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**Discharge of solubilized and Dectin-1-reactive  $\beta$ -glucan from macrophage cells phagocytizing insoluble  $\beta$ -glucan particles: involvement of reactive oxygen species (ROS)-driven degradation**

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## **Abstract**

Phagocytes engulf pathogenic microbes, kill them and degrade their cellular macromolecules by hydrolytic enzymes in phagolysosomes. However, such enzymes are unable to degrade some microbial polysaccharides, and fate of such indigestible polysaccharides in phagocytes remains uncertain. Using the extracellular domain of Dectin-1 as  $\beta$ -glucan-specific probes, we succeeded in detection of soluble and Dectin-1-reactive  $\beta$ -glucan discharged from mouse RAW264.7 and human THP-1 macrophage cell lines as well as mouse peritoneal macrophages, which had phagocytized insoluble  $\beta$ -glucan particles. The RAW264.7 cell culture-supernatant containing the discharged  $\beta$ -glucan stimulated naïve RAW264.7 cells, resulting in the induction of cytokine expression. Such discharge of Dectin-1-reactive  $\beta$ -glucan from macrophage cells was inhibited by either NADPH oxidase inhibitors (apocynin and diphenylene iodonium) or radical scavengers (*N*-acetyl cysteine and MCI-186). Moreover, reactive oxygen species (ROS) produced by a  $\text{Cu}^{2+}$ /ascorbic acid (AsA) system solubilized insoluble  $\beta$ -glucan particles *in vitro*, and a part of the solubilized  $\beta$ -glucan was Dectin-1 reactive and biologically active in macrophage activation. The soluble and biologically active  $\beta$ -glucan was degraded further during prolonged exposure to ROS. These results suggest that degraded but Dectin-1-reactive  $\beta$ -glucan is discharged from macrophage cells phagocytizing insoluble  $\beta$ -glucan particles and stimulates the other phagocytes, leading to the effective elimination of infected microbes and the ultimate breakdown and inactivation of metabolically resistant  $\beta$ -glucan.

(209 words)

## Introduction

Phagocytes such as macrophages, dendritic cells and neutrophils possess anti-microbe systems consisting of phagocytosis, killing and degradation. After being phagocytized, microbes are found in phagosome, which undergoes a maturation process into a hydrolase-rich phagolysosome [1, 2]. Most of microbial macromolecules such as proteins, lipids, nucleic acids are degraded metabolically with lysosomal hydrolytic enzymes [3-5], whereas microbial polysaccharides are indigestible and resistant to the lysosomal enzymes. Mechanisms of microbial polysaccharide degradation within phagocytes are not well understood.

When phagocytes are exposed to or engulf microbes, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated, resulting in production of reactive oxygen species (ROS) in phagosomes [6, 7]. ROS kills microbes by generating toxic ROS, which gives damages to microbial respiratory chains, and by activating hydrolytic enzymes in phagolysosomes [8]. ROS, especially hydroxyl radical, reacts chemically with microbial macromolecules, gives oxidative damages to them, and degrades them [3-5, 8]. Such oxidative damages of macromolecules by ROS have been investigated mostly for proteins, nucleic acids and fatty acids, e.g., methionine and reactive cysteine residues in the active sites of some enzymes, bases of DNA, and double bonds in unsaturated fatty acids [3-5]. However, ROS-dependent damages to microbial polysaccharides are not well understood. In an *in vivo* mouse model, *Candida* cells intravenously injected to mice was immediately deposited and remained for months afterward in liver, and insoluble  $\beta$ -glucan of the cell wall was gradually solubilized in the liver, probably by oxidative stress of macrophages [9]. Moreover, in a cellular level, the zwitterionic polysaccharide from *Bacteroides fragilis* was degraded by nitric oxide to lower molecular fragments within endosomes/lysosomes of antigen presenting cell before being presented to CD4<sup>+</sup> T cells [10, 11]. However, no study has been reported on subsequent fate of microbial polysaccharides degraded within cellular vesicles such as phagolysosomes and endosome/lysosome.

$\beta$ -Glucans are neutral and water-insoluble polysaccharides of fungal cell walls and bacterial capsular materials, consisting of  $\beta$ -(1,3)-linked  $\beta$ -D-glucopyranosyl units that form a backbone with or without randomly dispersed  $\beta$ -1,6-linked side chains. They are recognized as pathogen associated molecular patterns (PAMPs) by immune system and stimulate innate immune system of vertebrates [12]. In mammals, immune effector cells such as macrophages, dendritic cells and neutrophils recognize  $\beta$ -glucan-bearing microbes by  $\beta$ -glucan receptors, Dectin-1 and complement receptor 3 (CD11b/CD18, CR3) [12, 13]. The binding of  $\beta$ -glucan to Dectin-1 results in activation of the NF- $\kappa$ B signaling pathway, leading to the transcription of innate immune response-related genes such as cytokines and chemokines [14, 15]. Concurrently, macrophages phagocytizing  $\beta$ -glucan-bearing microbes generate ROS in a Dectin-1 dependent manner [16].

