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A Pattern Recognition Receptor, SIGN-R1, Mediates ROS Generation against Polysaccharide Dextran, Resulting in Increase of Peroxiredoxin-1 and Its Interaction to SIGN-R1

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Abstract − *Streptococcus pneumoniae* is the major pathogen that frequently causes serious infections in children, the elderly and immunocompromised patients. *S. pneumoniae* is known to produce reactive oxygen species (ROS) and *S. pneumoniae*-produced ROS is considered to play a role in pneumococci pathogenesis. SIGN-R1 is the principal receptor of capsular polysaccharides (CPSs) of *S. pneumoniae*. However, there is a considerable lack of knowledge about the protective role of SIGN-R1 against *S. pneumoniae*-produced ROS in SIGN-R1$^+$ macrophages. While investigating the protective role of SIGN-R1 against ROS, we found that SIGN-R1 intimately bound to peroxiredoxin-1 (Prx-1), one of small antioxidant proteins *in vitro* and *in vivo*. This interaction was increased with ROS generation which was produced by stimulating SIGN-R1 with dextran, a polysaccharide ligand of SIGN-R1. Also, SIGN-R1 crosslinking with 22D1 anti-SIGN-R1 antibody increased Prx-1 *in vitro* or *in vivo*. These results suggested that SIGN-R1 stimulation with CPSs of *S. pneumoniae* increase the expression level of Prx-1 through ROS and its subsequent interaction to SIGN-R1, providing an important antioxidant role for the host protection against *S. pneumoniae*.

Keywords: Pattern recognition receptors, SIGN-R1, Prx-1, ROS, Polysaccharides, Dextran

INTRODUCTION

Streptococcus pneumoniae is a microorganism that frequently causes serious infections in children, the elderly, and immunocompromised patients (Musher, 1992; Moens *et al*., 2007). *S. pneumoniae* is a major cause of otitis media, pneumonia, meningitis, and septicemia in humans (Martner *et al*., 2008). Despite antibiotic therapy and supportive intensive care, the morbidity and mortality of pneumococcal meningitis remain unacceptably high (Finch, 2001). Its significance as a pathogen can be illustrated by the that approximately one million children every year in developing countries die from pneumococcal diseases (Martner *et al*., 2008).

The oxidative burst is essential to the antibacterial activity against *S. pneumoniae* especially in neutrophils, which generates reactive oxygen species (ROS) through an NADPH oxidase system (Segal, 2005). ROS acts as intracellular and extracellular signaling molecules, participating in regulation of the functions of immune cells (Hellstrand *et al*., 1994; Olofsson *et al*., 2003; Hultqvist and Holmdahl, 2005). However, ROS damages the host's own cells and tissue components partly responsible for the tissue destruction associated with many microbial infections and inflammatory diseases (Martner *et al*., 2008), since ROS exerts a vast variety of toxic reactions, for instance, through lipid peroxidation, DNA strand breakage followed by poly (ADP-ribose) polymerase activation and subsequent cellular energy depletion, production of inflammatory cytokines, and activation of matrix metalloproteinases (Klein *et al*., 2006).

The cell is armed with a powerful antioxidant defense system to combat excessive production of ROS, including enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic scavengers

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such as glutathione, ascorbic acid and carotenoids (Neumann *et al*., 2003). Peroxiredoxins (Prxs), which are a group of scavengers for H_2O_2 (Chae *et al*., 1993), also play a protective antioxidant role in cells through its peroxidase activity, whereby H_2O_2, peroxynitrite, and a wide range of organic hydroperoxides (ROOH) are reduced and detoxified (Bryk *et al*., 2000; Hofmann *et al*., 2002). The mammalian Prx family members can be classified into six distinct groups (types I through VI) (Rhee *et al*., 2001). Although different Prx family proteins exhibit distinct tissue and organellar distributions (Kang *et al*., 1998), they have been shown to have strong antioxidant activities *in vitro* (Rhee *et al*., 2001). In addition to their antioxidant activities, Prxs participate in various biological functions, such as cell proliferation, differentiation, apoptosis, gene expression, and intracellular signaling (Wen and Van Etten, 1997; Kim *et al*., 2000; Fujii and Ikeda, 2002).

