Modulation of some cell surface antigens in the monocyte-like cell line U937 in response to β-1,3-1,6 glucan derived from *Aureobasidium pullulans*

Nobunao IKEWAKI Hidekazu TAMAUCHI * Hidetoshi INOKO **

Abstract

We examined the modulation of cell surface antigens in a human monocyte-like cell line, U937, cultured with β-1,3-1,6 glucan, produced by the black yeast *Aureobasidium pullulans (A. pullulans)*, using various monoclonal antibodies (mAbs) to human cluster of differentiation (CD) antigens and flow cytometry. The expressions of CD11b (complement component receptor type-3; CR3), CD54 (intercellular adhesion molecule-1; ICAM-1) and CD93 (receptor for complement component 1, subcomponent q phagocytosis; C1qRp) in the U937 cells cultured with β-1,3-1,6 glucan were significantly ($P<0.01$) enhanced, compared with those in the control cells (cultured without β-1,3-1,6 glucan), at 48 hrs. In addition, the cells cultured with β-1,3-1,6 glucan significantly ($P<0.01$) enhanced the production of interleukin-8 (IL-8) and an apoptosis-related molecule, soluble Fas (sFas), into the culture supernatants. Together, these findings strongly indicate that the β-1,3-1,6 glucan derived from A. *pullulans* has some immunopotentiator effects for some human immune responses.

Key words : β-1,3-1,6 glucan, CD antigens, interleukin-8 (IL-8), soluble Fas (sFas), U937 cells

Introduction

Polysaccharides, formed by the linking of numerous glucose molecules, have a variety of unique physical properties and physiological activities that have been exploited by the health food, medical and pharmaceutical industries [1]. Glucan is one such polysaccharide that has two types (α and β) of structural forms that are synthesized by many species of mushrooms, black yeast *(Aureobasidium pullulans)*, and several kinds of fungi. Beta glucan is a powerful immune stimulator known to activate macrophages and to have positive immune actions on B lymphocytes, T lymphocytes and natural killer (NK) cells [2, 3]. For example, zymosan is a yeast-derived particle principally composed of β-glucan that, when administered orally, has a number of effects on immune function, including the ability to confer resistance against cancers and infections and to stimulate the production of cytokines, such as interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) [4, 5]. Previously, the complement component type-3 receptor (CR3) (CD11b/CD18) on leukocytes, monocytes, macrophages, granulocytes, and dendritic cells (DCs) was identified as a β-glucan receptor [6]. However, recent studies using specific carbohydrate inhibitors, genetic CD11b/CD18-deficient animals, and several blocking monoclonal antibodies (mAbs) have provided evidence that Dectin-1, and not CR3 (CD11b/CD18), is the main β-glucan receptor [7]. Dectin-1

Kyushu University of Health and Welfare School of Health Science, and Institute of Immunology, Takahashi Educational Institute, 1714-1 Yoshino-cho, Nobeoka, Miyazaki 882-8508, Japan

*Department of Microbiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

**Department of Molecular Life Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan

is a type II transmembrane receptor containing a single extracellular C-type lectin domain that has a phagocytic function against β-glucan-containing particles [8].

In general, the actions of β-glucans are not due to direct actions on cancer cells, as is the case with chemical anti-cancer drugs; instead, β-glucans act as biological response modifiers (BRMs) to enhance the immunity of living organisms [9, 10]. Several β-glucans containing polysaccharides have been identified, studied and developed for use as supplements in the health food, medical and pharmaceutical industries. However, detailed analyses, particularly of their ability to modulate cell surface antigens on cells related to several immune responses, have yet to be performed.

Recently, we succeeded in establishing a β-1,3-1,6 glucan produced by the black yeast *Aureobasidium pullulans (A. pullulans)* strain AFO-202 by developing and using the latest microbial cultural techniques. In this study, we investigated some of the immunological actions of this β-1,3-1,6 glucan, such as its ability to modulate various human cluster of differentiation (CD) antigens and to produce soluble factors, using a human monocyte-like cell line, U937.

Materials and Methods

Reagents

β-1,3-1,6 glucan, synthesized by *Aureobasidium pullulans (A. pullulans)* strain AFO-202, was prepared by Sophy Co. (Kochi, Japan) using the latest biological culturing and preparative techniques and is currently available commercially as a health food supplement. The amount of β-1,3-1,6 glucan was measured using two enzymatic methods: the β-1,3 glucanase and the β-1,6 glucanase methods [11]. Enzyme-linked immunoassay (EIA) kits for interleukin-8 (IL-8) and an apoptosis-related molecule, soluble Fas (sFas), were purchased from MBL Co. (Nagoya Japan).