Microbial cell-wall  $\beta$ -glucans are insoluble and highly polymerized in physiological solutions and, therefore, quantitative handling and analysis are difficult in biochemical and cell biological studies. In *in vitro* cell culture systems size and solubility of  $\beta$ -glucan particles added to the culture are presumed to be critical for exhibiting biological activity as a Dectin-1 ligand [17]. In

the course of studies on solubilization of insoluble  $\beta$ -glucan particles in a cell culture system, we found that macrophage cells pulse-charged once with  $\beta$ -glucan particles discharged Dectin-1-reactive  $\beta$ -glucan in the culture medium. The aims of the present study are to quantitatively analyze Dectin-1-reactive and soluble  $\beta$ -glucan discharged from macrophage cells, to clarify ROS-driven  $\beta$ -glucan degradation *in vitro* and within the cells, and to evaluate biological activity of the degraded and discharged  $\beta$ -glucan to naïve macrophage cells. Mechanisms for gradual degradation of insoluble  $\beta$ -glucan particles by macrophage and biological meaning of the Dectin-1-reactive  $\beta$ -glucan discharge from macrophage were discussed.

## **Materials and methods**

### ***$\beta$ -glucan preparation***

Curdlan ( $\beta$ -(1,3) glucan) from *Alcaligenes faecalis* was purchased from SIGMA, Tokyo. The curdlan was suspended in distilled water (1:50, w/vol.) and centrifuged at 4,000 $\times g$  for 10 min, and the supernatant was used as soluble  $\beta$ -glucan. The precipitate was re-suspended in distilled water and centrifuged as above. This washing procedure was repeated 10 times, and the final precipitate was used as insoluble  $\beta$ -glucan. Neutral carbohydrate content in both fractions was determined by phenol-sulfuric acid method.

### ***Cell culture***

Resident peritoneal-macrophages (pM $\phi$ ) were isolated by peritoneal lavage from 6-7 week old female DBA/2 mice (SLC, Hamamatsu, Japan). Collected cells were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), and adherent cells were used. RAW264.7, THP-1 and pM $\phi$  prepared from DBA/2 mice were grown in RPMI-1640 medium with 10% FBS. THP-1 cells were differentiated to macrophage by phorbol myristate acetate (100 ng/ml, SIGMA) for 2 days before use. HEK 293 cells were grown in DMEM medium with 10% FBS. Insect S2 cells were cultured in Sf-900 II medium (Invitrogen) supplemented with 5% FBS.

### ***Construction of expression plasmids for soluble Dectin-1***

Total RNA was prepared from RAW 264.7 with TRIZOL reagent (Invitrogen). A total cDNA pool of RAW 264.7 was prepared from the RNA using Superscript II RT kit (Invitrogen) with a random hexanucleotide primer (Invitrogen). A cDNA fragment encoding the carbohydrate recognition domain of murine Dectin-1 (DnCRD) was amplified by PCR using a primer set, 5'-aaaaaagatctctagcattttggcgac-3' and 5'-aaaatctagacagttccttctcacagatac-3', containing Bgl II and Xba I restriction sites, respectively, digested with the enzymes, and inserted into the site of an insect cell expression vector, pMT/BiP/V5-HisA (Invitrogen). Another cDNA fragment was amplified using a primer set, 5'-aaaaaagatcttaccatacagatgtccagattacgctctagcattttggcga-3' and 5'-aaaaaagatctcagttccttctcacagatac-3', containing the Bgl II site with a HA-tag coding sequence, and inserted into the Bgl II site of a mammalian cell expression vector, pFUSE-SEAP-hIgG1-Fc1 (InvivoGen, San Diego, CA), to make a recombinant protein conjugated to the human IgG1 Fc

region.

### ***Soluble Dectin-1 preparation***

Insect S2 cells were transfected with pMT/BiP/DnCRD/V5His and pMK33/hygomycin using Cellfectin (Invitrogen) as described previously [18]. Stably transformed S2 cells were selected in hygromycin B (300 µg/ml, Invitrogen) medium for four weeks. DnCRD-V5His expression was induced in Sf-900 II supplemented with 0.7 mM CuSO<sub>4</sub>. DnCRD-V5His secreted in the culture medium was determined by immunoblotting with V5-tag specific antibody (Invitrogen) and affinity purified using His-bind resin (Novagen). The concentration of purified protein was detected by using Micro BCA Protein Assay Kit (Thermo-Scientific).

HEK293 cells were transfected with pFUSE-SEAP/HA-DnCRD/hIgG1-Fc1 using HilyMax (Dojindo, Tokyo). Stable transfectants were selected in medium with zeocin (200 µg/ml, Invitrogen) for 2 weeks. Stable transfectants were cultured in DMEM with ITS (SIGMA) to obtain medium containing DnCRD-hG1Fc. DnCRD-hG1Fc expressions were determined by immunoblotting with HA-tag-specific antibody (MBL, Nagoya).