SIGN-R1, a C-type lectin, is expressed at high levels in the splenic marginal zone (MZ), which lies at the junction of the white pulp nodule with the red pulp and lymph node medulla (Geijtenbeek *et al*., 2002; Kang *et al*., 2003; Kang *et al*., 2004). SIGN-R1 is the principal receptor for polysaccharide antigens such as dextran and the CPSs of *S. pneumoniae* (Kang *et al*., 2003; Kang *et al*., 2004). SIGN-R1 also mediates the new activation pathway of complement against *S. pneumoniae* on SIGN-R1$^+$ MZ macrophages by interacting with C1q (Kang *et al*., 2006), participating the protection processes against lethal pneumococcal infections in mice (Lanoue *et al*., 2004; Kang *et al*., 2006). Here, we show that SIGN-R1 stimulated by polysaccharide dextran mediates ROS generation and subsequently leads to increase of intracellular Prx-1 level, resulting in the increase of SIGN-R1 binding to Prx-1. All of these results suggest that the intimate SIGN-R1 binding to Prx-1 plays an important role for the antioxidant protection from *S. pneumoniae*-produced ROS.

MATERIALS AND METHODS

Mice, cells, and reagents

C57BL/6 mice were purchased from B&K Universal Ltd. (United Kingdom). Mice of both sexes were kept under specific pathogen-free conditions until use at 6-10 weeks of age. SIGN-R1 TKO mice were generated with an i.v. injection of 22D1 anti-SIGN-R1 antibody (100 μg, 24 h) (Kang *et al*., 2004). All experiments were conducted according to institutional guidelines. Stably transfected mouse fibroblasts (DCEK) or CHO cell line expressing SIGN-R1, mouse DC-SIGN were cultured in RPMI 1640 or DMEM medium supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin, respectively. Expression vectors containing the ORF of the respective cDNAs were transfected into HEK293 with Lipofectamine 2,000 reagent (Invitrogen, Carlsbad, CA). Two days after transfection, cells were harvested and lysed for analysis. The following materials were purchased: dextran 2,000 kDa (Sigma Chemical Co., St. Louis, Mo.), transferrin and Bovine Serum Albumin (BSA), hamster IgG (Jackson ImmunoResearch Laboratories), FITC and PE conjugated antibodies (Jackson ImmunoResearch Laboratories), Streptavidin (SA)-beads, Alexafluor-conjugated antibodies (Molecular Probes).

Antibodies

The generation of rabbit polyclonal antibody against the C-terminal 13 amino acid peptide of SIGN-R1 (PAb-C13) was described previously (Kang *et al*., 2003). 22D1 (anti-SIGN-R1) hamster monoclonal antibody was purified from hybridoma culture supernatants (Kang *et al*., 2003). We purchased antibodies to Prx-1 and Prx-2 (Santa Cruz, CA), actin (Abcam, Cambridge, MA). As secondary reagents, we used HRP-, FITC-, PE-, or Alexafluore-conjugated goat anti-hamster IgG, donkey anti-rabbit IgG, goat anti-rat IgG, and HRP-conjugated streptavidin from Jackson ImmunoResearch Laboratories, or Molecular Probes. Omission of the primary antibody served as negative controls.

Immunoprecipitation and immunoblot analysis

Antibodies were biotinylated with EZ-Link^{TM} NHS-Biotin (PIERCE) for 2 hours at 4°C. Biotinylated materials were incubated with 1 mg of tissues or cells for 12 hours at 4°C. 20 μl of SA-beads were further incubated for 2 hours at 4°C in PBS. Bound proteins were eluted and separated on a SDS-PAGE gel followed by immunoblot analysis. Tissues or cells were lysed in RIPA buffer (150 mM NaCl/50 mM Tris-HCl, pH 8.0/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS) supplemented with protease inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Each lysed sample was mixed with an equal volume of 2× SDS sample buffer with mercaptoethanol and boiled at 95°C for 10 minutes. Then, the lysates were separated in SDS-PAGE gel and transferred onto PVDF membranes, followed by incubation with antibodies. Antibody-reactive bands on the blots were visualized by incubation with peroxidase-labeled secondary antibodies followed by treatment with West-ZOL plus (Intron) and developed by LAS-4,000 (Fuji film).

Determination of reactive oxygen species (ROS) and FACS analysis

Generation of reactive oxygen species (ROS) was as-

sessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, Oregon, USA) as described previously (Chandel *et al*., 2001). ROS in cells cause oxidation of DCFH, yielding the fluorescent product 2',7'-dichlorofluorescein (DCF). Cells were incubated with DCFH-DA (10 μM) under various experimental conditions. Cell monolayers were subsequently rinsed with PBS/BSA and fixed with 1% paraformaldehyde in PBS for 10 minutes at room temperature. The monolayers were rinsed twice with PBS/BSA and scraped with rubber policemen. Cell suspensions were centrifuged at 1,500 rpm for 5 minutes, the supernatants discarded, and the pellets resuspended in 0.5 ml PBS/BSA. FACS analysis was performed using a Becton Dickinson FACScan (San Jose, CA). Acquisition was set at 5,000 gated cells and mean fluorescent intensities as well as the percentage of cells were measured in all samples using CellQuest software version 1.2 (Becton Dickinson).