Antibodies

The characteristics of the monoclonal antibodies (mAbs) used in this study are summarized in Table 1. A fluorescence isothiocyanate (FITC)-conjugated goat anti-mouse Ig (IgG+IgM+IgA) antibody was purchased from Cosmo Bio Co. (Tokyo Japan).

Cell line

The U937 cells used in this study were supplied by the Japanese Research Resource Bank (JRRB) (Tokyo, Japan) and were cultured in RPMI 1640 medium (GIBCO) supplemented with 10 mM HEPES buffer, 2 mM glutamine, and 10% fetal calf serum (FCS) (GIBCO) (subsequently referred to as the complete medium).

Preparation of U937 cells cultured with β-1,3-1,6 glucan

U937 cells ($1x10^5$) in complete medium were cultured in 96-well microplates (Sumitomo Co. Tokyo Japan) with or without β-1,3-1,6 glucan (50 μg/ml); plates were incubated at 37℃ for 48 hrs. After culturing, the cells were harvested and the modulation of cell surface antigens was examined using various mAbs, summarized in Table 1, and flow cytometry.

Flow cytometry

The cells were washed in cold phosphate-buffered saline (PBS) containing 0.1% NaN_3 (subsequently referred to as the washing buffer) and were then incubated in PBS containing 25% normal goat serum, 1 mg/ml of normal human IgG, and 0.1% NaN_3 for 10 min on ice to block the Fc receptor (FcR) for IgG. The cells were then incubated with an optimal concentration of various mAbs for 40 min at 4℃. After washing with the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse Ig (IgG+IgM+IgA) for 20 min at 4℃. Following a final wash with the washing buffer and resuspension in PBS containing 2% FCS and 0.1% NaN_3, the percentages of positively stained cells for various mAbs were determined using a FACScan (Becton Dickinson) system. The expression index was calculated using the following formula: (Expression index) = (percentage of positively stained cells for mAb cultured with β-1,3-1,6 glucan)/(percentage of positively stained cells for mAb cultured without β-1,3-1,6 glucan). The experiments were repeated three times.

Assay for interleulin-8 (IL-8) and soluble Fas (sFas) in the culture supernatants

The detection of interleukin-8 (IL-8) and soluble Fas (sFas) in the culture supernatants of the U937 cells cultured with or without β-1,3-1,6 glucan (50μg/ml) were performed using EIA kits. The experiments were repeated three times.

Statistical analysis

The statistical analysis was performed using the Student *t*-test. Differences were considered significant when the *P* value was less than 0.05.

Results and Discussion

In this study, we investigated the immunological actions, at the cellular level, of a β-1,3-1,6 glucan synthesized by *A. pullulans* strain AFO-202. First, the percentages of positively stained cells for various human CD antigen mAbs were analyzed using flow cytometry. As shown in Figs. 1 and 2, the expressions of CD11b (CR3), CD54 (ICAM-1) and CD93 (C1qRp) on the U937 cells cultured with β-1,3-1,6 glucan were significantly ($P<0.01$) enhanced at 48 hrs.

CD11b is a member of the β2 integrin family of adhesion molecules and is mainly expressed on certain lymphocytes, monocytes, and macrophages. It plays an important role as a receptor for bacterial phagocytosis and the regulation of various immune responses, such as cell activation and immunoregulatory cytokines production [12,13].

CD54 is a member of the immunoglobulin (Ig) superfamily of adhesion molecules and is expressed on various cells such as lymphocytes, monocytes, granulocytes, and epithelial cells. It plays important roles as a ligand for CD11a (lymphocyte function-associated antigen-1α; LFA-1α) in various human immune responses [14]. Furthermore, the expression of CD54 is reportedly regulated by several stimulants mediated by protein kinase C (PKC), protein tyrosine kinase (PTK), calcium/calmodulin, STAT6, and multiple intracellular signal transductions. In fact, we also found that cytochalasin E (a drug that disrupts actin filaments) strongly up-regulated CD54 in HeLa epithelial cells in a PKC-mediated manner [15].