### ***ELISA-based assay for soluble β-glucan***

β-Glucan-binding activity of the soluble Dectin-1 preparations, DnCRD-V5His and DnCRD-hG1Fc, were evaluated by ELISA-based assay. ELISA 96-well plates (Nunc) were coated with 0-125 µg/ml of curdlan solubilized in NaOH solution. The microplates were washed with PBS containing 0.05% Tween-20 (PBST) and blocked with PBST containing 1% BSA (PBST/BSA). The plates were incubated with conditioned medium containing DnCRD-hG1Fc, or purified DnCRD-V5His (10ng/ml). Bound DnCRDs were detected with HRP-conjugated rabbit anti-human IgG (Abcam) or HRP-conjugated mouse anti-V5 (Invitrogen) as detection antibodies, respectively. In β-glucan quantification, sandwich ELISA using Dectin-1 proteins were performed as below. DnCRD-V5His (1 µg/ml) were coated and then blocked with PBST/BSA. Samples containing soluble β-glucan were incubated and bound soluble β-glucan was detected by DnCRD-hG1Fc and HRP-conjugated rabbit anti-human IgG. The concentration of β-glucan was calculated from a standard curve using the soluble β-glucan solution with known neutral carbohydrate concentration determined by phenol-sulfuric acid method.

### ***β-glucan degradation assay***

In cell based assay,  $1 \times 10^6$  of macrophages were incubated with insoluble β-glucan (0.01-1 mg neutral carbohydrate/ml of DMEM) for 1 hour. After washing with fresh medium 5 times to remove β-glucan, the cells were maintained for up to 72 hours. The culture medium was harvested and centrifuged at  $4,000 \times g$  for 10 min before analyses. RAW264.7 cell conditioned medium collected 72 hours after the pulse charge with insoluble β-glucan (1mg/ml) for 1 hour was concentrated 6-fold by ultrafiltration using Amicon Ultra-4 filter, MWCO 30 kDa (Millipore). The concentrated medium was added to naïve RAW264.7 cells and cultured for 8 hours.

For inhibition studies in ROS-production, macrophage cells were treated with apocynin (APO, 100  $\mu$ M, Calbiochem), diphenyleneiodonium sulfate (DPI, 10  $\mu$ M, Calbiochem), *N*-acetyl cysteine (NAC, 3 mM, Calbiochem), or 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186, 200  $\mu$ M, Calbiochem) for one hour before incubation with insoluble  $\beta$ -glucan (1 mg/ml). After exposure to insoluble  $\beta$ -glucan in the presence of the inhibitors and complete washing, the medium was changed to fresh one containing each of the inhibitors and further incubated for 6 h. Then soluble  $\beta$ -glucan content in the conditioned medium was quantified by the sandwich ELISA-based assay.

For *in vitro* oxidative degradation experiments, suspension of insoluble  $\beta$ -glucan (0-25 mg neutral carbohydrate/ml in PBS) was treated with 50 mM AsA and 0.5 mM CuSO<sub>4</sub> for 0-24 hours at 37°C with gentle shaking, as described [19]. After the oxidative reaction was terminated by adding EDTA equivalent to Cu<sup>2+</sup>, the reaction mixture was centrifuged at 15,000 $\times$  *g* for 10 min to remove the insoluble  $\beta$ -glucan and the supernatant was subjected to the  $\beta$ -glucan and neutral sugar quantification analyses as above and macrophage activation assay as below. Differentiated THP-1 macrophage cells in 6-well plates were cultured in the presence of the supernatant for 9 hours and IL-1 $\beta$  in the medium was determined using an ELISA kit (Human IL-1B Platinum ELISA, eBioScience).

### ***RT-PCR***

Total RNA was prepared from RAW 264.7 cells stimulated for 8 hours with the concentrated conditioned medium as above. First-strand cDNAs were synthesized as described above. The resultant cDNA mixtures were amplified by PCR to isolate cDNAs for IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , TGF- $\beta$ , GM-CSF and  $\beta$ -actin. Primers used in PCR were following.

IL-1 $\beta$ ; 5'-gacagtgatgagaatgacc-3' and 5'-tgttcactctggagcctgtag-3',  
IL-6; 5'-ccagagatacaaagaatgatgg-3' and 5'-actccagaagaccagaggaaat-3',  
IL-10; 5'-accgactccttaatgc-3' and 5'-gtcctcactgccttgctc-3',  
TNF- $\alpha$ ; 5'-ttatggctcagagtccaact-3' and 5'-aaggtctgaaggtaggaacg-3',  
TGF- $\beta$ 1; 5'-gtggatactgagacacc-3' and 5'-ttggacaggatctggc-3',  
GM-CSF; 5'-ggaagcatgtagaggccatc-3' and 5'-gagtggcctgggcttcctc-3',  
 $\beta$ -actin; 5'-taaccaactgggacgatag-3' and 5'-atacagggacagcacagcct-3'.