RESULTS

SIGN-R1 specifically binds to Prx-1 *in vitro*

To determine the protective role of SIGN-R1 against *S. pneumoniae*-produced ROS in SIGN-R1$^+$ splenic marginal macrophages, we have investigated SIGN-R1 binding proteins in the spleen or lymph nodes. After incubating biotinylated 22D1 anti-SIGN-R1 antibody with tissue lysates, SIGN-R1-22D1 antibody complexes were immunoprecipitated with streptavidin beads. The bound proteins to the complexes were eluted and separated on a 4-15% gradient SDS page gel and developed with silver staining (Appendix 1A). From the gel separation, the 23 kDa band was repeatedly isolated and identified by MALDI-TOF peptide sequence analysis to be Peroxiredoxin-1 (Prx-1), a key component of antioxidant enzyme family that scavenge ROS and are thought to be involved in the cellular response to ROS (Neumann *et al*., 2003) (Appendix 1B). Since SIGN-R1 is known to recognize and uptake polysaccharides such as dextran (Kang *et al*., 2003) or capsular polysaccharides (CPSs) of *S. pneumoniae* (Kang *et al*., 2004) in spleen and lymph node, it was expected that SIGN-R1 binding to Prx-1 would have important roles in scavenging ROS or involved in the cellular response to ROS.

To verify SIGN-R1 binding to Prx-1, biotinylated hamster IgG (Mock) or 22D1 anti-SIGN-R1 antibody were incubated with the cell lysates from stable DCEK transfectants of mouse DC-SIGN (DCEK-mDS) or SIGN-R1 (DCEK-SR1). And the subsequent immunoprecipitation was performed with streptavidin beads, followed by the immunoblotting analysis for Prx-1, Prx-2 or SIGN-R1. Mouse DC-SIGN is one of a family of C-type lectin genes homologous to SIGN-R1 and DCEK-mDS was used as negative control cell lines. The results clearly show that Prx-1 is the binding partner for SIGN-R1, but not Prx-2 (Fig. 1A). To confirm these results, the same immunoprecipitation assay was performed in HEK 293 cells which were transiently transfected with no insert (EV in Figure), SIGN-R1 (SR1 in figure) or mouse DC-SIGN (mDS in figure) cDNA. Expectedly, only Prx-1 was observed from the immunoprecipitate from HEK293-SR1 transfectant cell line, whereas

