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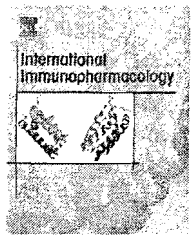

Contribution of dectin-1 and granulocyte macrophage–colony stimulating factor (GM-CSF) to immunomodulating actions of β-glucan

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Abstract

β-Glucans are major cell wall structural components in fungi. As they are not found in animals, these carbohydrates are considered to be classic pathogen-associated molecular patterns (PAMPs), and are recognized by the innate immune system. Although their immunomodulating activities have been shown to be associated with the recognition of some fungi, and with their medicinal properties in the field of cancer immunotherapy, it is still unclear how β-glucans mediate their effects. Recent studies have started to shed some light on their cellular receptors, such as dectin-1, and their molecular mechanisms of action. We have extensively investigated the response of leukocytes to β-glucan, focusing on cytokine induction by SCG, which is a major 6-branched 1,3-β-D-glucan in *Sparassis crispa* Fr. There is a strain difference in the reactivity of mice to SCG, and DBA/1 and DBA/2 mice are highly sensitive strains. In the process of research on cytokine induction by SCG in DBA/2 mice, we found that GM-CSF plays a key biological role in this activity. Cytokine induction by SCG was completely abolished in dendritic cells from dectin-1 knockout mice. On the other hand, controlling the level of endogenous GM-CSF production and/or dectin-1 expression could regulate the reactivity to β-glucan. These results indicate that the key factors in the responsiveness to β-glucan are GM-CSF production and dectin-1 expression. In this review, we describe how the key molecules related to the expression of the immunomodulating activities of β-glucan were identified, and how the response to β-glucan is controlled.

1. Introduction

β-Glucans are major cell wall structural components in fungi and are also found in plants and some bacteria. Among β-glucans, 6-branched 1,3-β-glucan is the best characterized. These polysaccharides are a heterogeneous group of glucose

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polymers, consisting of a backbone of β(1→3)-linked β-D-glucopyranosyl units with β(1→6)-linked side chains of varying distribution and length. As they are not found in animals, these carbohydrates are considered to be classic pathogen-associated molecular patterns (PAMPs) [1] and are recognized by the innate immune system of vertebrates, as well as invertebrates. Some β-glucans are well-known biological response modifiers. We and others have demonstrated that the immunomodulating activity of β-glucans is mainly related to their effects on immune effector cells, such as macrophages, mononuclear cells, and neutrophils, involved in innate immunity, resulting in the production of cytokines [2,3]. The body's defense against microbial attack and against spontaneously arising malignant tumor cells comprises a dynamic orchestrated interplay of innate and acquired immune responses, and the effectors of innate immunity can activate these systems. The host defense system has receptors for β-glucans that function to recognize and eliminate fungi, such as *Pneumocystis* and *Candida*, which generally contain β-glucan in their cell walls [4]. These findings indicate that β-glucan is an important player in both host defense against fungi and cancer immunotherapy. Although the therapeutic benefits of these compounds have been demonstrated, it is still unclear how β-glucans mediate their effects.

A number of biologically active β-glucans have been prepared from various sources (Table 1). Purity, homogeneity, structural characterization, and molecular mechanism as well as their clinical application vary significantly. In some studies crude extracts from fungi and/or mushrooms have been used and described just as function of β-glucans. In addition, the unfortunately, majority of the materials have not been clinically applied and/or not clinically approved with appropriate statistics. Mushrooms, yeasts, fungi, as well as algae have been used as foods or fermentation products all over the world for thousands of years, thus, these materials may not cause serious problems for human health. Indeed, curdlan, β-glucan without side chains from Gram-negative bacteria, has been applied as an ingredient of various foods for many years. The safety of curdlan has been approved by Food and Drug Administration in the US. Lentinan from *Lentinus edodes* [5] and Sonifilan (SPG) from *Schizophyllum commune* [6] were developed in the 1980s and have been used clinically for cancer therapy in Japan. β-Glucans derived from yeasts, such as *Saccharomyces cerevisiae* and *Candida albicans*, have been prepared and used for basic as well as clinical studies. Double blind clinical trials of yeast β-glucans have been examined extensively for cancer immunotherapy, as immunopotentiators for immunocompromised patients, and for lipid lowering effects since the 1990s [7–9]. Some clinical trials (Phase I Study) using β-glucan in combination with monoclonal antibodies were performed in the Memorial Sloan-Kettering Cancer Center in patients with metastatic neuroblastoma, relapsed CD20-positive lymphoma, leukemia, or post-transplant lymphoproliferative disease. We have prepared particulate and soluble β-glucans, OX-CA and CSBG, from *Candida* and analyzed molecular mechanisms [10].

