nM FITC-labeled Prx1 and PE-conjugated anti-TLR4 to the media of TG-elicited macrophages and kept at 37°C for the indicated times before being transferred to ice, fixed, and analyzed. Immunostaining to detect the nuclear translocation of NF-κB was performed in the following manner. TG-elicited macrophages obtained from C3H/HeNCr (TLR4+/+) and C3H/HeNJ (TLR4−/−) were treated with 200 nM Prx1. After the indicated times at 37°C, the cells were then scraped and collected in tubes, washed twice in wash buffer (2% FBS in PBS), and then fixed in fixation buffer (4% paraformaldehyde in PBS) for 10 min at room temperature. After washing, the cells were resuspended in Perm Wash buffer (0.1% Triton X-100, 3% FBS, and 0.1% sodium azide in PBS) containing 10 μg/ml anti-NF-κB p65 Ab (Santa Cruz Biotechnology) for 20 min at room temperature. The cells were then washed with Perm Wash buffer and resuspended in Perm Wash buffer containing 7.5 μg/ml FITC-conjugated F(ab′)2 donkey anti-rabbit IgG for 15 min at room temperature. Cells were washed twice in Perm Wash buffer and resuspended in 1% paraformaldehyde containing 5 μM DRAQ5 nuclear stain (BioStatus, Leicestershire, UK) for 5 min at room temperature.

Image analysis

Colocalization of Prx1 and TLR4 and nuclear translocation of NF-κB were analyzed with the ImageStream multispectral imaging flow cytometer (34) (Ammis, Seattle, WA). At least 5000 events were thus acquired for each
experimental condition, and the corresponding images were analyzed using the IDEAS software package. A hierarchical gating strategy was employed using image-based features of object contrast (gradient RMS) and area versus aspect ratio to select for in-focus, single cells. Colocalization and nuclear translocation was determined in each individual cell using the IDEAS similarity feature, which is a log-transformed Pearson’s correlation coefficient of the intensities of the spatially correlated pixels within the whole cell, of the Prx1 and TLR4 images or NF-κB and DRAQ5 images, respectively. The similarity score is a measure of the degree to which two images are linearly correlated.

EMSA

EMSA was performed as described previously (35). Briefly, 10 μg nuclear protein was incubated with γ-[32P]-labeled double-stranded NF-κB oligonucleotide (5’-AGTTGAGGGGACTTTCCCAGGC-3’) in 20 μl binding solution containing 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, and 100 μg/ml poly(dexohydroinosinic-deoxythymydilic acid). The DNA-protein complexes were resolved on a 6% polyacrylamide gel under nondenaturing conditions at 200 V for 2 h at 4˚C. Gels were dried and then subjected to autoradiography.

Statistical analysis

Statistical analyses were performed using a standardized t test with Welch’s correction, where equal variances were not assumed, to compare experimental groups. Differences were considered significant when p values were ≤0.05.

Results

Prx1 stimulation of cytokine secretion from DCs and TG macrophages and maturation of DCs is dependent on TLR4

TG-elicited murine macrophages were used to assess the ability of Prx1 to stimulate cytokine secretion. Macrophage phenotype was assessed by analysis of peritoneal exudate cell populations for CD11b, Gr1, and F4/80 expression. The isolated populations were >99% CD11b+ and, of the CD11b+ cell population, a majority were Gr1+, F4/80+ (Fig. 1A). Stimulation of TG-elicited macrophages with Prx1 resulted in the dose-dependent secretion of TNF-α and IL-6 that was significantly greater than that observed in unstimulated cells at all doses (p ≤ 0.01; Fig. 1B). Preincubation of Prx1 with the endotoxin inactivator polymyxin B had no significant effect on Prx1 stimulation of cytokine secretion (Fig. 1C); in contrast, denaturing of Prx1 significantly reduced its ability to stimulate cytokine secretion (p < 0.01).

Stimulation of cytokine secretion by TG-elicited macrophages following incubation with Prx1 was significantly diminished in the absence of serum (p ≤ 0.01; Fig. 1D); however, even in serum-free conditions, incubation of TG-elicited macrophages with Prx1 significantly increased IL-6 secretion (p ≤ 0.005 when compared with secretion by cells incubated in serum-free media). Prx1 was also able to stimulate cytokine secretion from the cultured DC line, DC1.2, and the murine macrophage cell line, RAW264.7 (data not shown).