CD93 is a receptor for complement component 1, subcomponent q phagocytosis (C1qRp) and was originally reported to be involved in the C1q-mediated enhancement of phagocytosis [16]. CD93 has a molecular weight of about 90 -100 kDa and is a heavily *O*-glycosylated type I transmembrane protein consisting of unique C-type lectin domains [17]. CD93 is selectively expressed on myeloid cells (granulocytes and monocytes), endothelial cells and stem cells, suggesting that this molecule may be involved in some important biological functions in several immune responses [18]. The modulation of CD93 expression has been investigated in a variety of cells, particularly in granulocytes and monocytes, and the rapid up-regulation of this molecule's expression by the inflammatory peptide FMLP has been reported [19]. We also previously reported that CD93 expression on a human monocyte-like cell line (U937), a human NK-like cell line (KHYG-1) and a human umbilical vein endothelial cell line (HUV-EC-C) was strongly up-regulated by exposure to a PKC activator, phorbol myristate acetate (PMA); this up-regulation was controlled by a PKC delta isoenzyme, but not by PKA or PTK [20]. *O*-glycosylation of the CD93 molecule has been reported to be a very important factor in the stability of this molecule's expression [17], suggesting that the modulation of CD93 expression by β-1,3-1,6 glucan may be associated with unglycosylation. Together, the modulation of C11b, CD54 and CD93 on the U937 cells by β-1,3-1,6 glucan may provide important information on cell-to-cell interactions in various human immune responses.

Next, we measured IL-8 and sFas in the culture supernatant of U937 cells cultured with or without β-1,3-1,6 glucan using EIA kits. Figure 3 shows that β-1,3-1,6 glucan strongly induced IL-8 production into the culture supernatant of the U937 cells at 48 hrs, and the difference between the supernatant from cells cultured in the presence or absence of β-1,3-1,6 glucan was significant ($P<0.01$). By contrast, no other cytokines, including IL-1β, IL-2, IL-4, IL-6 or IFN-γ, were detected in the culture supernatant under the same conditions (data not shown). IL-8 is one of the most potent chemoattractants for neutrophils and is released by

several human cells stimulated by various substances to regulate inflammatory responses in humans [21]. In addition, the regulation of IL-8 production by various stimulatory substances is reportedly controlled mainly by protein kinases, such as PKC or PKA [15, 22]. We also showed that the production of IL-8 by cytochalasin E is regulated by PKC [15]. Furthermore, the production of IL-8 also appears to depend on NF-κB activation and intracellular signaling mediated by Toll-like receptor-2 (TLR-2) and the β-glucan receptor, Dectin-1 [23]. Although the detailed mechanism(s) of IL-8 production by U937 cells in response to β-1,3-1,6 glucan is (are) unknown, we are vigorously re-analyzing IL-8 production mechanism(s).

β-1,3-1,6 glucan also significantly ($P < 0.01$) induced the production of soluble Fas (sFas) by U937 cells (Fig. 4). Fas (APO-1) is a 45-kDa type I membrane protein that belongs to the TNF-α receptor and ligand families [24, 25]. The activation of Fas via mechanisms mediated by Fas ligands (FasLs), such as anti-Fas antibodies, induces the apoptosis of various Fas-expressing cells through the activation of the caspase cascade [26]. The Fas/FasL apoptosis induction system constitutes one of the main systems regulating immune responses and homeostasis in living organisms [24, 25]. The β-1,3-1,6 glucan-induced sFas production by U937 cells observed in this study suggests that β-1,3-1,6 glucan might prevent apoptosis by suppressing the Fas/FasL apoptosis induction system for the maintenance of homeostasis in living organisms.

In conclusion, β-1,3-1,6 glucan produced by *A. pullulans* strain AFO-202 significantly enhanced the expressions of CD11b, CD54 and CD93 on U937 cells, ultimately leading to the production of IL-8 and sFas. These findings strongly indicate that the action of this β-1,3-1,6 glucan is highly critical to immunological responses. Further analyses are needed to demonstrate the detailed mechanism(s) of this finding at the molecular level.

References

1 Wasser, S.P.: Medicinal mushrooms as a souce of antitumor and immunomodulating polysaccharides. Appl. Microbiol. Biotechnol. 60: 258-274, 2002.

2 Brown, G.D., Gordon, S. :Fungal beta-glucans and mammalian immunity. Immunity 19: 311-315, 2003.

3 Cramer, D.E., Allendorf, D.J., Baran, J.T., et al.:Beta-glucan enhances complement-mediated hematopoietic recovery after bone marrow injury. Blood 107: 835-840, 2006.

4 Langeggen, H., Namork, E., Johnson, E., et al. :HUVEC take up opsonized zymosan particles and secrete cytokines IL-6 and IL-8 *in vitro*. FEMS Immunol. Med. Microbiol. 15: 55-61, 2003.

5 Young, S.H., Ye, J., Frazer, D.G., et al.:Molecular mechanism of tumor necrosis factor-alpha production in 1-->3-beta-glucan (zymosan)-activated macrophages. J. Biol. Chem. 276: 20781-20787, 2001.