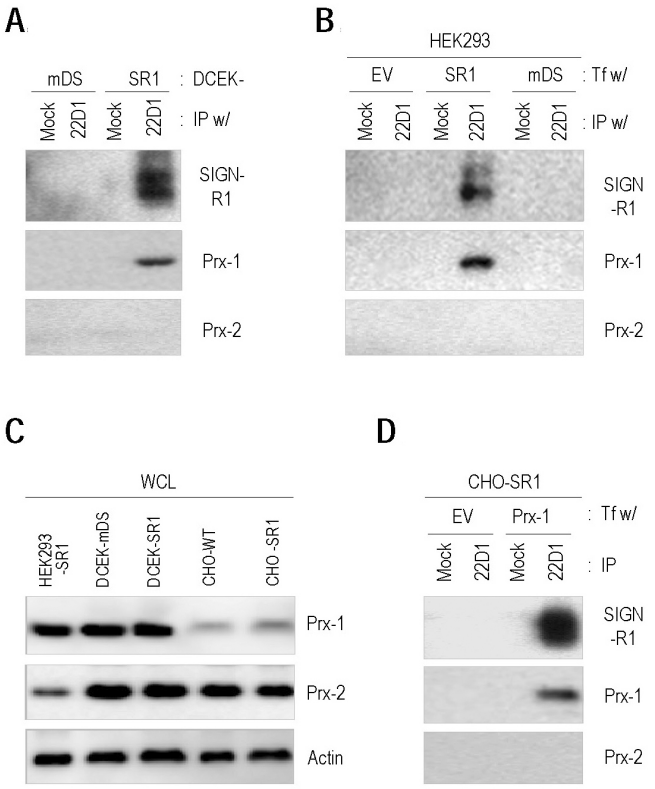

Fig. 1. SIGN-R1 specifically binds to Prx-1 *in vitro*. (A) 1 mg of lysates from DCEK-mDS or -SR1 were incubated with 2 μg of biotinylated hamster IgG (mock) or 22D1 for 12 hours at 4°C, followed by the incubation of streptavidin beads for 2 hours at 4°C. Bead extracts were immunoblotted for Prx-1, Prx-2, or SIGN-R1. The monomer form of SIGN-R1 is shown in all figures. (B) As in (A), but eukaryotic expression vectors containing no insert (EV in Figure), SIGN-R1 (SR1 in Figure) or mouse DC-SIGN (mDS in Figure) cDNA were transfected into HEK293 cells and cell lysates were used 2 days later. (C) 10 μg of cell lysates from HEK293, DCEK-mouse DC-SIGN or DCEK-SIGN-R1, CHO-EV or CHO-SIGN-R1 were immuobloted for Prx-1 or Prx-2. (D) As in (A), but eukaryotic expression vectors containing no insert (EV in Figure) or Prx-1 cDNA were transfected into CHO cells and cell lysates were used 2 days later.

no Prx-1 was found in HEK293-EV or -mDS (Fig. 1B, middle). The expression of SIGN-R1 in the immunoprecipitates of HEK293-SR1 transfectant cell line was checked by immunoblotting analysis with PAb-C13 anti-SIGN-R1 antibody (Fig. 1B, top).

In the process to confirm SIGN-R1 binding with Prx-1 *in vitro*, we found that the basal expression level of Prx-1 in CHO wild type (-WT) or －SIGN-R1 transfectant cell lines was much lower than in HEK293-WT, DCEK-mDS or －SIGN- R1 cell lines (Fig. 1C, top). On the other hand, Prx-2 was expressed abundantly in all cell lines except in HEK293-WT cell line (Fig. 1C, middle or bottom). Therefore, Prx-1 cDNA was transiently transfected into stable CHO-SR1 cell line and followed by the same immunoprecipitation assay in Fig. 1A. Prx-1 binding to SIGN-R1 was also evident in this alternative transfection condition (Fig. 1D), suggesting the intimated interaction between SIGN-R1 and Prx-1.

SIGN-R1 preferentially binds to Prx-1 in non-reducing condition

To emphasize the physiological role of SIGN-R1 binding to Prx-1, biotinylated hamster IgG (Mock) or 22D1 anti-SIGN-R1 antibody were incubated with the tissue lysates from spleen or lymph nodes where SIGN-R1 are exclusively expressed (Kang *et al*., 2003). Then the subsequent immunoprecipitation was performed with streptavidine beads and followed by the immunoblotting analysis for Prx-1, Prx-2 or SIGN-R1. It was demonstrated that the interaction between SIGN-R1 and Prx-1 was obvious in both spleen or lymph nodes (Fig. 2A). In contrast, there was no interaction between Prx-2 and SIGN-R1 in both tissues (Fig. 2A, bottom). These results strongly suggested that Prx-1, but not Prx-2, is a binding partner for the physiological role of SIGN-R1 in SIGN-R1$^+$ splenic macrophages.

To understand the mechanism of SIGN-R1 binding to Prx-1, the immunoprecipitation assay was performed with the same lysate of spleen useed in Fig. 2A in the existence of dithiothreitol (DTT), which reduces disulfide bonds quantitatively and maintain monothiols in the reduced state. In the reduced condition with DTT, SIGN-R1 binding to Prx-1 was significantly decreased (Fig. 2B), indicating that SIGN-R1 would preferentially bind Prx-1 in oxidative status.

SIGN-R1 binding to Prx-1 is increased with ROS generation by dextran uptake *in vivo*

Next, we investigated whether exposure to dextran, a ligand of SIGN-R1, affect in generation of ROS in SIGN-R1$^+$ macrophages. Dextran was exposed to DCEK-WT, mDS or SIGN-R1 transfectant cell lines and generation of intracellular ROS was assessed using DCFH-DA, which is oxidized by intracellular ROS to yield the fluorescent product DCF. DCF fluorescence was not detected in DCEK-WT and DCEK-mDS cell lines, but observed in DCEK-SIGN-R1 cell line (Fig. 3A), indicating that SIGN-R1 stimulated by dextran generates intracellular ROS.