An interesting and important concept from these basic as well as clinical studies is that the activities of β-glucans are very diverse in each case, thus it has been necessary to develop our molecular understanding of these compounds. SCG is a purified soluble β-glucan from a cold NaOH extract of *Sparassis crispa*, which is an edible mushroom. SCG is 6-branched 1,3-β-glucan, with one branch approximately every third main chain unit [11]. SCG and extracts from *S. crispa* show antitumor activity in tumor bearing mice and cancer patients in combination with lymphocyte transplantation immunotherapy [12–14]. SCG enhanced the hematopoietic response in cyclophosphamide-induced leukopenic mice when administered by the intraperitoneal (i.p.) and oral (p.o.) routes over a wide range of concentrations [15,16], and the effect was augmented by combination with isoflavone aglycone [17]. SCG stimulated leukocytes to produce cytokines in preparations of human peripheral blood mononuclear cells [18] and splenocytes from mice [19]. These results show that SCG could enhance immune responses *in vivo* and *in vitro*. The field of glucan research has been confounded by the presence of endotoxin in glucan preparations. Endotoxin was not detected in SCG by determination using endospecy (<30 pg/mg) [14], so SCG has also been used as purified soluble β-glucan in investigations on cellular receptors and molecular mechanisms [20,21].

In the process of research on the cytokine induction of SCG, we found that GM-CSF and dectin-1 play key biological

Table 1 Chemical diversity of glucans

Glucan	Source	Main components
Lentinan	*Lentinus edodes*	6-branched 1,3-β-glucan
Sonifilan (SPG)	*Schizophyllum commune*	Soluble 6-branched 1,3-β-glucan, having one branch chain every third main chain unit
Grifolan (GRN)	*Grifola frondosa*	6-branched 1,3-β-glucan, having one branch chain every third main chain unit
Zymosan	*Saccharomyces cerevisiae*	Crude cell wall extract containing 1,3-β-glucan, 1,6-β-glucan, mannan, and chitin
Yeast whole β-glucan particule (WPG)	*Saccharomyces cerevisiae*	Crude preparation 6-branched 1,3-β-glucan
SSG	*Sclerotinia sclerotiorum*	6-branched 1,3-β-glucan, having one branch chain every second main chain unit
Curdlan	*Alcaligenes faecalis*	Linar
Laminarin	*Laminoria digitata*	1,3-β-glucan, 1,6-β-glucan
Paramylon	*Euglena gracilis*	1,3-β-glucan
OX-CA	*Candida albicans*	Particle 1,3-β-glucan with long 1,6-β-glucan segments
CSBG	*Candida albicans*	Soluble 1,3-β-glucan with long 1,6-β-glucan segments
SCG	*Sparassis crispa*	Soluble 6-branched 1,3-β-glucan, having one branch chain every third main chain unit

roles in this activity. Our findings indicated that both GM-CSF production and dectin-1 expression are key factors in responsivity to β-glucan. In this review, we describe the identification of the key molecules related to the expression of the immunomodulating activities of β-glucan, and discuss how the response to β-glucan is controlled.

2. Strain differences in reactivity to β-glucan

Many animal experiments have demonstrated remarkable effects of β-glucans on a range of tumors. Several human clinical trials have also shown possible treatment benefits. However, there is still not much scientific evidence to explain the mechanisms of the actions of β-glucan or the differences of responsiveness to β-glucan in humans. The study of animal strain differences of reactivity to β-glucan is important for the clarification of the individual variation in humans. Research on the reactivity to β-glucan could provide clues for developing more effective cancer immunotherapies using β-glucans.

There are strain differences of the reactivity of mice to β-glucan, and that DBA/1 and DBA/2 mice are highly sensitive to β-glucan *in vitro* [19] and *in vivo* [22]. First, SCG-induced leukocytes from DBA/1 and DBA/2 mice produce high amounts of interferon-γ (IFN-γ) (Fig. 1), tumor necrosis factor-α (TNF-α), interleukin-12p70 (IL-12p70), and granulocyte macrophage–colony stimulating factor (GM-CSF) following SCG stimulation *in vitro* [19,23]. This fact surprised us, because generally the level of cytokine induction by pure soluble β-glucan from leukocytes in naïve mice was lower than that induced by other PAMPs. Actually, the induction of IFN-γ by SCG *in vitro* in leukocytes was not detected or was at a low level in almost all strains of mice [19]. Second, DBA/1 and DBA/2 mice also produce significantly higher titers of antibody to SCG than other inbred naive mice *in vivo* [22]. Previously we thought that β-glucans would be a weak immunogen in mice. Indeed, the titers of anti-SCG antibody were low in almost all strains of mice. On the other hand, in humans, almost all serum samples contain detectable concentrations of anti-β-glucan antibody, and the activity is modulated depending on the presence of infectious and some other diseases [24]. Such antibodies would be important for the recognition of β-glucan by Fc receptors and complement receptors. The alternative pathway of the complement system is also well known to be activated by β-glucan, and it functions as the initiation reaction of β-glucan-mediated activity. These findings suggest that DBA/2 and DBA/1 mice would be useful model mice for β-glucan studies.