6 Elstad, M.R., Parker, C.J., Cowley, F.S., et al.: CD11b/CD18 integrin and beta-glucan receptor act in concert to induce the synthesis of platelet-activating factor by monocytes. J. Immunol. 52: 220-230, 1994.

7 Brown, G.D., Gordon, S.:Immune recognition. A new receptor for beta-glucans. Nature 413: 36-37, 2001.

8 Grunebach, F., Weck, M.M., Reichert, J., et al.: Molecular and functional characterization of human Dectin-1. Exp. Hematol. 30: 1309-1315, 2002.

9 Hong, F., Hansen, R.D., Yan, J., et al.: Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. Cancer Res. 63: 9023-9031, 2003.

10 Lehne, G., Haneberg, B., Gaustad, P., et al.: Oral administration of a new soluble branched beta-1,3-D-glucan is well tolerated and can lead to

increased salivary concentrations of immunoglobulin A in healthy volunteers. Clin. Exp. Immunol. 143: 65-69, 2006.

11 Prosky, L., Asp, N.G., Schweizer, T.F., et al.: Determination of soluble dietary fiber in foods and food products: collaborative study. JAOAC Int. 77:690-694, 1994.

12 Ikewaki, N., Tamauchi, H., Inoko, H.: Modulation of cell surface antigens and regulation of phagocytic activity mediated by CD11b in the monocyte-like cell line U937 in response to lipopolysaccharide. Tissue Antigens. 42:125-132, 1993.

13 Nilsson, M., Weineisen, M., Andersson, T., et al.: Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of *Streptococcus pyogenes* by neutrophils in human immune serum. Eur. J. Immunol. 35:1472-1481, 2005.

14 Wang, P., Váky, F., Li, S.L., et al.: Functional characteristics of the intercellular adhesion molecule-1 (CD54) expressed on cytotoxic human blood lymphocytes. Cell. Immunol. 131:366-380, 1990.

15 Ikewaki, N., Yamada, A., Inoko, H: Depolymerization of actin filament by cytochalasin E induces interleukin-8 production and up-regulates CD54 in the HeLa epithelial cell line. Microbiol. Immunol. 47:775-783, 2003.

16 Tenner, A.J.: C1q receptors regulating specific functions of phagocytic cells. Immunobiology 199: 250-264, 1998.

17 Nepomuceno, R.R., Ruiz, S., Park, M., et al.: C1qRP is a heavily *O*-glycosylated cell surface protein involved in the regulation of phagocytic activity. J.Immunol. 162:3583-3589, 1999.

18 McGreal, E.P., Ikewaki, N., Akatsu, H., et al.: Human C1qRp is identical with CD93 and the mNI-11 antigen but does not bind C1q. J. Immunol. 168:5222-5232, 2002.

19 Jack, R.M., Lowenstein, B.A., Nicholson-Weller, A.: Regulation of C1q receptor expression on human polymorphonuclear leukocytes. J. Immunol. 153:262-269, 1994.

20 Ikewaki, N., Kulski, J.K., Inoko, H.: Regulation of CD93 cell surface expression by protein kinase C isoenzymes. Microbiol. Immunol. 50: 93-103, 2006.

21 Driscoll, K.E. : Macrophage inflammatory proteins: biology and role in pulmonary inflammation. Exp. Lung Res. 20: 473-490, 1994.

22 Ikewaki, N., Inoko, H.: A very late activating antigen-alpha4 (CD49d) monoclonal antibody, BU49 induces phosphorylation of a cAMP response element-binding protein (CREB), resulting in induction of homotypic cell aggregation and enhancement of interleukin-8 (IL-8) production. Microbiol. Immunol. 46:685-695, 2002.

23 Ikeda, Y., Adachi, Y., Ishibashi, K., et al. :Activation of toll-like receptor-mediated NF-kappa beta by zymosan-derived water-soluble fraction: possible contribution of endotoxin-like substances. Immunopharmacol. Immunotoxicol. 27: 285-298, 2005.

24 Maher, S., Toomey, D., Condron, C., et al.:Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. Immunol. Cell Biol. 80: 131-137, 2002.