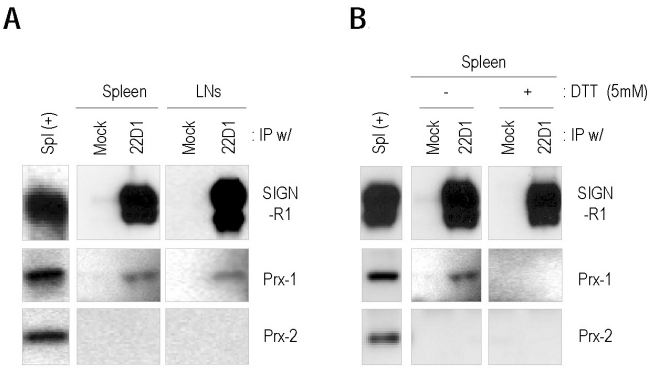

Fig. 2. SIGN-R1 preferentially binds to Prx-1 in non-reducing condition *in vivo*. (A) 1 mg of tissue lysates from spleen or lymph nodes were incubated with 2 μg of biotinylated hamster IgG (mock) or 22D1 for 12 hours at 4°C, followed by the incubation of streptavidin beads for 2 hours at 4°C. Bead extracts were immunoblotted for Prx-1, Prx-2, or SIGN-R1. The monomer form of SIGN-R1 is shown in all figures. (B) As in (A), but the preparation of tissue lysates and immunoprecipitation were prepared with 5 mM of dithiothreitol (DTT).

It has been reported that Prx1 was upregulated by exposure to ROS (Schreibelt *et al*., 2008). Therefore, it was examined whether ROS generation followed by the uptake of dextran leads to increase the expression of Prx-1 in SIGN+ cells *in vitro* or *in vivo*. Firstly, 1 μg of dextran was applied to DCEK-mDS or DCEK-SIGN-R1 for 6 hours and the cell lysates were immunoblotted for SIGN-R1, Prx-1 or Prx-2. Actin was immunoblotted as quantitative control. Although the expression level of SIGN-R1 or Prx-2 was not changed at all, the expression of Prx-1 was increased (Fig. 3B, top, Appendix 2A). To further verify these results *in vivo*, 200 μg of dextran was intravenously administered into the control mouse and 6 hours later, the tissue lysates of spleen or lymph nodes were immunoblotted for Prx-1 or Prx-2. As shown in Fig. 3B, Prx-1 was significantly increased in both the spleen and the lymph nodes, but the level of Prx-2 was not significantly changed (Fig. 3C, Appendix 2B).

Since SIGN-R1 preferentially bound Prx-1 in the oxidative status (Fig. 2B), it was speculated that ROS generation by dextran would increase the interaction of SIGN-R1 and Prx-1. To confirm this, the immunoprecipitation assay was performed using the the tissue lysates of lymph

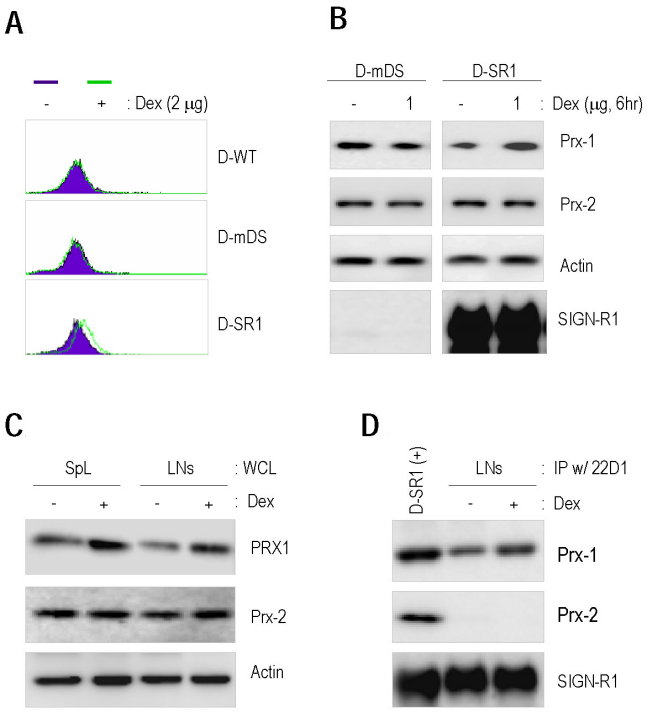

Fig. 3. SIGN-R1 binding to Prx-1 is increased with the uptake of polysaccharide dextran by SIGN-R1$^+$ macrophages. (A) Dextran enhances intracellular ROS generation only in DCEK-SR1 transfectant. 2 μg of dextran was exposed to DCEK transfectants for 10 min and intracellular ROS was detected by the DCF fluorescence as described in the Methods and Materials. (B) DCEK-mDS or -SIGN-R1 transfectants were pre-incubated with 1 μg of dextran for 6 hours and the cell lysates were immunoblotted for Prx-1, Prx-2, Actin or SIGN-R1. (C) 200 μg of dextran were intravenously adminstered for 7 hours and the tissue lysates from spleen or lymph nodes were immunoblotted for Prx-1, Prx-2 or Actin. (D) 1 mg of tissue lysate from lymph nodes in (C) was incubated with 2 μg of biotinylated hamster IgG (mock) or 22D1 for 12 hours at 4°C, followed by the incubation of streptavidin beads for 2 hours at 4°C. Bead extracts were immunoblotted for Prx-1, Prx-2, or SIGN-R1. The monomer form of SIGN-R1 is shown in all figures.