The cytokine induction by SCG was not dependent on gender or breeding status [19]. These results suggest that DBA/1 and DBA/2 mice are highly sensitive to β-glucan in a hereditary manner. The genetic differences between DBA/1 and DBA/2 mice are probably too large to be accounted for by mutation, and are probably due to substantial residual heterozygosity following the crosses between substrains. DBA/1 and DBA/2 differ at least at the following loci: Car2, Ce2, Hc, H2, If1, Lsh, Tla, and Qa3. With such large differences, they should probably be regarded as different strains rather than substrains of the same strain. DBA strains are inbred strains widely used for immunological research *in vivo* and *in vitro*. Collagen-induced arthritis is a well-characterized model for human rheumatoid arthritis that was developed in DBA/1 mice [25]. As compared with the DBA strain from the viewpoint of complement (C), DBA/1 mice are C5a normal, and DBA/2 mice are C5-deficient, similar to AKR/N mice [26]. The MHC genotype restrains the antigen specificity of T cells, and major histocompatibility complex (MHC) restraint is a key feature in the antigen recognition mechanism of the T cells. Some diseases, such as collagen-induced arthritis, Crohn's disease, and ulcerative colitis, have certain susceptibility genes in the MHC region. The MHC haplotype in DBA/2 mice is H2d, the same as that in BALB/c mice, while that in DBA/1 mice is H2q. These findings show that there is no MHC restraint on the sensitivity to β-glucan. Since large immunological differences are observed between DBA/1 and DBA/2 mice, it might be not easy to identify the susceptibility gene to the β-glucan response by using these mice. C57BL/6 × DBA/2 F1-hybrid (BDF1) mice exhibited little to no IFN-γ production (Fig. 1) [23]. The titer of anti-SCG antibody in the sera from BDF1 mice was lower than that from DBA/2 mice (Fig. 2). These results indicate that the susceptibility to SCG is conferred as a recessive genetic trait. We believe that further analysis of the

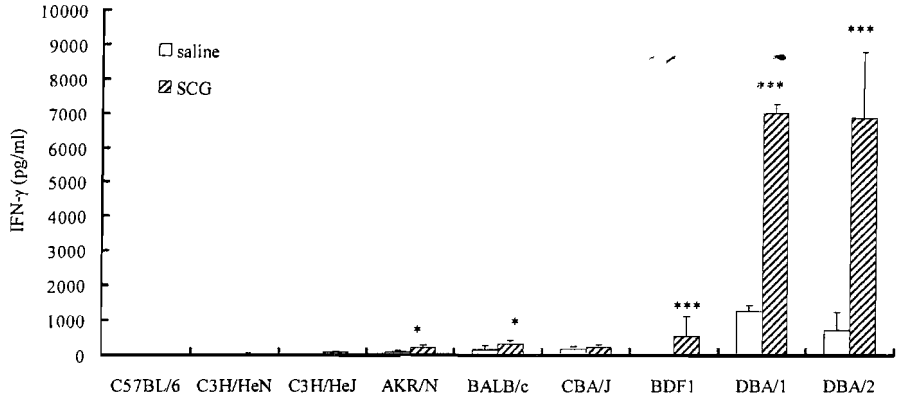

Figure 1 Cytokine production by splenocytes stimulated with SCG in various strains of mice. Splenocytes were isolated from C57BL/6, C3H/HeN, C3H/HeJ, AKR/N, BALB/c, CBA/J, BDF1, DBA/1, and DBA/2 mice. Cell suspensions were adjusted to 5×10^6 cells/ml in 10% FCS/RPMI medium and incubated for 48 h with SCG (100 μg/ml). After incubation, the supernatant was collected, and the concentration of IFN-γ in the supernatant was determined by ELISA. The data represent the mean of four samples, and were reproducible in three independent experiments. Significant difference from the control, $^*P<0.05$, $^{***}P<0.001$.

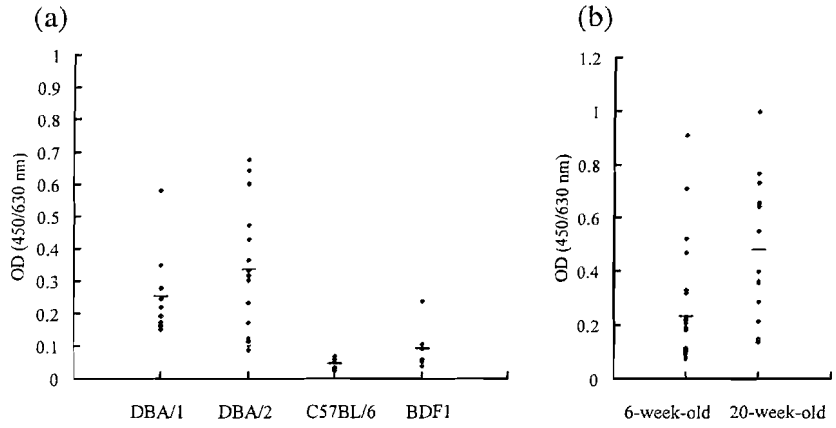

Figure 2 Anti-SCG antibody of sera from various strains of mice. The sera were obtained from naive 8 week-old DBA/1, DBA/2, C57BL/6, and BDF1 mice (a), and 6-week-old and 20-week-old DBA/2 mice (b). One-thousand-fold diluted sera were prepared and applied to the SCG-pre-coated plate (25 μg/ml). Binding of Ig was detected using peroxidase-conjugated anti-mouse IgG + IgM. Enzyme activity was measured by the addition of TMB substrate. Horizontal lines show mean absorbance.