### ***Immunohistochemical analysis***

RAW264.7 cells were seeded on type I collagen coated glass slip and incubated with insoluble  $\beta$ -glucan (1mg/ml). The cells were blocked with NETG [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.05% Triton X-100, 2.5 mg/ml gelatin] after fixing with paraformaldehyde. To stain phagocytized  $\beta$ -glucan, DnCRD-hG1Fc were used as a specific probe. DnCRD-hG1Fc bound to  $\beta$ -glucan was visualized by rabbit anti-HA antibody and Alexa488-conjugated goat anti-rabbit IgG (Invitrogen). Nuclei were stained with Propidium iodide (PI, SIGMA). Immunofluorescences were observed with a laser scanning confocal microscope (LSM510, Carl Zeiss).

## Results

### ELISA-based quantitative analysis of apparently soluble $\beta$ -glucan

Two kinds of soluble recombinant proteins containing extracellular carbohydrate recognition domain and stalk regions of Dectin-1 (DnCRD) were generated using insect and mammalian cell expression systems. Schematic structures of the recombinant proteins were shown in **Fig. 1A**. Both DnCRD-V5His and DnCRD-hG1Fc showed expected molecular sizes, respectively, under SDS-PAGE with silver staining and immunoblotting (Data not shown), and exhibited  $\beta$ -glucan binding activity as measured by two types of ELISA-based assay (**Fig. 1B**). Using a sandwich-type assay, in which absolute  $\beta$ -glucan concentration was determined by the phenol-sulfuric acid method, soluble  $\beta$ -glucan could be determined at the level of 10-200 ng/ml. As the  $\beta$ -glucan was detected on the basis of Dectin-1 binding, it was considered biologically active.

### Discharge of degraded but Dectin-1-reactive $\beta$ -glucan from macrophage cells phagocytizing insoluble $\beta$ -glucan particles

First, presence of  $\beta$ -glucan in RAW264.7 cells, which had been pulse-charged with insoluble  $\beta$ -glucan particles, was confirmed by immunofluorescence staining using the soluble Dectin-1 as a probe (**Fig. 2A**). Under a confocal microscope, fluorescence signals of engulfed  $\beta$ -glucan were clearly observed in the vesicle-like granular structures in the macrophage cells. Then, discharge of engulfed and intracellularly degraded  $\beta$ -glucan was chased by the measurement of soluble  $\beta$ -glucan in the cell culture supernatant (**Fig. 2B**). The  $\beta$ -glucan discharged into the medium increased with the chase time and plateaued 20 to 50 hours later under the experimental condition. The amount of discharged  $\beta$ -glucan depended on  $\beta$ -glucan concentration of the culture medium used for pulse-charge of the cells, but this dose dependency was not necessarily linear. Discharge of soluble and Dectin-1 reactive  $\beta$ -glucan was observed also from a human macrophage cell, differentiated THP-1, and mouse peritoneal macrophage. Compared with the RAW264.7 mouse macrophage cell, mouse peritoneal macrophages and the human THP-1 macrophage cell discharged a larger amount of  $\beta$ -glucan within shorter periods, e.g.,  $\beta$ -glucan concentration in the medium reached the level of 100 ng/ml for the chase period of 1 hour (**Fig. 2C**). These discharged and Dectin-1 reactive  $\beta$ -glucan preparations were not passed through a 30 kDa-cutoff membrane filter.

### Stimulation of naïve macrophage by the soluble and Dectin-1-reactive $\beta$ -glucan discharged from macrophage

Presence of soluble and Dectin-1-reactive  $\beta$ -glucan in the culture supernatant of macrophage cells prompted us to assume that discharged soluble  $\beta$ -glucan effectively activate other phagocytes living around through the Dectin-1 signaling pathway. Naïve RAW 264.7 cells were cultured in the conditioned medium containing the discharged soluble and Dectin-1-reactive  $\beta$ -glucan, and cytokine expression was analyzed at an mRNA level using RT-PCR (**Fig. 3**). Expression of all six cytokines tested was induced or enhanced by adding the discharged soluble



$\beta$ -glucan. Pre-treatment by boiling of the conditioned medium to inactivate cell-derived proteinous factors showed no significant changes on the cytokine expression.

### **Involvement of ROS in biological degradation of $\beta$ -glucan particles by macrophage cells**

Assuming that insoluble  $\beta$ -glucan particles are non-enzymatically degraded and solubilized within macrophage cells, effect of ROS suppression or elimination substances was analyzed on discharge of soluble  $\beta$ -glucan from RAW264.7 cells (**Fig. 4A**). Both of the NADPH oxidase inhibitor (APO and DPI) and radical scavenger (NAC and MCI-186) significantly reduced discharge of soluble  $\beta$ -glucan from the macrophage and, especially, NAC suppressed it to about 20% of the control level.