25 Nagata, S., Golstein, P.: The Fas death factor. Science 267: 1449-1456, 1995.

26 Chen, M., Wang, J. :Initiator caspases in apoptosis signaling pathways. Apoptosis 7: 313-319, 2002.

Table 1. Characteristics of various mouse mAbs used in this study

mAb	Ig class	CD antigen	Molecule
mNI-58A	IgG1	CD11a	LFA-1 α
Bear-1	IgG1	CD11b	CR3
MY-4	IgG2b	CD14	LPS-R
AHN1.1	IgM	CD15	Lewis-X
Leu11b	IgG1	CD16	Fc γ R III
IB4	IgG2a	CD18	LFA-1 β
H107	IgG2b	CD23	Fc ε R II
4B7R	IgG1	CD29	VLA-β 1
L133.1	IgG1	CD31	PECAM-1
2H4	IgG1	CD45R	LCA
L25.3	IgG2b	CD49d	VLA-4 α
LB-2	IgG2b	CD54	ICAM-1
mNI-11	IgG1	CD93	C1qRp
E1/6	IgG1	CD106	VCAM-1

LFA-1 α, lymphocyte function-associated antigen-1 α; CR3, complement component type-3 receptor; LPS-R, lipopolysaccharide receptor; Fc γ R III, Fc γ receptor III; LFA-1 β, lymphocyte function-associated antigen-1 β; VLA-β 1, very late antigen-β 1; PECAM-1, platelet endothelial cell adhesion molecule-1; LCA, lymphocyte common antigen; VLA-4 α, very late antigen-4 α; ICAM-1, intercellular adhesion molecule-1; C1qRp, receptor for complement component 1 subcomponent q phagocytosis; VCAM-1, vascular cell adhesion molecule-1.