homologous genes between DBA/1 and DBA/2 could clarify which genes control the susceptibility to β-glucan. The titers of anti-SCG antibody in individual mice differ significantly. The level of IFN-γ induction by SCG [19] and the titer of anti-SCG antibody (Fig. 2) are also dependent on the age of mice. If the SCG-responsiveness of DBA/1 and DBA/2 mice were due to only genetic factors, the phenotype might not show these differences. Environmental factors, including feeding and microbial flora, are expected to be important factors.

In the process of studying the mechanism of cytokine induction by SCG in DBA/1 and DBA/2 mice, we discovered that GM-CSF is one of the key factors in the reactivity to β-glucan [23]. As described in detail below, GM-CSF induction by SCG is the specific key step for the induction of other cytokines in DBA/2 mice *in vitro*. In fact, neutralizing GM-CSF in splenocyte cultures significantly inhibited the production of these cytokines elicited by SCG in DBA/2 mice. The splenocytes in various strains of mice show similar patterns of production of cytokines in response to SCG treatment in the presence of recombinant GM-CSF. The reason for the high sensitivity to β-glucan shown by DBA/1 and DBA/2 mice may be related to differences of their regulation of GM-CSF compared with that in other mice. GM-CSF is a 23 kDa glycoprotein known to be a hematopoietic growth factor required for the proliferation and survival of hematopoietic cells committed to granulocytic and macrophage cell lineages and myeloid leukemic cells [27]. It is also required for the differentiation of these cells into neutrophilic or eosinophilic granulocytes, macrophages, bone marrow macrophages, or dendritic cells [27–30]. In addition to these physiological roles, a growing body of evidence indicates that GM-CSF has important functions in host responses to external stimuli and inflammatory/autoimmune conditions [31]. Numerous studies in rodents have indicated effects of GM-CSF against many types of bacteria and fungi [32]. The role of GM-CSF in protection against fungal infection is thought to be related to the enhancement of reactivity to β-glucan by GM-CSF. GM-CSF-deficient mice develop normally and show no major perturbation of hematopoiesis. On the other hand, studies with GM-CSF-deficient mice showed that GM-CSF action in the steady state was important for alveolar macrophage maturation in mice [33]. β-Glucan appears to be responsible at least in part for the stimulation of GM-CSF production by fungal infection in the lung [34]. These results imply a strong relationship between alveolar macrophages and β-glucan in fungal infections in the lung. Willment et al. [35] reported that the expression and function of the β-glucan receptor, dectin-1, are enhanced in GM-CSF-treated macrophages. These findings suggest that the level of expression of β-glucan receptor on leukocytes regulated by GM-CSF would modulate the responsiveness to β-glucan. These functions of GM-CSF would enhance the reactivity to β-glucan. However, it has not yet been clarified how GM-CSF enhances the response to β-glucan. Further analysis of the role of GM-CSF in the response to β-glucan would help to clarify the details of the mechanism of the response to β-glucan.

3. Characterization of cytokine induction by β-glucan in vitro

The structural features of glucans, including the primary structure, solubility, degree of branching, conformation and molecular weight (MW), could be dependent on both the source and methods of isolation. All these factors play an important role in various kinds of glucan-associated biological activities [2]. The method of preparation of the extract fractions from *S. crispa* is related to the structural features of these fractions, and the immunomodulating activities *in vivo* and *in vitro* are significantly modulated by the method of preparation [14]. In general, *in vitro* studies have suggested that high MW or particulate β-glucans, such as OX-CA and zymosan, can directly activate leukocytes, inducing macrophages, to produce several mediators, including inflammatory cytokines IL-1, IL-6, TNF-α, and nitric oxide (NO) [36,37]. Low MW or soluble β-glucans, such as SPG, possess biological activity *in vivo*, but their cellular effects are less clear. In understanding the molecular mechanisms underlying the cellular effects of soluble β-glucans e.g. SCG, will help clarify the mechanism of its biological activity *in vivo*.