To evaluate ROS-driven production of soluble and Dectin-1-reactive  $\beta$ -glucan *in vitro*, insoluble  $\beta$ -glucan particles were treated with a typical ROS generating substance,  $\text{Cu}^{2+}/\text{AsA}$  solution (**Fig. 4B**). When a  $\beta$ -glucan/ROS ratio was high (5 and 25 mg/ml of  $\beta$ -glucan), soluble  $\beta$ -glucan was abruptly produced after a few hours lag period, reached a maximum level 6 to 9 hours later, and then gently decreased. In the case of a low  $\beta$ -glucan/ROS ratio (2.5 and 0.5 mg/ml of  $\beta$ -glucan), on the other hand, soluble and Dectin-1-reactive  $\beta$ -glucan was at almost undetectable levels. The solubilized  $\beta$ -glucan was chemically determined as neutral sugar by phenol-sulfuric acid method using glucose as a standard (**Fig. 4C**). The solubilized  $\beta$ -glucan including those without Dectin-1 binding ability was produced from the beginning of the  $\text{Cu}^{2+}/\text{AsA}$  treatment without lag period, increased and reached a maximum level 12 to 24 hours later. This production profile of solubilized  $\beta$ -glucan as measured by the phenol-sulfuric acid method was different from that of Dectin-1 reactive  $\beta$ -glucan (see **Fig. 4B**), indicating that all of the solubilized  $\beta$ -glucan molecules were not necessarily Dectin-1 reactive.

Biological activity as a Dectin-1 ligand of the  $\beta$ -glucan solubilized by the  $\text{Cu}^{2+}/\text{AsA}$  treatment was evaluated using THP-1 human macrophage cells (**Fig. 4D**). By the incubation with THP-1 cells for 9 h, the  $\beta$ -glucan solubilized by 9 h-treatment with  $\text{Cu}^{2+}/\text{AsA}$  induced IL-1 $\beta$  secretion from THP-1 cells. On the other hand, the solubilized  $\beta$ -glucan preparations by the treatment for 12 and 24 hours did it much more weakly, despite that these preparations contained neutral sugar comparable to that of the 9 hour-treatment (see **Fig. 4C**).

### **Discussion**

$\beta$ -Glucans are polysaccharides constructing fungal cell walls and bacterial capsular materials and, therefore, they are essentially water-insoluble. Technically, it is difficult to quantitatively handle insoluble materials suspended in aqueous solution. Nevertheless, in the present study,  $\beta$ -glucan suspended in the  $4000\times g$  centrifugation supernatant was regarded as apparently soluble  $\beta$ -glucan and used as a standard solution for quantitative analysis of solubilized  $\beta$ -glucan (**Fig. 1B**). The linear relationship between ELISA value and chemically determined carbohydrate concentration indicates that this ELISA assay using the standard solution is useful for

quantitative analysis of apparently soluble  $\beta$ -glucan, though size distribution of  $\beta$ -glucan aggregates or particles is uncertain and molar concentration is unavailable.

Macrophage cells phagocytize microbes or microbial polysaccharides and subsequently degrade or depolymerize them through non-enzymatic processing [9-11]. Indeed, when macrophage cells were charged with a lower dose of  $\beta$ -glucan (10  $\mu\text{g/ml}$ ), very low or almost undetectable levels of Dectin-1-reactive  $\beta$ -glucan was detected in the culture supernatant for the chase period of 72 h (**Fig. 2B**). However, when macrophage cells phagocytized theoretically 10 and 100 times more  $\beta$ -glucan than the cells with the low-dose, the cells discharged  $\beta$ -glucan as soluble but Dectin-1-reactive forms into culture medium. These results suggest that macrophage cells can process and degrade a certain amount of  $\beta$ -glucan completely within the cell but there is an upper limit to the completely degradable amount of  $\beta$ -glucan within a cell. It is most likely that excessive amounts of  $\beta$ -glucan was partially degraded within a cell and tentatively discharged from the cell into the medium. It seems interesting to note that  $\beta$ -glucan concentration in the culture medium of the cell with lower doses (0.01 and 0.1 mg/ml) reached a gentle peak after the 48h chase. The slight but significant decrease during the later stage of chase time in the  $\beta$ -glucan concentration of the culture medium suggest that the  $\beta$ -glucan discharged once was phagocytized or pinocytized again by the macrophage cells. Such repetitive phagocytosis and discharge by the cells are further suggested by the result that the conditioned medium from pulse-charged macrophage cells activated naïve macrophage cells as effective as the authentic  $\beta$ -glucan preparation (**Fig. 3**). Macrophage activation by other unknown protein factor(s) derived from the macrophage cells is unlikely because the activity was not affected by heating and the cytokine induction profile was similar to that of the authentic  $\beta$ -glucan.

As reported in some previous studies on microbial polysaccharide degradation by phagocytes [10, 11], the discharge of degraded and solubilized  $\beta$ -glucan from macrophage cells was inhibited by either of the NADPH oxidase inhibitors or free radical scavengers (**Fig. 4A**), indicating that the phagocytized  $\beta$ -glucan particles were processed at least partially in ROS-dependent mechanisms prior to the discharge from the cell. Effective inhibition of  $\beta$ -glucan discharge by NAC (N-acetyl cysteine) compared to MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) and the NADPH oxidase inhibitors could be ascribed to the fact that NAC plays roles as not only a free radical scavenger but also a precursor of glutathione, which exhibits anti-oxidative effect to a variety of ROS.