Exogenous Prx1 was able to induce maturation and activation of iBMDCs. iBMDCs were incubated with increasing concentrations of Prx1 for 24 h and examined for cell surface expression of costimulatory molecules and secretion of TNF-α. Addition of Prx1 led to significant dose-dependent increase in cell surface expression of the costimulatory molecule, CD86 (Fig. 2A) and TNF-α expression (Fig. 2B) at all doses tested (p ≤ 0.01 when compared with control).

It is possible that enhanced secretion of cytokines from iBMDCs and TG-elicited macrophages upon addition of exogenous rPrx1 is a phenomena of the recombinant protein and not physiologically

FIGURE 1. Prx1 stimulates cytokine secretion from macrophages. A, TG-elicited macrophages were analyzed by flow cytometry for expression of CD11b, Gr1, and F4/80. A representative histogram of three independent isolations is shown and depicts Gr1 and F4/80 expression by CD11b+ cells. Numbers in the insets indicate the percentages of CD11b+ cells in each quadrant. B, TG-elicited macrophages were incubated with stimulants for 24 h: supernatants were harvested and analyzed for TNF-α (○) and IL-6 levels (gray bars). Results are shown as picograms per milliliter and are representative of three independent experiments; error bars represent SD. C, TG-elicited macrophages were incubated for 24 h with media only (gray bars), 100 nM LPS or 2000 nM Prx1 (○), 100 nM LPS, or 2000 nM Prx1 preincubated for 20 min with 10 μg/ml polymyxin B (hatched bars), or 100 nM LPS or denatured 2000 nM Prx1 (●). Asterisks indicate p ≤ 0.01 as compared with cells treated with Prx1 or LPS alone. D, TG-elicited macrophages were incubated with media alone, Prx1 (50 nM), or LPS (100 nM) for 24 h in the presence (gray bars) or absence (●) of 10% FBS. Supernatants were harvested and analyzed for IL-6 levels. Results are shown as picograms per milliliter; error bars represent SD.
Prx1 stimulates DC maturation and activation. 

A and B, iBMDCs were incubated with media alone, 20–200 nM Prx1 or 100 nM LPS for 24 h. A, Following incubation cells were analyzed by flow cytometry for expression of CD11c and CD86. Results are shown as percent total cells; error bars represent SD. B, Supernatants were harvested and analyzed for TNF-α. Results are shown as picograms per milliliter and are representative of three independent experiments; error bars represent SD.

C, TG-elicited macrophages were incubated with media harvested from prostate tumor cell lines that were transfected with cDNA encoding for either control shRNA (Scramble) or shRNA specific for Prx1 (shPrx1) or in media harvested from cells expressing Prx1-specific shRNA to which 50 nM exogenous Prx1 had been added (shPrx1 + Prx1). Following 24 h of incubation, supernatants were harvested and analyzed for TNF-α. Results are shown as picograms per milliliter and are representative of three independent experiments; error bars represent SD. #p ≤ 0.01 when compared with TNF-α levels secreted by cells incubated with media alone; ##p ≤ 0.01 when compared with TNF-α levels secreted by cells incubated with media from cells expressing control shRNA; †p ≤ 0.01 when compared with TNF-α levels secreted by cells incubated with media from cells expressing shRNA specific for Prx1.

Relevant. To begin to determine whether Prx1 could promote cytokine secretion in a physiologic context, TG-elicited macrophages were incubated for 24 h in the presence of supernatant collected from Prx1-secreting tumor cells or supernatant collected from tumor cells engineered to express shRNA specific for Prx1. Expression of shRNA resulted in reduced expression of Prx1 but not Prx2 (Supplemental Fig. 1A). Incubation of TG-elicited macrophages with supernatants of tumor cells engineered to express shRNA specific for Prx1 resulted in reduced expression of Prx1 but not Prx2 (Supplemental Fig. 1A). Incubation of TG-elicited macrophages with supernatants of tumor cells engineered to express shRNA specific for Prx1 resulted in reduced expression of Prx1 but not Prx2 (Supplemental Fig. 1A).

To further demonstrate the interaction Prx1 and TLR4/MD2/CD14, TG-elicited macrophage cell lysates were incubated with isotype control Abs or Abs specific for TLR4 or TLR2 (Fig. 4B). The Abs complexes were isolated and immunoblotting was performed using Abs to Prx1; Prx1 was only found in the lysates immunoprecipitated with TLR4 (Fig. 4B). The TLR4/Prx1 complexes isolated from Prx1-treated cells also contained CD14 and MD2 (Fig. 4C), confirming the finding that Prx1 interacts with TLR4 in a complex that contains both CD14 and MD2.