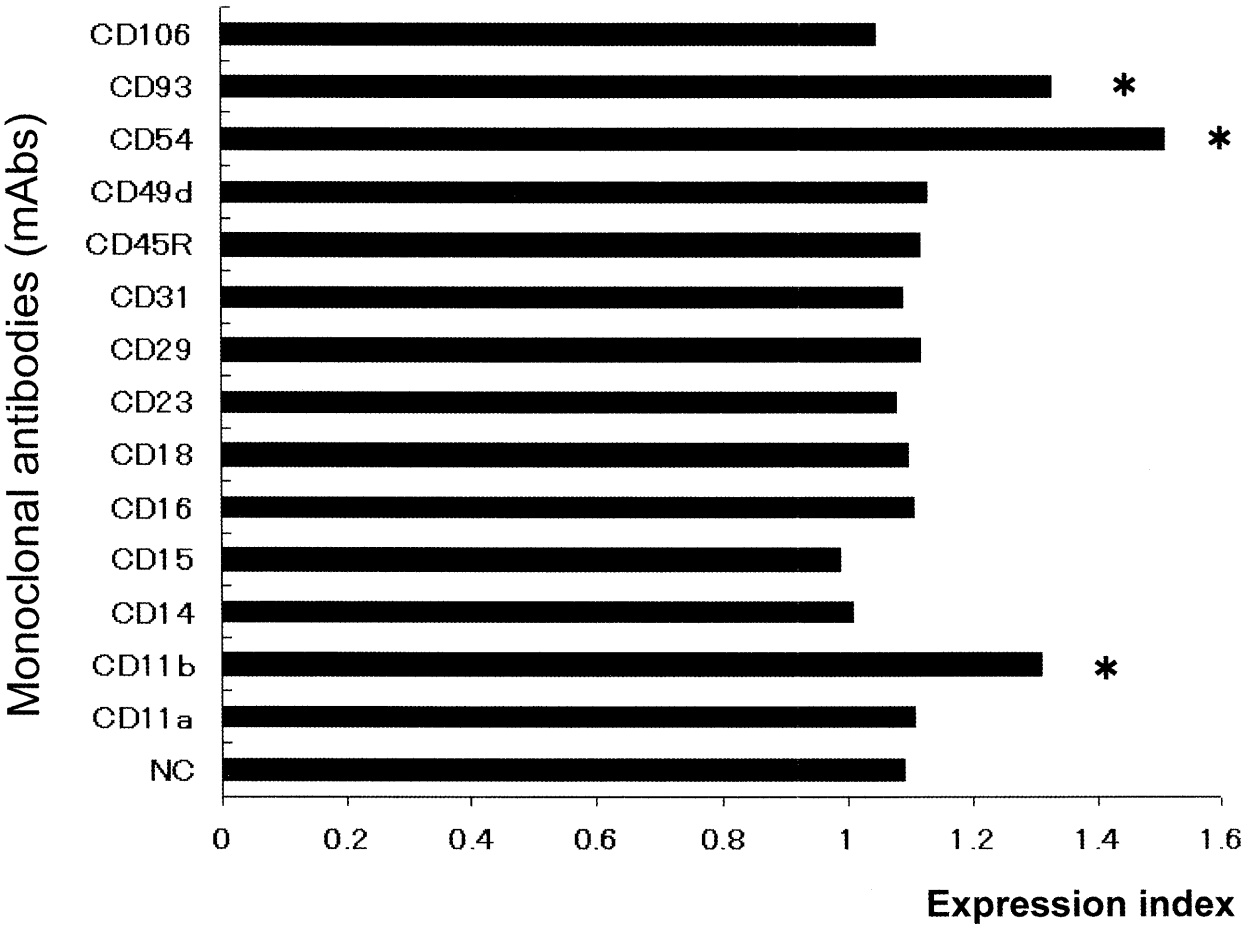

Figure 1. Modulation of some cell surface antigens on U937 cells cultured with β-1,3-1,6 glucan. The U937 cells cultured with or without β-1,3-1,6 glucan (50 μg/ml) were incubated with an optimal concentration of various mAbs for 40 min at 4℃. After washing with the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse Ig (IgG+IgM+IgA) for 20 min at 4℃. The percentages of positively stained cells for various mAbs were determined using a FACScan system. The expression index was calculated using the following formula: (Expression index) = (percentage of positively stained cells for mAb cultured with β-1,3-1,6 glucan)/(percentage of positively stained cells for mAb cultured without β-1,3-1,6 glucan). *$P<0.01$ (NC vs. CD11b, CD54 or CD93 mAb, respectively). The experiments were repeated three times. NC: negative control.

