Developmental and Comparative Immunology 88 (2018) 173–182

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Debaryomyces hansenii CBS 8339 β-glucan enhances immune responses and down-stream gene signaling pathways in goat peripheral blood leukocytes

Miriam Angulo, Martha Reyes-Becerril, Dariel Tovar-Ramírez, Felipe Ascencio, Carlos Angulo*

Immunology & Vaccinology Group, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Av. Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, La Paz B.C.S. 23096, Mexico

ARTICLE INFO

Keywords:
Marine yeast
β-glucan
Receptors
Intracellular regulation
Goat
Immune response

ABSTRACT

Debaryomyces hansenii-derived β-glucan has shown immunostimulant effect on aquaculture species and recently on goat peripheral blood leukocytes. Moreover, the marine yeast *D. hansenii* CBS 8339 has demonstrated to enhance fish immune response. Nonetheless, the associated immune signaling pathways induced by β-glucan from this marine yeast have not been characterized yet. This study described the effects of β-glucan from *D. hansenii* CBS 8339 against challenge with *Escherichia coli* and activation of possible mechanisms on goat peripheral blood leukocytes. The proton nuclear magnetic resonance spectra showed that *D. hansenii* had β-(1,3)(1,6)-glucan. The phagocytic ability enhanced after *E. coli* challenge, and nitric oxide production increased before and after challenge in leukocytes stimulated with *D. hansenii* β-glucan. In addition, an early gene expression stimulation was found related to β-glucan recognition by TLR2 and Dectin-1 receptors, intracellular regulation by Syk, TRAF6, MyD88 and transcription factor NFκB, and effector functions of pro-inflammatory cytokine, such as IL-1β and TNF-α. Interestingly, simulation with *D. hansenii*-derived β-glucan increased leukocyte viability after *E. coli* challenge. In conclusion, β-glucan from *D. hansenii* CBS 8339 reduced cytotoxic effects of *E. coli* and modulated signaling pathways and innate immune response in goat peripheral blood leukocytes.

1. Introduction

The β-glucans found in the cell wall of yeasts, fungi, and certain bacteria are non-digestible oligosaccharides that have immunomodulatory effects (Abel and Czop, 1992; Novak and Vetvicka, 2008). The yeast cell wall consists mainly of (1,3)-β-linked backbone with small numbers of (1,6)-β-linked side chains. β-glucans induce cellular immune responses by cell surface receptors, such as Dectin 1 and TLR2/6 dimer, which activate down-stream regulatory Syk and Raf-1 and MyD88 pathways, respectively (Batbayar et al., 2012) and consequently activate transcription factors, such as NFκB and AP-1 (Kerrigan and Brown, 2010; Roy et al., 2011; Fang et al., 2012). Altogether, this signaling activation stimulates phagocytic activity, reactive oxygen and nitrogen species, as well as pro-inflammatory cytokine production by monocytes, macrophages and neutrophils (Williams et al., 1988; Rubin-Bejerano et al., 2007; Du et al., 2015).

Yeast-derived β-glucans have been the focus of study because of their immunostimulant effects on humans (Lehne et al., 2006; Harnack et al., 2011), mice (Javmen et al., 2015), fish (Selvaraj et al., 2005) and shrimp (Scholz et al., 1999) and protection enhancement against many pathogens (Stuyven et al., 2009; Chethan et al., 2017). In addition, marine yeast β-glucans from *Candida tropicals, C. parapsilosis, C. oceani, Hortaea werneckii, Debaryomyces fabryi, D. nepalensis* and *D. hansenii* have shown immunostimulant effects in aquatic animals (Sukumaran et al., 2010; Wilson et al., 2015; Angulo et al., 2017). On the other hand, few studies are available regarding the immunostimulant potential of yeast β-glucans (i.e. *Saccharomyces cerevisiae*) in the livestock sector, including chickens (Zhang et al., 2008), pigs (Sonck et al., 2010; Vetvicka et al., 2014), cows (Tao et al., 2015), sheep (Zabek et al., 2013) and goats (Benda and Mádr, 1991). *Saccharomyces cerevisiae* was the first yeast investigated and clearly not suitable for all biotechnological purposes. Instead, extremophilic yeasts aroused great interest, including the genera *Debaryomyces* that proved to be a genetically and biochemically suitable yeast with biotechnological potential (Breuer and Harms, 2006). Recently, our research group found that β-glucans from marine *D. hansenii* strains CBS003, CBS005 and CBS006, significantly increased cell immune parameters, such as phagocytic ability, reactive oxygen species (ROS) and nitric oxide production and peroxidase activity in goat peripheral blood leukocytes (Medina-Córdova et al., 2018). Those β-glucans activated Toll-like receptor 4 (TLR4) and