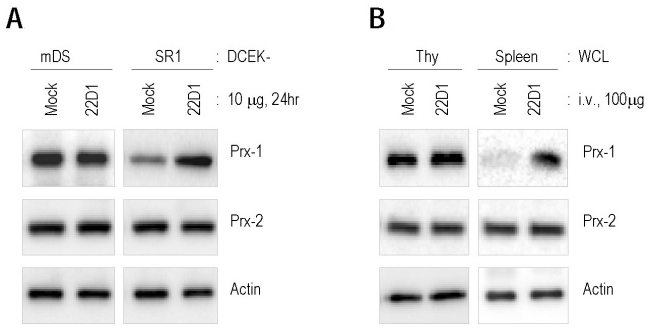

Fig. 4. SIGN-R1 crosslinking with 22D1 anti-SIGN-R1 antibody increase Prx-1 *in vitro* or *in vivo*. (A) DCEK transfectants of mouse DC-SIGN or SIGN-R1 were incubated with 10 μg hamster IgG (mock) or 22D1 anti-SIGN-R1 antibody for 1 day and 10 μg of cell lysates were immunoblotted for SIGN-R1, Prx-1, Prx-2 or Actin. The monomer form of SIGN-R1 is shown in all figures. (B) Mice were given 200 μg of hamster IgG (mock) or 22D1 anti-SIGN-R1 antibody. One day later, 10 μg of tissue lysates from thymus or spleen were immunoblotted for SIGN- R1, Prx-1, Prx-2 or Actin. The monomer form of SIGN-R1 is shown in all figures.

nodes in Fig. 3C and the immunoprecipitates were immunoblotted for Prx-1, Prx-2 or SIGN-R1. Expectedly, SIGN-R1 binding to Prx-1 was significantly increased in the lymph nodes with the *i.v.* injection of dextran (Fig. 3D, Appendix 2C).

SIGN-R1 crosslinking with 22D1 results in increase of Prx-1 *in vitro* or *in vivo*

The previous studies have reported that the receptor stimulated by cross-linking with the specific antibodies induced ROS generation (Jackson *et al*., 2004; Jung *et al*., 2004) and caused the upregulation of Prx-1 (Schreibelt *et al*., 2008). Therefore, it was expected that stimulation of SIGN-R1 with 22D1 anti-SIGN-R1 antibody is likely to increase intracellular Prx-1 level through the generation of ROS. After the 24 hour-incubation of DCEK-mDS or -SIGN-R1 with 20 μg of 22D anti-SIGN-R1 antibody to crosslink SIGN-R1, the cell lysates were immunoblotted for Prx-1 or Prx-2. The level of Prx-1 was significantly increased by SIGN-R1 crosslinking only in DCEK-SIGN-R1 cell line, but not in DCEK-mDS cell line (Fig. 4A, top, Appendix 3A). On the other hand, the level of Prx-2 was not changed (Fig. 4A, middle).

To further confirm these results *in vivo*, 200 μg of 22D anti-SIGN-R1 antibody were intravenously injected into the mice for 24 hours to stimulate SIGN-R1 on splenic MZ macrophages, and the tissue lysates from thymus or spleen were prepared for immunoblotting for Prx-1 or Prx-2. As a negative control, hamster IgG was intravenously applied into the mouse. The level of Prx-1 was dramatically increased only in spleen, but not in thymus (Fig. 4B, Appendix 3B), indicating that SIGN-R1 crosslinking by 22D1 anti-SIGN-R1 antibody on SIGN-R1$^+$ MZ macrophages specifically mediated the increase of Prx-1.

DISCUSSION

Specialized macrophage populations in the mammalian innate immune system are ideally situated within secondary lymphoid tissues such as spleen and lymph nodes to first encounter pathogens that enter the blood and lym-

phatics (Phillips *et al*., 2010). These macrophages recognize pathogens through pattern recognition receptors (PRRs) that interact with pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide and capsular polysaccharides (Farnell *et al*., 2003). It is well known that PRRs such as dectin-1, L-selectin and Toll-like receptors (TLRs) activates production of ROS against various pathogens (Waddell *et al*., 1994; Gantner *et al*., 2005).