As mentioned above, the splenocytes from naive DBA/1 and DBA/2 mice were potently stimulated by SCG to produce IFN-γ, TNF-α, GM-CSF, and IL-12p70. Polymyxin B is well known to form an inactive complex of LPS *in vitro*. The result that treatment with Polymyxin B did not affect cytokine induction by SCG showed that the reaction was β-glucan specific. GM-CSF induction by SCG is the specific step up-regulated in DBA/2 mice *in vitro* and accounts for the enhancement of cytokine induction by SCG. The mechanism of cytokine induction by SCG in DBA/2 mice is, in part as follows (Fig. 3): SCG induces GM-CSF production through cell–cell contact mediated by the association of $CD4^+$ T cell-expressed LFA-1 and APC-expressed ICAM-1 molecules. GM-CSF enhances the response to SCG. TNF-α contributes to inducing sufficient release of GM-CSF. In the presence of a sufficient concentration of GM-CSF, SCG directly induces adherent cells to produce TNF-α and IL-12p70. IFN-γ induction requires both IL-12p70 and the cell–cell contact mediated by the association of ICAM-1–LFA-1. GM-CSF production mediated through ICAM-1 and LFA-1 interaction triggers further cytokine induction by SCG, while reciprocal actions of the cytokines are essential for enhancement of the overall response to SCG in DBA/2 mice.

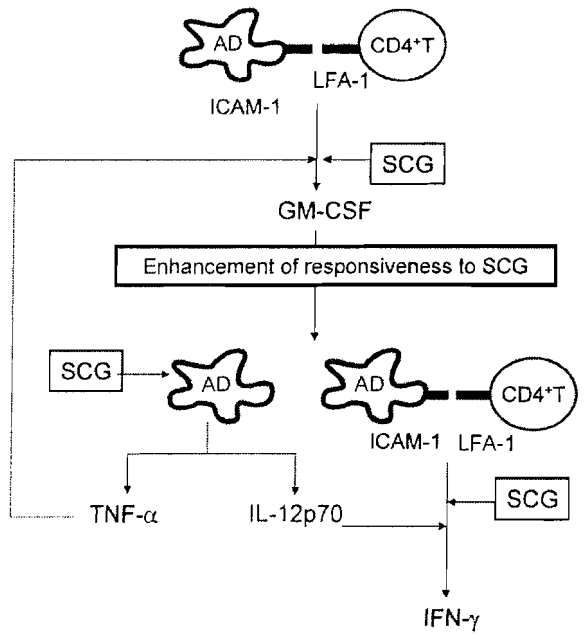

Figure 3 A model for the mechanism of cytokine induction by SCG in DBA/2 mice. Based on the sum of our findings, part of the mechanism of cytokine induction by SCG in DBA/2 mice is as follows: SCG induces GM-CSF production through cell–cell contact mediated by the association of $CD4^+$ T cell-expressed LFA-1 and APC-expressed ICAM-1 molecules. GM-CSF enhances the response to SCG. TNF-α contributes to the production of a sufficient concentration of GM-CSF. In the presence of sufficient concentrations of GM-CSF, SCG directly induces adherent cells to produce TNF-α and IL-12p70. IFN-γ induction requires IL-12p70 and the cell–cell contact mediated by the association of ICAM-1–LFA-1. GM-CSF production mediated through ICAM-1 and LFA-1 interaction triggers further cytokine induction by SCG, while reciprocal actions of cytokines are essential for the enhancement of the overall response to SCG in DBA/2 mice.

Various kinds of leukocytes in the spleen take part in this series of steps involved in cytokine induction by SCG, and the effects on these leukocytes are mediated through cell–cell contact and soluble factors. First, cell–cell contact between adherent cells and T cells was required for the induction of GM-CSF and IFN-γ by SCG. On the other hand, SCG directly induced adherent cells to produce TNF-α and IL-12p70. The finding that B cell depletion from splenocytes did not affect the induction of these cytokines by SCG suggested that B cells and specific antibodies against SCG are not be involved in the mechanism of cytokine induction by SCG. IFN-γ production was not induced by SCG in splenocytes from nu/nu mice. Blocking of CD4 using an antibody inhibited the induction of GM-CSF and IFN-γ elicited by SCG in splenocytes from DBA/2 mice, but blocking of CD8 had no effect on cytokine induction (Fig. 4). These results suggest that $CD4^+$ T cells may play an important role in cytokine induction by SCG. Cell–cell interactions mediated by the engagement of adhesion molecules, such as ICAM-1, B7.1/B7.2, and CD40, on adherent cells and their ligands, such as LFA-1, CD28, and CD40L, on T/natural killer cells, are involved in the production of IL-12, TNF-α, and IFN-γ in acquired and innate immune responses [38]. Blocking of ICAM-1 and LFA-1 significantly inhibited the induction of GM-CSF and IFN-γ elicited by SCG in splenocytes from DBA/2 mice. ICAM-1, a 95 kDa member of the Ig superfamily found on lymphocytes, vascular endothelium, high endothelial venules, epithelial cells, macrophages, and DC, is a ligand for LFA-1 [39]. The optimal activation of T cells in many responses may also require the interaction of T cells expressing LFA-1 with ICAM-1 or ICAM-2 molecules on the surface of APCs [40–42]. Immature DCs owe part of their remarkable antigen-independent T cell stimulatory ability to chemokines and ICAM-1 [42]. These findings suggest that cell–cell contacts mediated by the association of APC-expressed ICAM-1 and $CD4^+$ T cell-expressed LFA-1 molecules is an essential step in the induction of GM-CSF and IFN-γ by SCG.