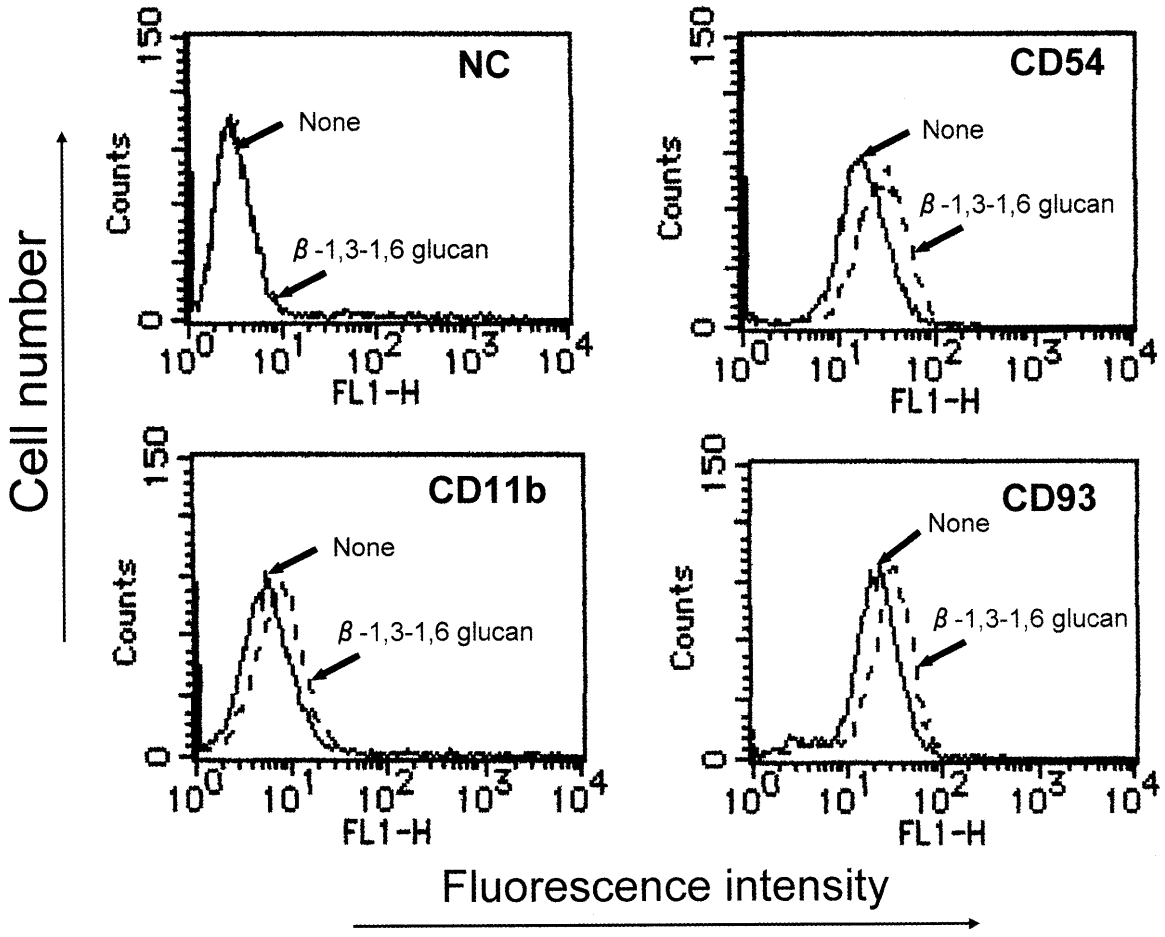

Figure 2. Modulation of CD11b, CD54 and CD93 on U937 cells cultured with β-1,3-1,6 glucan. The U937 cells cultured with or without β-1,3-1,6 glucan (50μg/ml) were incubated with an optimal concentration of CD11b, CD54 and CD54 mAbs for 40 min at 4℃. After washing with the washing buffer, the cells were incubated with an FITC-conjugated goat anti-mouse Ig (IgG+IgM+IgA) for 20 min at 4℃. The percentages of cells that stained positive for CD11b, CD54 or CD93 mAbs were analyzed using a FACScan system. Solid line: without β-1,3-1,6 glucan; dotted line: with β-1,3-1,6 glucan. The experiments were repeated three times. NC: negative control.

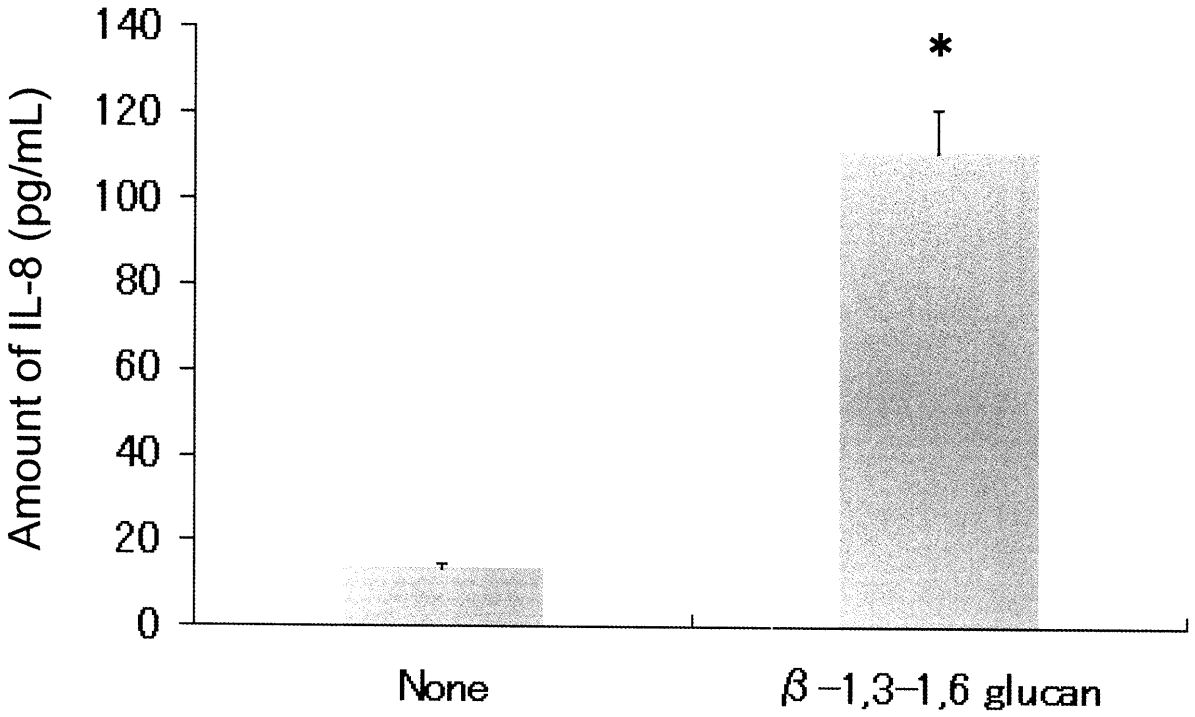

Figure 3. Induction of IL-8 production by U937 cells cultured with β-1,3-1,6 glucan. Detection of IL-8 in the culture supernatants from U937 cells cultured with or without β-1,3-1,6 glucan (50 μg/ml) was performed using an EIA kit. The experiments were repeated three times. *$P < 0.01$ (None vs. β-1,3-1,6 glucan).