The kinetics of the Prx1 and TLR4 interaction was determined using image stream analysis (Aminis) to examine colocalization of the two molecules. TG-elicited macrophages were incubated with FITC-labeled Prx1 and PE-conjugated anti-TLR4 Abs. The merged images of representative cells indicate that Prx1 and TLR4 localize together on the membrane of the macrophage within 5 min, and that by 30 min, TLR4 and a portion of the Prx1 molecules have been internalized (Fig. 5A). The histograms to the right of the merged images are a statistical analysis of the similarity of FITC-Prx1 and PE-anti-TLR4 in 5000 cells on a pixel-by-pixel basis. A shift of this distribution to the right indicates a greater degree of similarity. The average similarity coefficient at each time point was demonstrated in Fig. 5B. At all time points there was a high similarity of Prx1 and TLR4 staining (similarity coefficients > 1), indicating a colocalization of Prx1 and TLR4. These results confirm that Prx1 and TLR4 interact on the cell surface and that at least portion of the Prx1 is internalized with TLR4.

Stimulation of cytokine secretion and binding to TLR4 depends on Prx1 structure

Prx1 acts as both a peroxidase and a protein chaperone (1). To determine whether the ability of Prx1 to stimulate cytokine secretion from TG-elicited macrophages was related to its peroxidase activity and/or chaperone activity, two Prx1 mutants were examined. The Prx1C52S mutant lacks peroxidase activity but
retains the decamer structure needed for chaperone activity; Prx1C83S exists mainly as a dimer and has reduced chaperone activity and intact peroxidase activity (32, 33, 36). Cytokine secretion following Prx1C83S stimulation of TG-elicited macrophages was not significantly distinct from that observed following stimulation with Prx1 (Fig. 6A); however, TG-elicited macrophages stimulated with Prx1C83S displayed a significant reduction in cytokine secretion ($p \leq 0.01$).

Prx1 binding to TG-elicited macrophages was dependent on the presence of TLR4 as binding of Prx1 and the enzymatic null mutant (Prx1C52S) was significantly decreased in the absence of TLR4 (Fig. 6B). Prx1C83S binding was minimal to either TLR4 expressing or nonexpressing macrophages, confirming that Prx1 interaction with TLR4 is peroxidase independent and structure dependent.

Saturation binding (Fig. 6C) and competition analyses (Fig. 6D) were used to determine the $K_a$ and $K_i$ values for Prx1 binding to the surface of TG-elicited macrophages. The $K_d$ for Prx1 binding to TG-elicited macrophages was 1.6 mM, and the $K_i$ was 4.1 mM (Table I).

**Prx1 stimulation of cytokine secretion is MyD88 dependent and leads to TLR4-dependent translocation of NF-κB to the nucleus**

The consequential downstream signaling events of ligand-mediated activation of TLR4 can be MyD88 dependent or independent. Prx1 was used to stimulate cytokine expression from RAW264.7 cells expressing DN MyD88 protein. IL-6 secretion following Prx1 stimulation is dependent on MyD88 function (Fig. 7A), indicating that Prx1 activates the MyD88 signaling cascade, which can lead to activation of NF-κB (37).

To determine whether Prx1/TLR4 interaction leads to NF-κB activation, NF-κB translocation following Prx1 stimulation was analyzed in macrophages isolated from C3H/HeNCr and C3H/HeNJ mice. C3H/HeNCr mice have a mutation in the TLR4 ligand binding domain that prevents ligand binding (37). TG-elicited macrophages from C3H/HeNCr and C3H/HeNJ mice were incubated with 200 nM Prx1 at 37°C for the indicated times, transferred to ice, and incubated with Abs against NF-κB p65; the nuclear stain DRAQ5 was added 15 min prior to image stream analysis. Prx1 incubation with macrophages isolated from C3H/HeNCr mice triggered NF-κB translocation within 5 min, and nuclear localization was apparent for up to 60 min (Fig. 7B). In contrast, Prx1 incubation with macrophages isolated from C3H/HeNJ mice did not trigger NF-κB translocation (Fig. 7B). The histogram to the right of the merged image column depicts the similarity of NF-κB and the nuclear stain on a pixel-by-pixel basis. Prx1 stimulation led to NF-κB translocation to the nucleus in a TLR4-dependent manner as demonstrated by the positive similarity coefficient observed following Prx1 stimulation of C3H/HeNCr TG-elicited macrophages, which was decreased following Prx1 stimulation of C3H/HeNJ TG-elicited macrophages (Fig. 7C). The ability of Prx1 to activate NF-κB was confirmed by EMSA, which indicated that incubation of macrophages with Prx1 resulted in a dose-dependent increase in NF-κB DNA-binding activity (Fig. 7D).