* Corresponding author.
E-mail address: eangulo@cibnor.mx (C. Angulo).

https://doi.org/10.1016/j.dci.2018.07.017
Received 5 June 2018; Received in revised form 18 July 2018; Accepted 18 July 2018
Available online 18 July 2018
0145-305X/

Dectin-1 receptor. Nevertheless, neither in that study nor other studies have down-stream regulatory signaling pathways been investigated. On the other hand, *D. hansenii* CBS 8339 is a halotolerant and non-pathogenic marine yeast that has demonstrated to enhance fish immune response, including increase in IgM, respiratory burst and phagocytic and cytotoxic activities (Reyes-Becerril et al., 2008a, 2008b; Tovar-Ramírez et al., 2010).

Notably, *D. hansenii* CBS 8339 β-glucan immunostimulant potential and immunomodulatory mechanisms for goats and other ruminants have never been investigated. Therefore, this study isolated and characterized β-glucan from *D. hansenii* CBS 8339, as well as assessed its immunostimulant potential and possible activation mechanisms in goat peripheral blood leukocytes.

2. Materials and methods

2.1. Yeast strains used for glucan extraction

The *Debaryomyces hansenii* strain CBS 8339 was isolated from the gut of rainbow trout (Andlid et al., 1995; Reyes-Becerril et al., 2008a, 2011) and used in this work to extract β-glucan as described below.

2.2. Glucan extraction

Yeast biomass was obtained inoculating the culture (1:10) in Yeast Peptone Dextrose broth supplemented with chloramphenicol (1 μl/ml) and incubated at 30 °C for 48 h under agitation (150 rpm). Biomass was recovered by centrifugation (2457 g at 4 °C for 15 min) and lyophilized (FreeZone 18, LABCONCO, Kansas City, MO, USA) for 24–48 h. The glucan was extracted according to the methodology performed by Wilson et al. (2015). Briefly, two grams of dried biomass were suspended in 40 ml, 3% NaOH and incubated at 100 °C for 3 h in water bath and then kept at room temperature (RT) over night. The glucan suspension was centrifuged at 6297 g for 15 min to collect the supernatant and again resuspended with 3% NaOH. This procedure was repeated twice. Then, the supernatant was resuspended with 0.5 N acetic acid, maintained at 75 °C for 6 h and centrifuged (6297 g rpm for 15 min). The insoluble fraction was resuspended in ethanol, brought to boil and centrifuged (6297 g for 15 min). This procedure was repeated thrice. The residue was washed (thrice) with distilled water and lyophilized for 48 h. The final mass (glucan) was used for the assays, and the yield of extracted glucan was recorded.

2.3. Nuclear magnetic resonance (NMR) analysis

Ten to twenty five grams of β-glucan sample were dissolved in DMSO-d_6 and subjected to ^{1}H NMR analyses. The analysis was run at 80 °C on a Bruker Avance 600 Mhz spectrometer (Bruker, Billerica, Massachusetts, USA). NMR chemical shifts were referenced to the residual DMSO-d_6 multiplet proton resonance at 2.50 ppm. The NMR spectral data and setup conditions were: 25 ppm spectral width centered at 5.0 ppm, 32768 data points, 15 s relaxation delay, 32 scans, and 0.2 Hz exponential apodization.

2.4. Content of β-1,3-1,6-glucan

To determine the β-1,3-1,6-glucan content, a standard reference curve with Laminarin (Sigma, Saint Louis, MO, USA) was performed. The stock solution was dissolved in 1-mol/L sodium hydroxide. This stock solution was used in the range of 50–150 μg/ml. A defined volume of sample solution or Laminarin stock solution was dispensed into Eppendorf tubes and diluted with distilled water to a total volume of 700 μl. Then 600 μl of 0.2 mol/L citric acid/sodium hydroxide buffer (pH 7) and 100 μl of dye solution (0.08% Congo red) were added. Subsequently, the samples were mixed and analyzed at 523 nm in a microplate spectrophotometer Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). Appropriate control buffers were used. All analyses were performed in triplicate.