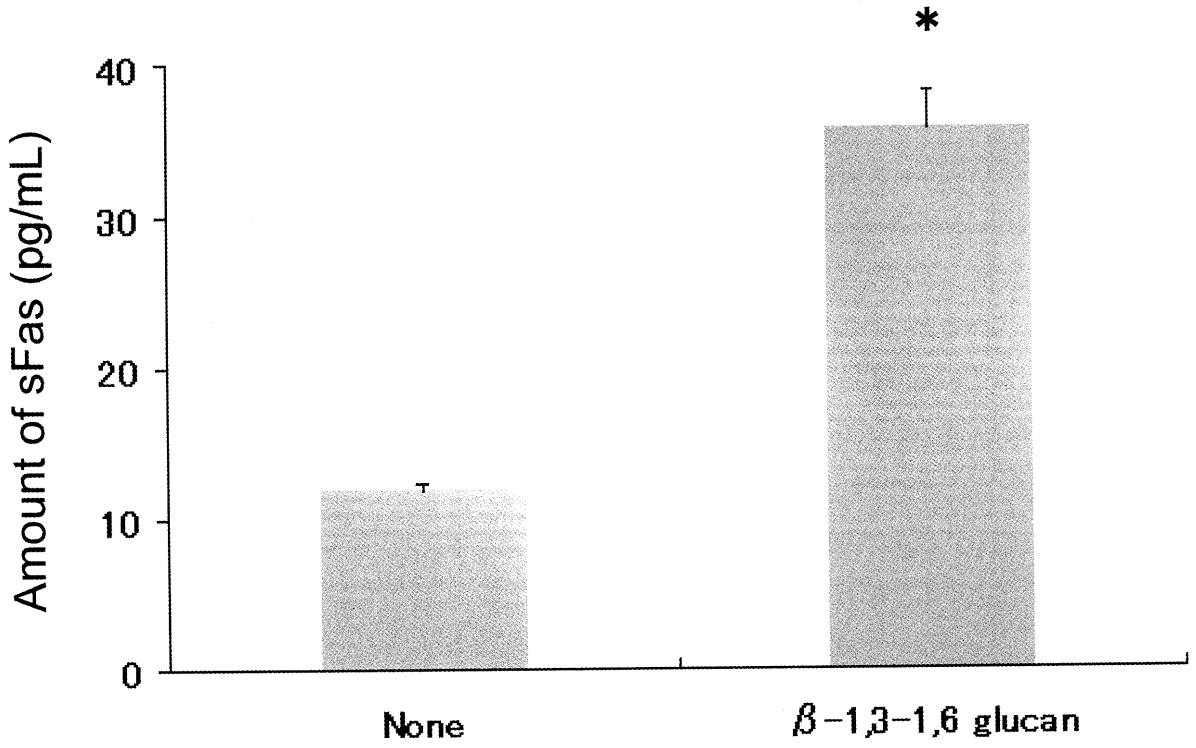

Figure 4. Induction of sFas production by U937 cells cultured with β-1,3-1,6 glucan. Detection of sFas in the culture supernatants from U937 cells cultured with or without β-1,3-1,6 glucan (50μg/ml) was performed using an EIA kit. The experiments were repeated three times. *$P<0.01$ (None vs. β-1,3-1,6 glucan).

黒酵母 (*Aureobasidium plulluns*) 由来のβ-1,3-1,6 glucanによる単球系細胞株(U937)表面上のCD11b、CD54およびCD93の発現増強

池脇信直　玉内秀一 *　猪子英俊 **

九州保健福祉大学保健科学部・高梁学園免疫研究所
〒882-8508　宮崎県延岡市吉野町1714-1
*北里大学医学部微生物学
〒228-8555　神奈川県相模原市北里1-15-1
**東海大学医学部分子生命学
〒259-1193　神奈川県伊勢原市望星台

要旨

黒酵母 (*Aureobasidium plulluns*) 由来のβ-1,3-1,6 glucanによる単球系細胞株(U937)表面上のCD抗原の動態を各種モノクローナル抗体とフローサイトメトリー法を用いて解析した。その結果、β-1,3-1,6 glucanで培養したU937細胞はCD11b (C3R)、CD54(ICAM-1)、CD93(C1qRp)の発現が有意に増強した($P<0.01$)。さらに、β-1,3-1,6 glucanで培養したU937細胞はインターロイキン－8(IL-8)およびアポトーシス関連分子である可溶性Fas分子(sFas)の産生も有意に増強した($P<0.01$)。以上の結果から、*Aureobasidium plulluns*由来のβ-1,3-1,6 glucanはヒトの免疫応答を増強する作用があることがわかった。

キーワード：β-1,3-1,6グルカン、CD 抗原、インターロイキン－8(IL-8)、可溶性Fas (sFas)、U937細胞
2007.11.12受理