SIGN-R1 is one of PRRs and expressed at high levels in the MZ which lies at the junction of the white pulp nodule with the red pulp and lymph node medulla (Geijtenbeek *et al*., 2002; Kang *et al*., 2003; Kang *et al*., 2004). Within the MZ, SIGN-R1$^+$ MZ macrophages, which are avidly phagocytic, are well-characterised as facilitating clearance of *S. pneumoniae* from the blood stream (Kang *et al*., 2004; Lanoue *et al*., 2004; Koppel *et al*., 2005b). Therefore, it is speculated that SIGN-R1$^+$ MZ macrophages are excessively expose to oxidative stress by ROS generation against *S. pneumoniae.* However, it remains under the cover whether SIGN-R1 stimulates ROS generation against its ligands such polysaccharide dextran or capsular polysaccharide of *S. pneumoniae* and SIGN-R1$^+$ MZ macrophages contain specialized antioxidant systems to effectively remove ROS to protect themselves.

To pursue these questions, we investigated SIGN-R1 binding proteins and Prx-1 was identified as one of SIGN-R1 binding partners. Prx-1 is an antioxidant enzyme that can eliminate hydrogen peroxide *in vivo* and can regulate ROS induced by growth factor signaling (Kang *et al*., 1998). The interaction between SIGN-R1 and Prx-1 was confirmed with the several SIGN-R1 transfectant cell lines (Fig. 1) and with the tissues such as spleen and lymph nodes which exclusively express SIGN-R1 (Fig. 2A). Furthermore, the results demonstrated preferential binding of SIGN-R1 to Prx-1 in oxidative status (Fig. 2B). Since *S. pneumoniae* clearly generate ROS production in neutrophils (Martner *et al*., 2008) and the interactions between C-type lectins with PAMPs induce ROS generation (Segal, 2005; Fu *et al*., 2006), it is hypothesized that ROS generation is induced by SIGN-R1 recognizing *S. pneumoniae*, resuling in increase of the interaction between SIGN-R1 and Prx-1 to protect SIGN-R1$^+$ macrophages.

In agreement with this hypothesis, SIGN-R1 stimulation with dextran generated intracellular ROS (Fig. 3A), and followed by the significant increase of Prx-1 *in vitro* (Fig. 3B) or *in vivo* (Fig. 3C), resulting in increase of SIGN-R1 binding to Prx-1 *in vivo* (Fig. 3D). These results were consistent with the previous results showing that C-type lectins such as TLR-2, TLR-4 and Dectin-1 activates production of ROS against their specific ligands (Brown and Gordon, 2001; Farnell *et al*., 2003; Steele *et al*., 2003; Gantner *et al*., 2005) and these oxidative stresses cause an upregulation of Prx1 in a variety of cell types, including macrophages, vascular smooth muscle cells, and pancreatic beta cells (Mitsumoto *et al*., 2001; Rhee *et al*., 2005; Conway and Kinter, 2006; Schreibelt *et al*., 2008). In addition, SIGN-R1 stimulation by crosslinking with 22D1 anti-SIGN-R1 antibody increased Prx-1 *in vitro* (Fig. 4A) or *in vivo* (Fig. 4B). Since receptor stimulation by cross-linking with the specific antibodies also induced ROS generation too (Waddell *et al*., 1994; Jackson *et al*., 2004; Jung *et al*., 2004), it seems likely that the increase of Prx-1 was caused from the exposure of SIGN-R1$^+$ cells to ROS.

Furthermore, complements, the major humoral component of the innate immune system, is also involved in oxidative burst with release of ROS (Mollnes *et al*., 2002). Since SIGN-R1 mediates the new activation pathway of complements against *S. pneumoniae* on SIGN-R1$^+$ MZ macrophages (Lanoue *et al*., 2004; Kang *et al*., 2006), it is expected that SIGN-R1$^+$ MZ macrophages are especially well-equipped with anti-oxidant systems to protect themselves from the toxic effects of ROS.

The production of ROS seems, at least in part, to be responsible for the poor outcome of patients with pneumococcal infections especially including pneumococcal meningitis (Klein *et al*., 2006). In consequence, ROS has been investigated as potential targets to develop novel antioxidant strategies (Guzik and Harrison, 2006) or adjunctive therapy in pneumococcal infections (Klein *et al*., 2006). Therefore, further studies are required for better understanding the role of SIGN-R1 binding to Prx-1 in SIGN-R1$^+$ MZ macrophages, leading development of novel methods to protect the host from lethal pneumococcal infections. Since SIGN-R1 is a murine homolog of human DC-SIGN (Saunders *et al*., 2010) and both SIGN-R1 and human DC-SIGN interact with viral and bacterial pathogens directly through their specificity for high mannose oligosaccharides (Feinberg *et al*., 2001; Koppel *et al*., 2005a), further studies on the role of SIGN-R1 bound to Prx-1 in the clearance of ROS will be the first step in unraveling the pathophysiological roles of human DC-SIGN against various viral or bacterial infection.