The  $\text{Cu}^{2+}/\text{AsA}$  system was used for ROS-induced  $\beta$ -glucan degradation *in vitro*. In the presence of transition metal ions, such as  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$ , the ascorbic acid is known to generate superoxide ( $\text{O}_2^-$ ) under physiological conditions and the produced superoxide triggers oxidative depolymerization of several soluble polysaccharides, including hyaluronate, alginate and  $\beta$ -cyclodextrin [20-22]. Like these soluble polysaccharides, insoluble  $\beta$ -glucan particles were depolymerized in the  $\text{Cu}^{2+}/\text{AsA}$  ROS generation system, resulting in the solubilization of  $\beta$ -glucan (**Fig. 4B and 4C**). Almost no detection of solubilized  $\beta$ -glucan in the lower dose (0.5 and 2.5 mg/ml) conditions indicates that in these conditions the generated ROS was large enough to degrade  $\beta$ -glucan particles completely to lower molecular mass compounds, which were too small to be

recognized by the Dectin-1. By contrast, when larger doses (5 or 20 mg/ml) were given, partially degraded and Dectin-1 reactive  $\beta$ -glucan was generated, probably because generated ROS was not large enough in amount for complete degradation of  $\beta$ -glucan particles. Moreover, the soluble  $\beta$ -glucan produced by ROS-induced degradation *in vitro* showed Dectin-1 reactivity and macrophage activation ability (**Fig. 4B and 4D**). These results shown in **Fig. 4** suggested that ROS-dependently degraded but Dectin-1-reactive  $\beta$ -glucan is discharged from macrophage cells phagocytizing excessive amounts of insoluble  $\beta$ -glucan particles and stimulates the other phagocytes.

Oxidative depolymerization of  $\beta$ -glucan similar to those found in the *in vitro* ROS-induced degradation model may be induced within macrophage cells, which phagocytized  $\beta$ -glucan particles and, subsequently, incompletely degraded but partially depolymerized  $\beta$ -glucan fine particles may be discharged from the cells. Superoxides generated by NADPH oxidase reactions in phagocytes are converted to hydrogen peroxide and finally to hydroxyl radical by the reaction with reduced metal ions (Fenton reaction). The hydroxyl radical might mainly play a role in the oxidative depolymerization of  $\beta$ -glucan as previously suggested for  $\beta$ -cyclodextrin oxidative depolymerization [22]. Although biological meanings of discharging incompletely degraded  $\beta$ -glucan by the macrophage cells is uncertain at this moment, it is likely that the Dectin-1 reactive  $\beta$ -glucan fine particles discharged from one macrophage cell diffuses and activates the other phagocytes inhabiting around, resulting in the effective elimination of infected microbes as well as metabolically resistant  $\beta$ -glucan.

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## Figure legends

### **Figure 1 ELISA-based assay for soluble and Dectin-1-reactive $\beta$ -glucan using recombinant proteins containing the Dectin-1 carbohydrate recognition domain (CRD)**

**(A)** Schematic drawings of mouse Dectin-1 and two kinds of recombinant proteins used for ELISA-based assay. Intermolecular disulfide bond is formed within the Fc region of DnCRD-hG1Fc. V5-, His-, and HA-tags were shown with light, medium, and dark gray boxes, respectively. **(B)** Binding of the Dectin-1 recombinant proteins was analyzed to curdlan ( $\beta$ -glucan) solubilized in NaOH solution and coated on ELISA plates (left). Each data was represented by mean $\pm$ SD of three wells. A standard curve for soluble  $\beta$ -glucan determination was obtained by an ELISA-based sandwich assay using the two kinds of the recombinant Dectin-1 proteins (right). Concentration of the soluble  $\beta$ -glucan was chemically determined by the phenol-sulfuric acid method.

### **Figure 2 Detection of phagocytized insoluble $\beta$ -glucan particles within macrophage and the determination of soluble and Dectin-1-reactive $\beta$ -glucan discharged from the macrophage**

**(A)** RAW 264.7 cells were culture in the presence of insoluble  $\beta$ -glucan (1mg/ml) for 60 min. After being washed and permeabilized, the cells were stained with DnCRD-hG1Fc for the phagocytized  $\beta$ -glucan particles and propidium iodide. **(B)** RAW 264.7 cells were puls-charged by the 60 min culture in the presence of insoluble  $\beta$ -glucan (0.01, 0.1 and 1mg/ml) and, after being washed, chased for 72 hours. The culture supernatant harvested with time was subjected to soluble  $\beta$ -glucan determination using the ELISA-based sandwich assay. **(C)** Differentiated human THP-1 macrophages and mouse peritoneal macrophages were puls-charged by the 60 min culture in the presence of insoluble  $\beta$ -glucan (1 mg/ml) and chased similarly. Each value in **B** and **C** represented mean $\pm$ SD from three independent conditioned medium.