**Discussion**

We present compelling evidence that Prx1 stimulates TLR4-dependent secretion of TNF-α and IL-6 from TG-elicited macrophages and DCs. Cytokine secretion was the result of TLR4 stimulation of the MyD88-dependent signaling cascade and resulted in activation and translocation of NF-κB. Prx1 is an intracellular protein that is secreted from tumor cells and activated T cells (12, 13, 38). The ability of Prx1 to interact with TLR4 and stimulate the release of proinflammatory cytokines suggests that it may also act as an endogenous damage-associated molecular pattern molecule (DAMP).

HSP72 and HMGBl, which have also been classified as endogenous DAMPs, have been shown to interact with TLR4 (17, 19, 39, 40). Saturation and competition studies indicate that Prx1 has a $K_d$ of ~1.3 mM and a $K_i$ of ~4.1 mM; extraction of data presented by Binder...
et al. (41) implies that HSP72 has a $K_d$ of 2.1–4.4 mM and a $K_i$ of 10–21.8 mM, suggesting that Prx1 interaction with TLR4 is stronger than that of HSP72. Binding affinities are not available for HMGB1.

Identification of TLR4 as a receptor for a recombinant protein is complicated by the potential of the presence of LPS within the recombinant protein preparation. To account for this possibility in the results presented here, two controls were included in all of the performed studies. In the first control, recombinant proteins were combined with polymixin B prior to their addition to immune cells. Polymixin B is a powerful inactivator of LPS; preincubation of rPrx1 with polymixin B had no effect on the ability of Prx1 to stimulate cytokine expression (Fig. 1). However, preincubation of LPS with the same concentration of polymixin B significantly inhibited its ability to stimulate cytokine release. As a second control, Prx1 and LPS were boiled prior to addition to immune cells; denaturing Prx1 significantly inhibited its ability to stimulate cytokine release, but boiling had no effect on the ability of LPS to stimulate cytokine release. Finally, all of the recombinant proteins used in this study were prepared in the same fashion, and following purification, all were found to have equivalent levels of endotoxin (∼14 EU/ml), yet Prx1C83S stimulated significantly lower cytokine secretion and did not appear to bind to TLR4-expressing cells. Thus, it appears as though the results demonstrating that Prx1 interacts with TLR4 are not due to the presence of LPS contamination.

Prx1, HSP72, and HMGB1 not appear to have significant structural similarity, nor do these molecules appear to share homology with LPS (22, 42). Prx1, HSP72, and HMGB1 are molecular chaperones, and the lack of structural homology between HSP72/HMGB1 and other TLR4 ligands has led some to speculate that the chaperone cargo rather than the chaperone is being recognized by TLR4 (43, 44). In support of this hypothesis, recent studies have shown that HMGB1 binding to TLR9 is a result of TLR9 recognition of HMGB1/DNA complexes (45). Extracellular Prx1 is present as a decamer, which is associated with Prx1 chaperone activity (46), and our studies indicate that Prx1 binding to TLR4 was dependent on the ability to form decamers (Figs. 3, 4B). Thus, it is possible that Prx1 binding of TLR4 is due to recognition of its cargo rather than of Prx1 itself.

The Prx1C83S mutant, which lacks chaperone activity and exists primarily as a dimer (46), did not appear to bind to TLR4 (Fig. 4B); however, the purified mutant protein was able to stimulate cytokine secretion from macrophages (Fig. 4A). Assays for...
biological function are traditionally more sensitive than binding assays, and it is possible that the interaction of the dimeric form of Prx1 with TLR4 was below the level of detection in the binding assay used in these studies. A small portion of Prx1C83S is present as a tetramer (46), which may also be able to interact with TLR4 at a level that is below detection, but that is sufficient to stimulate cytokine secretion.