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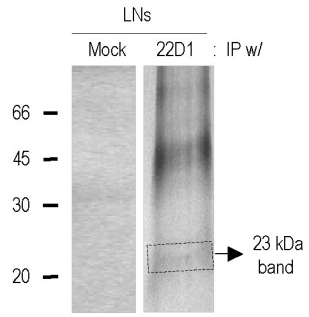
APPENDIX

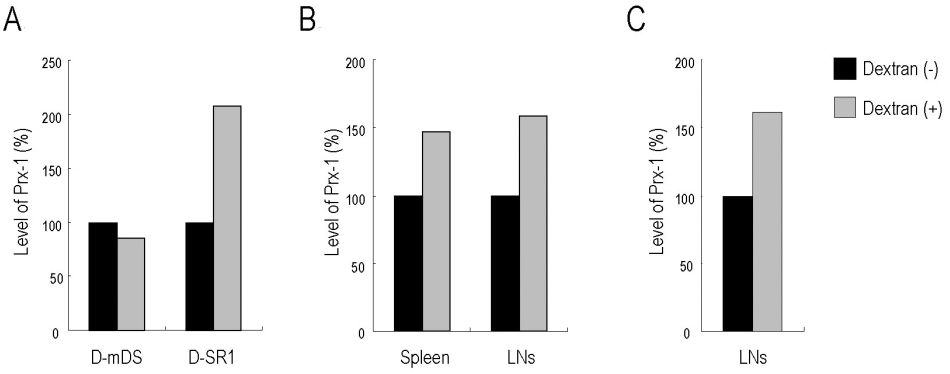
A



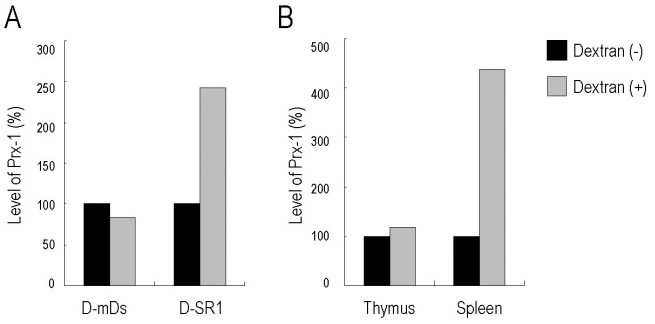
B

No	Expect	M.W. (kDa)	Name
1	2.6x10E15	22.4	peroxiredoxin 1; macrophage stress protein 23 kDa
2	3.2x10E6	28.4	hypothetical protein 4833421E05Rik
3	5.7x10E6	18.6	DJ-1 protein; RNA-binding protein regulatory subunit
4	8.3x10E6	24.8	hypoxanthine guanine phosphoribosyl transferase
5	4.1x10E4	21.9	PDX2_MOUSE Peroxiredoxin 2 (Thioredoxin peroxidase 1)
6	6.5X10E4	22.8	unnamed protein product
7	4.0x10E2	24.1	RAB14, member RAS oncogene family
8	0.24	26.3	complement component 1, q subcomponent, gamma polypeptide; complement component 1, q subsomponent, c polypeptide
9	0.74	25.3	RIKEN cDNA C230094F14 gene
10	0.74	24.6	RIKEN cDNA 2310012G06
11	0.74	26.2	RAB33B, member of RAS oncogene family
12	0.85	21.9	unnamed protein product

Appendix 1. The 23 kDa band identified by MALDI- TOF peptide sequence analysis to be Peroxiredoxin-1 (Prx-1). After incubating biotinylated 22D1 anti-SIGN-R1 antibody with tissue lysates, SIGN-R1-22D1 antibody complexes were immunoprecipitated with streptavidin beads. The bound proteins to the complexes were eluted and separated on a 4-15% gradient SDS page gel and developed with silver staining. The coomasie gel staining result that was used for the gel separation (A) and the MALDI-TOF data (B).



Appendix 2. Densitometric quantification of the expression level of Prx-1 was performed and (A), (B) and (C) showed the quantification results of Fig. 3B-D, respectively.



Appendix 3. Densitometric quantification of the expression level of Prx-1 was performed and (A) and (B) showed the quantification results of Fig. 4 A and B, respectively.