### **Figure 3 Activation of naïve RAW 264.7 macrophage cells by the conditioned medium containing soluble and Dectin-1-reactive $\beta$ -glucan discharged from the pulse-charged macrophage cells**

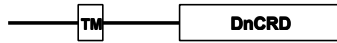
Naïve RAW 264.7 cells were cultured for 8 hours in the conditioned medium containing discharged  $\beta$ -glucan without (lane B) and with (lane C) boiling for proteinous factor inactivation, then, RNA was extracted from the cells and subjected to RT-PCR analysis for expression of various cytokines and  $\beta$ -actin as a control shown on the left. The cells were cultured also in the medium supplemented with  $\beta$ -glucan (125  $\mu$ g/ml) as a positive control (lane D) and the conditioned medium of RAW 264.7 cells without the charge with  $\beta$ -glucan as a negative control (lane A).

### **Figure 4 ROS-driven conversion of insoluble $\beta$ -glucan particles to soluble and Dectin-1-reactive $\beta$ -glucan within macrophage cells and in an *in vitro* model system**

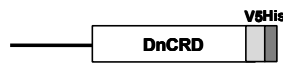
**(A)** Effect of the NADPH oxidase inhibitors (APO and DPI) and radical scavengers (NAC and MCI-186) was analyzed on discharge of soluble and Dectin-1-reactive  $\beta$ -glucan by RAW 264.7 cells, which had been pulse-charged with insoluble  $\beta$ -glucan (1 mg/ml). The soluble and Dectin-1-reactive  $\beta$ -glucan discharged for 6 hours in the culture medium was determined by the ELISA-based sandwich assay. Each value represented mean  $\pm$  SD from three independent conditioned medium. Significant difference ( $*p < 0.01$ ) between the two with and without the inhibitor was obtained by Student's t-test. **(B)** Insoluble  $\beta$ -glucan (0.5, 2.5, 5.0 and 20 mg/ml in final concentration) was incubated at 37°C for 24 hours in the presence of 0.5 mM CuSO<sub>4</sub> and 50 mM AsA. Soluble and Dectin-1-reactive  $\beta$ -glucan in the solution was determined with the incubation time by the ELISA-based assay. Each value represented mean  $\pm$  SD from three independent experiments. **(C)** Insoluble  $\beta$ -glucan (25 mg/ml) was incubated in the Cu<sup>2+</sup>/AsA solution as in (B) and neutral sugar in the soluble fraction was determined by phenol-sulfuric acid method. **(D)** IL-1 $\beta$  was determined for the culture medium of THP-1 macrophage cells, which had been stimulated for 9 h with the soluble fraction from the Cu<sup>2+</sup>/AsA-treated insoluble  $\beta$ -glucan.