Next, soluble factors contribute to the cytokine induction by SCG in DBA/2 mice. Cytokines are mutually and deeply influenced by a complex network of cytokines. TNF-α and IL-12 as well as GM-CSF are required for cytokine induction by SCG. Neutralizing TNF-α significantly inhibited the induction of GM-CSF, IFN-γ, and IL-12p70 elicited by SCG. On the other hand, in the presence of sufficient GM-CSF in the splenocyte culture medium, blocking of TNF-α had no influence on cytokine induction by SCG. These results suggested that the TNF-α induced by SCG could enhance GM-CSF induction, and thereby promote IFN-γ and IL-12p70 induction. The finding that the expression of adhesion molecules, including ICAM-1, was up-regulated upon stimulation by inflammatory mediators, such as TNF-α [43], suggests that TNF-α promoted GM-CSF induction by SCG through the enhancement of adhesion molecule expression. Interleukin-12 (IL-12) production is also important for this activity. IL-12 stimulates the production of IFN-γ from T cells, NK cells, B cells, and even antigen-presenting cells [44,45]. Even if a high level of GM-CSF was present, blocking of IL-12 inhibited IFN-γ induction of SCG, in contrast to the case of TNF-α. These results suggest that IL-12 production is essential for the induction of IFN-γ by SCG.

There were different mechanisms for the induction of each cytokine by SCG in DBA/2 mice. The fact that the concentration of SCG required for GM-CSF induction was

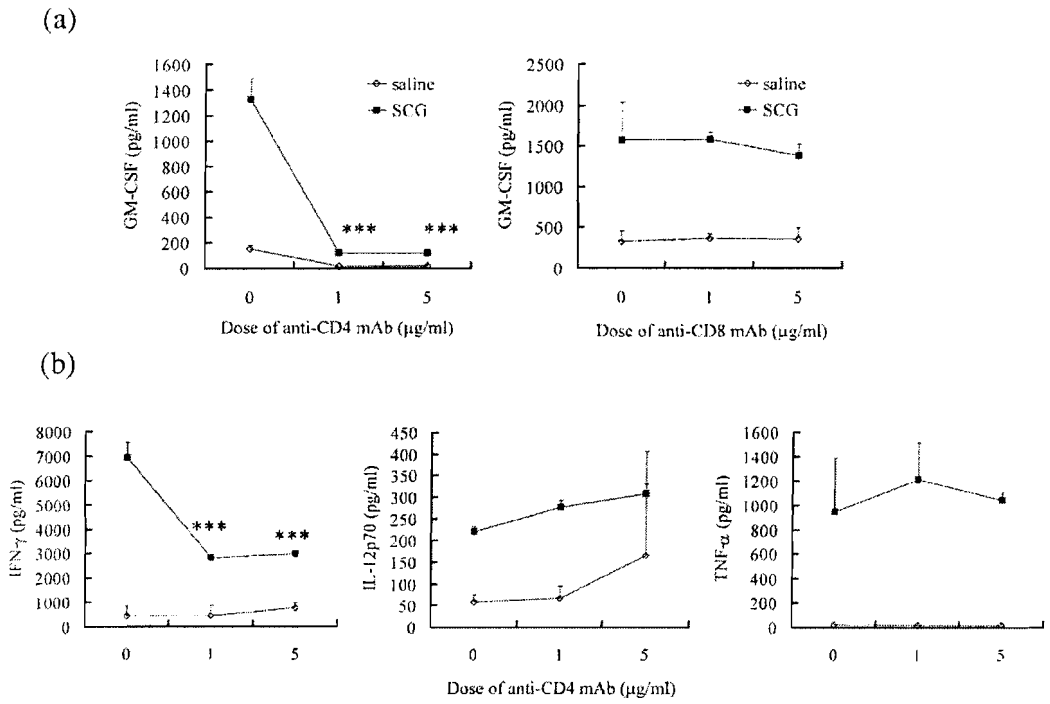

Figure 4 Cytokine production by splenocytes stimulated with SCG in DBA/2 mice in the presence of anti-CD4 mAb. Splenocytes were isolated from DBA/2 mice. (a) Cell suspensions were adjusted to 5×10^6 cells/ml in 10% FCS/RPMI medium and incubated for 48 h with SCG (100 μg/ml) in the presence of anti-CD4 mAb (BD Biosciences) or anti-CD8 mAb (BD Biosciences) (1 or 5 μg/ml). After incubation, the supernatant was collected, and the concentration of GM-CSF in the supernatant was determined by ELISA. (b) Cell suspensions were incubated for 48 h with SCG (100 μg/ml) in the presence of anti-CD4 mAb (1 or 5 μg/ml) and rmGM-CSF (1 ng/ml). After incubation, the supernatant was collected, and the concentrations of IFN-γ, IL-12p70, and TNF-α in the supernatant were determined by ELISA. The data represent the mean of four samples, and were reproducible in three independent experiments. Significant difference from the control, ***$P<0.001$.

higher than that required for TNF-α, IFN-γ, and IL-12p70 induction suggests that the receptor involved in GM-CSF induction had low affinity, while the receptor(s) regulated by GM-CSF had high affinity. We have not reported the results of the analysis of functional receptors involved in the cytokine induction of SCG in DBA/2 mice yet. In future studies, much effort will be required to precisely characterize the molecular mechanisms of β-glucan signaling in DBA/2 mice.