2.5. Measurement of carbohydrate content

β-glucan was resuspended in water (2.5 mg/ml) and heated in autoclave using glass tubes with screw caps to 135 °C for 4 h (the samples were taken at one and four hours), and the supernatant was filtered through a 0.20 μm disposable syringe filter (Adachi et al., 1990). The carbohydrate content of *D. hansenii* β-glucan was determined by phenol-sulfuric acid method according to Dubois et al. (1956). Then, 12.5 μl phenol (80% w/v) and 1.5 ml concentrated sulfuric acid were added to the samples. After that, the samples were incubated in water bath at 25 °C for 30 min. Optical density (OD) was measured at 490 nm in a microplate reader (BioRad, Model 3550 UV, Hercules, CA, USA). Glucose was used in standard curve as reference.

2.6. Isolation of leukocytes from caprine peripheral blood

Caprine peripheral blood samples were acquired from healthy goats and collected into sodium heparin-containing BD Vacutainer® (Franklin Lakes, NJ, USA) tubes to isolate leukocytes under sterile conditions following Azmi et al. (2006) with slight modifications. Blood was diluted with RPMI 1640 medium (3X heparin) (1:1 v/v); then 1.25 ml were placed in 15-mL falcon tubes containing 2 ml of Histopaque®-1077 (Sigma; St. Louis, MO, USA), and centrifuged at 222 g at 20 °C for 20 min. The buffy coat was collected and incubated at room temperature with 1 ml of ACK (Ammonium-Chloride-Potassium) Lysing Buffer for erythrocyte lysis for 10 min. The lysis reaction was stopped with 3 mL of PBS and centrifuged again under the same conditions. Then, leukocytes were suspended in RPMI 1640 supplemented with fetal bovine serum (3%) penicillin/streptomycin (100 IU/ml, 100 mg/ml) and glutamine (1%). Finally, leukocytes were checked for their viability with Trypan Blue Exclusion test using TC20 Automated Cell Counter (BioRad, Hercules, CA, USA) (Reyes-Becerril et al., 2016).

2.7. Stimulation test of peripheral blood leukocytes and Escherichia coli challenge

One milliliter of the peripheral blood leukocyte suspension was placed into flat-bottomed 24-well cell culture plates (Sigma, St. Louis, MO, USA) containing 1×10^6 cells per well. Then, leukocytes were incubated with 200 μl of *D. hansenii* β-glucan (200 μg/ml) at 37 °C and 5% CO_2 atmosphere for 24 h. As positive control, leukocytes were incubated with 200 μl of commercial β-glucan (200 μg/ml) (Curdlan, Sigma, St. Louis, MO, USA). As negative control, leukocytes incubated with RPMI medium were used and collected at 24 h post-incubation; β-glucan-stimulated leukocytes were challenged with pathogenic *Escherichia coli* 23 (20 μl; 1×10^8 cells/ml) at 6 h post-incubation and incubated for 18 h.

2.8. Cell viability

The resazurin assay was used to determine the effect of *D. hansenii* β-glucan and *E. coli* challenge on viability of goat peripheral blood leukocytes according to Riss et al. (2016). Briefly, leukocytes were dispensed in 96-well plates (100 μl 1×10^6 cells/ml) and incubated with 20 μl/well of β-glucan from *D. hansenii* (200 μg/ml). Six hours later, they were challenged with 2 μl per well of *E. coli* 23 (1×10^8 cells/ml) and cultured overnight (at 37 °C and 5% CO_2). Then, cells were stained with 10 μl resazurin solution (Sigma, St. Louis, MO, USA) and incubated at 37 °C and 5% CO_2 for 4 h. Fluorescence was measured in Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA) excitation at 530 nm and emission at 590 nm. Peripheral blood leucocytes without β-glucan and those incubated with DMSO (10% final concentration) were used as controls.