**A**

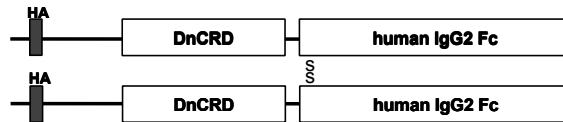
**Mouse Dectin-1**



**DnCRD-V5His from S2 cells**



**DnCRD-hG1Fc from HEK293 cells**



**B**

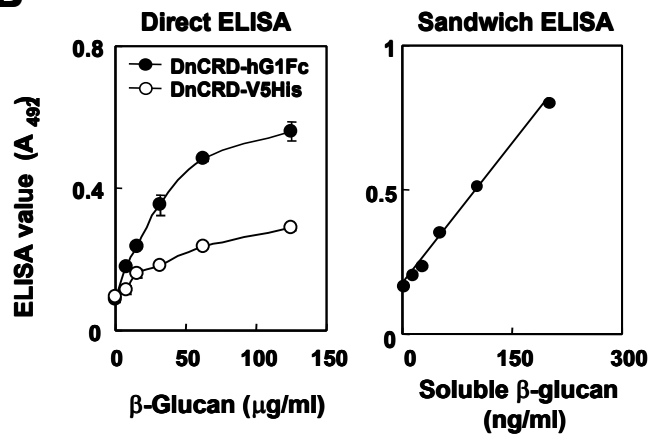


Fig. 1 Hino et al



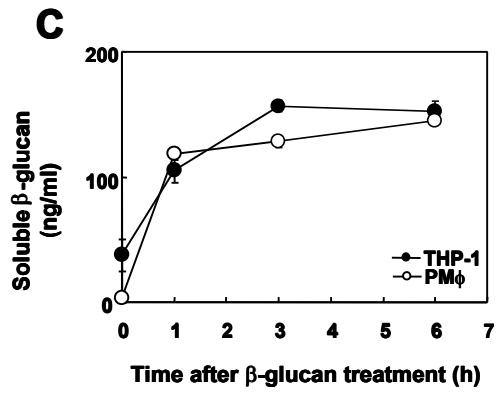
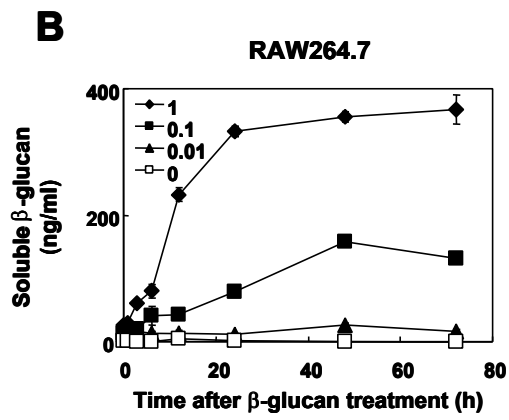
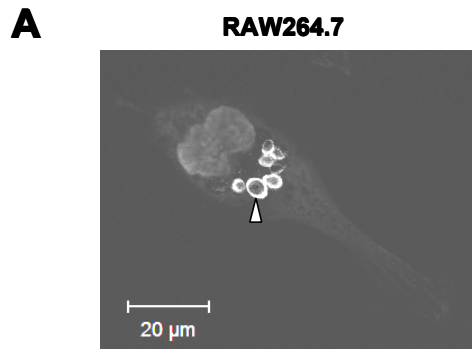


Fig. 2 Hino et al

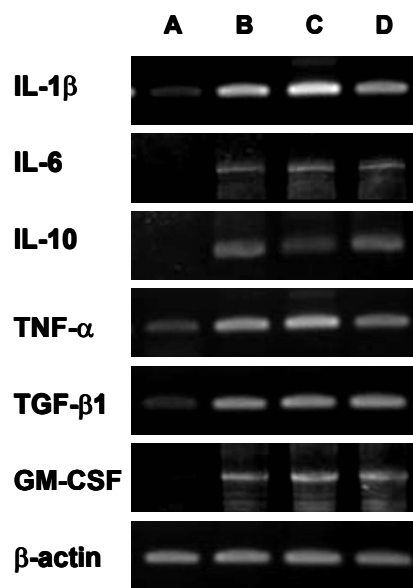


Fig. 3 Hino et al

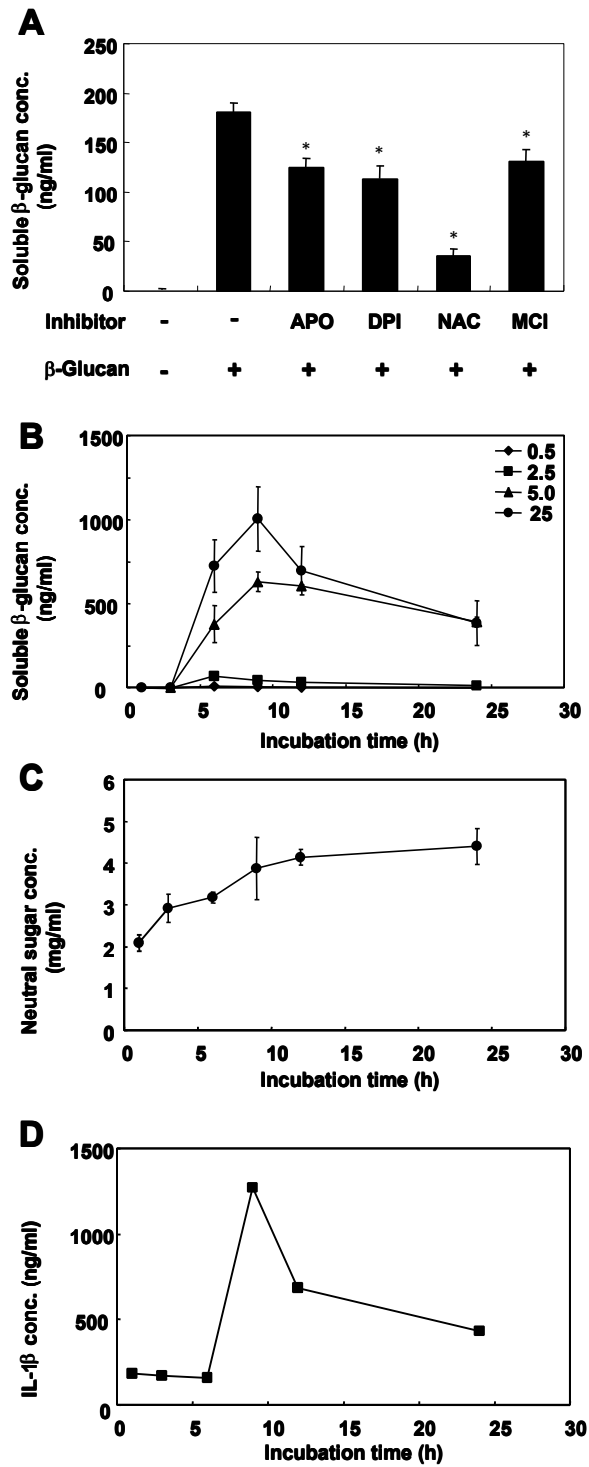


Fig. 4 Hino et al