4. Functional receptor for β-glucan

Evidence for β-glucan receptors first came from the demonstration that nonopsonic zymosan recognition by human monocytes was β-glucan dependent. However, it should be noted that some leukocyte populations also express other nonopsonic receptors for zymosan, such as mannose binding lectins [46]. β-Glucan receptor activity has subsequently been reported in a variety of other leukocytes, including macrophages, neutrophils, eosinophils, and NK cells. Nonopsonic recognition of β-glucans by these cells has been ascribed to multiple receptors [47], and indeed a number of β-glucan receptors have been identified, including CR3, lactosylceramide, scavenger receptors, and dectin-1. CR3 is a heterodimeric integrin receptor, consisting of the αm (CD11b) and β2 (CD18) chains and recognizes microbial cells and adhesion molecules. It is expressed mainly on neutrophils, monocytes, and NK cells, and less is present on macrophages [48]. CR3 functions as an adhesion molecule through recognition of endothelial ICAM-1, and as a phagocytic receptor for iC3b-opsonized particles, including opsonized particulate glucans. CR3 also possesses a lectin domain, which maps to a site C-terminal to the I-domain, and recognizes selected monosaccharides and a variety of β-glucans. Therapy failures of β-glucan treatment in C3- and CR3-deficient mice confirmed the requirement for iC3b on tumors and CR3 on leukocytes [49]. Therapeutic efficacy could be enhanced significantly by injecting mice with complement-activating antitumor mAbs that greatly increased the amount of tumor-bound iC3b. However, the efficacy of combining glucan with mAbs was not observed in mice deficient in either C3 or CR3 [50]. Lactosylceramide (LacCer; CDw17) is a glycosphingolipid found in the plasma membranes of many cells and was identified as a β-glucan receptor from biochemical analyses of the interactions between PGG-glucan and isolated human leukocyte membrane components [51]. It has been suggested that the interaction of β-glucan with this receptor can induce macrophage inflammatory protein (MIP)-2 and the activation of NFκB and can enhance the neutrophil oxidative burst and antimicrobial functions. β-1,6-long glucosyl side-chain-branched β-glucan, isolated from *C. albicans* (CSBG), but not SCG, binds to LacCer with a terminal galactose residue, and induces neutrophil migration through the activation of a Src family kinase/PI-3K/heterotrimeric G-protein signal transduction pathways [20].

Recently we and others demonstrated that dectin-1, which is a C-type lectin, plays a crucial role in the detection of β-glucan and live pathogenic fungi (*C. albicans*, *Aspergillus fumigatus*, *Pneumocystis carinii*) by macrophages and DCs and in the host defense mechanism against fungi by acting as a key coordinator of antimicrobial responses [21,52–55]. Cytokine induction by SCG was completely abolished in dectin-1 knockout BMDCs. In addition, DBA/2 mice highly express dectin-1 on BMDCs as compared with other strains of mice (in preparation). Yoshitomi et al. [56] reported that β-glucans could trigger arthritis in SKG mice to various degrees, and that blockade of dectin-1 could prevent SKG arthritis triggered by β-glucans. We confirmed the induction of arthritis in SKG mice by *Candida*-derived β-glucan: CSBG and OX-CA [57]. These results indicated that dectin-1 is one of the important β-glucan receptors in the activation of immune responses.

The binding of ligands to dectin-1 activates several signaling pathways to promote phagocytosis, ROS production, and cytokine production [58,59]. Dectin-1 signals through a 'hemITAM' motif, an immunoreceptor tyrosine-based activation motif-like sequence containing a single 'YxxL' motif, which becomes phosphorylated by Src family kinases after receptor engagement. This allows recruitment of the spleen tyrosine kinase Syk, which then activates down-stream signaling components, including the transcription factor NF-κB [60]. Activation of NF-κB by dectin-1 requires the CARMA1-related adaptor protein CARD9, which binds the adaptors MALT1 and Bcl-10 and promotes activation of the IKK kinase complex [61]. Leibundgut et al. [62] recently reported that dectin-1-Syk-CARD9 signaling promoted DC maturation and induced the secretion of proinflammatory cytokines, including IL-23, but little IL-12, and demonstrated that DCs activated by dectin-1 engagement strongly biased Th cell differentiation to a Th-17 fate. The engagement of human DC-SIGN, which is a C-type lectin, by *Mycobacterium tuberculosis* alters TLR-induced cytokine responses [63]. Some studies demonstrated that dectin-1 collaborates with TLRs, followed by activation of transcription factor NF-κB via MyD88, in the induction of proinflammatory cytokines. However, it remains unclear to what extent dectin-1, as well as DC-SIGN, signal directly or simply act as TLR coreceptors. Many of the published studies regarding β-glucan-mediated signal transduction were performed using commercially available β-glucan preparations, such as zymosan, a polysaccharide particle from the cell wall of *S. cerevisiae*. However, we have already shown that this zymosan is very crude and commonly contains other components, including mannans, other glucans, and chitins (Table 1) [64]. Cytokine induction by zymosan was significantly reduced by the lack of MyD88, but SCG-induced cytokines were not affected at all and cytokine induction by SCG was completely abolished in dectin-1 knockout BMDCs [21]. These findings indicate that cytokine induction via dectin-1 signaling is independent of MyD88.