Prx1 stimulation of cytokine secretion was dependent on TLR4 and MyD88 (Figs. 3–5); however, FITC-labeled Prx1 did bind to macrophages isolated from TLR4−/− (B10ScNJ) mice (Fig. 4B), albeit at a lower level than bound to macrophages isolated from TLR4+/+ (B6) mice. Examination of the interaction of Prx1 with TLR4 at a cellular level indicated that although a majority of the TLR4 was internalized upon Prx1 binding, at least a portion of the Prx1 remained on the cell surface (Fig. 3B,3C). These findings could be the result of excess Prx1 or alternatively that Prx1 is binding to additional receptors. Other TLR4-binding DAMPs have been shown to bind to multiple danger receptors (14, 17, 19, 28, 47–49) and, in some cases, DAMP binding to TLR4 requires coreceptors. PbA, the malaria homolog of Prx1, requires MD2 to bind to TLR4 (20); our studies indicate that Prx1 stimulation of cytokine secretion is optimal in the presence of serum and that Abs to CD14 and MD2 block cytokine secretion from Prx1-stimulated cells. Furthermore, immunoprecipitated complexes of TLR4 and Prx1 contain MD2 and CD14, suggesting that these proteins contribute to the binding of Prx1 to TLR4.

Numerous studies have shown that activation of TLRs expressed on tumor cells can act to promote tumor survival, chemoresistance, progression, and metastasis (50–52). Furthermore, inflammation, such as that which occurs during chronic infection, has been shown to promote carcinogenesis primarily through the generation of a tumor-permissive microenvironment and recruitment of tumor-promoting macrophages (52, 53). In contrast, there is evidence suggesting that TLR4 induction of IL-10–producing T cells acts to regulate the destructive tendencies of inflammation and that the incidence of gastric cancer is increased in the absence of TLR4 (54, 55). However, the presence of LPS, the prototypical TLR4 ligand, has been shown to accelerate tumor growth in both clinical and preclinical studies (50). Prx1 expression is elevated in various cancers and cancer cell lines (2–9, 26), and elevated Prx1 levels have been linked with poor clinical outcomes and diminished overall patient survival (4, 10, 11). Thus, it is possible that release of Prx1 from tumor cells, as has been shown to occur in lung cancer cells (12, 13), and subsequent interaction with both TLR4-expressing tumor cells and innate immune cells may promote tumor growth.

Table I.  Binding constants for Prx1

<table>
<thead>
<tr>
<th>Ligand(nM)</th>
<th>Bmax (MFI/FITC protein)</th>
<th>Kd (mM)</th>
<th>Ki (mM)</th>
<th>Log (Ki)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>0.6143</td>
<td>1.3</td>
<td>1.1 × 10^-4</td>
<td>10</td>
</tr>
<tr>
<td>Prx1</td>
<td>3.148</td>
<td>1.6</td>
<td>4.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Prx1C52S</td>
<td>3.607</td>
<td>2.5</td>
<td>5.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Prx1C83S</td>
<td>1.033</td>
<td>1.2</td>
<td>4.5 × 10^-5</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Materials and Methods
described in bars represent SD. The furthest right column shows a pixel-by-pixel statistical analysis of the similarity of NF-κB complete media. At the indicated time points, cells were stained with FITC-conjugated Abs to NF-κB p65 and DRAQ5 (nuclear stain) for 10 min and analyzed using Amnis technology. The furthest right column shows a pixel-by-pixel statistical analysis of the similarity of NF-κB expression.

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Disclosures
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Supplementary Figure 1. (A) Cell lysate isolated from PC-3M cells (right panel) engineered to express control (Scramble) shRNA or Prx1 specific shRNA (shPrx1) was separated by gel electrophoresis, blotted and probed with antibodies specific for Prx1. (B) Expression of shRNA specific for Prx1 leads to decreased Prx1 levels. PC3-M cell lines engineered to express either control shRNA (Scramble) or shRNA specific for Prx1 were harvested and analyzed for expression of Prx1 or Prx2 by Western analysis. The upper panel shows a representative blot; the lower panel shows the quantification of three separate experiments; open bars represent PC-3M cells expressing control (Scramble) shRNA; closed bars represent PC-3M cells expressing Prx1 specific shRNA. (C) TG-elicited macrophages were isolated from C57BL/6 mice and stimulated with LPS in the presence or absence of control or blocking antibodies to CD14 or MD2 for 24h. Supernatants were collected and analyzed by IL-6 ELISA kits. Results are presented as pg/ml; error bars represent SEM.