A β-glucan from *C. albicans*, OX-CA, is also used as a particle β-glucan in investigations of cellular receptors and molecular mechanisms [21]. OX-CA, as well as SCG, stimulates splenocytes in DBA/2 mice to produce cytokines (Fig. 5). Interestingly, cytokine production was also induced by OX-CA, but not by SCG, in splenocytes from C57BL/6 mice. It seems doubtful that all the pathways of cytokine induction by OX-CA in the spleen would be simply mediated through GM-CSF, like the pathways induced by SCG. In the research on cytokine induction by soluble β-glucan, SCG greatly clarified both the basis of the specific features of DBA/1 and DBA/2 mice and the mechanisms of reactivity to β-glucan mediated through GM-CSF. Dectin-1 plays a crucial role in the detection and expression of biological activities of both of SCG and OX-CA [21]. These findings suggest that the pattern of association of dectin-1 with other receptors, such as CR3, could be different between soluble β-glucan e.g. SCG and particle β-glucan e.g. OX-CA. Differences of cell signaling through association with dectin-1 versus other receptors could explain the basis of the structure–activity relationship.

5. External regulation of reactivity to SCG by GM-CSF and dectin-1

The high doses of cyclophosphamide (CY) that are required for effective chemotherapy lead to immunosuppression, a property that is the basis for its other clinical uses, such as preventing graft-versus-host disease in bone marrow transplantation, minimizing chances of organ rejection in organ transplantation, and treatment of autoimmune diseases such as Wegener's granulomatosis and rheumatoid arthritis [65]. It is well established that many chemotherapeutic drugs actively suppress cell-mediated immunity. SCG enhanced the hematopoietic response in CY-induced leukopenic mice when administered by intraperitoneal (i.p.) and oral (p.o.) routes. IL-6 production in spleen, bone marrow, and peritoneal exudate cells (PECs) was significantly increased by SCG in CY-treated mice *in vivo*. IL-6 is a multifunctional hematopoietic cytokine

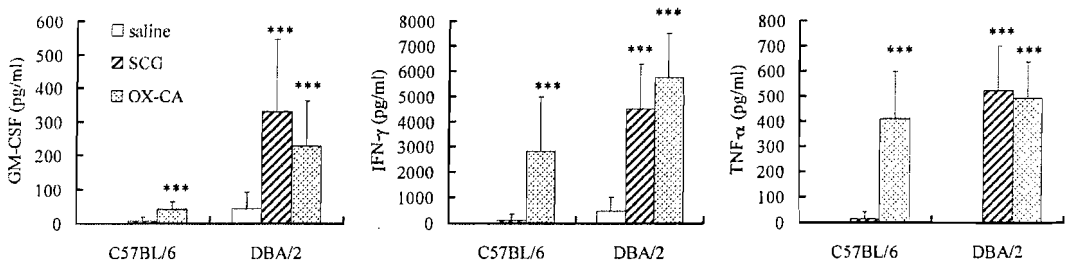

Figure 5 Cytokine production by splenocytes stimulated with soluble β-glucan (SCG) or particle β-glucan (OX-CA) derived from *Candida albicans*. Splenocytes were isolated from C57BL/6 and DBA/2 mice. Cell suspensions were adjusted to 5×10^6 cells/ml in 10% FCS/RPMI medium and incubated for 48 h with SCG (100 μg/ml) or OX-CA (1 μg/ml). After incubation, the supernatant was collected, and the concentrations of GM-CSF, IFN-γ, and TNF-α in the supernatant were determined by ELISA. The data represent the mean of six samples. Significant difference from the control, ***$P<0.001$.

involved in such processes as B cell differentiation, T cell activation, induction of acute phase proteins, and reduction of the G0-residence time of hematopoietic stem cells [66]. These findings indicate that at least a part of the hematopoietic activity of SCG could be explained by its induction of IL-6. Production of cytokines such as IL-6, IFN-γ, IL-12p70, TNF-α, and GM-CSF by splenocytes of CY-treated mice was enhanced by the addition of SCG, while these cytokines were not induced by SCG in splenocytes from control mice *in vitro* [67]. These results suggest that the number of cells responsive to SCG was increased in CY-treated mice. Interestingly, GM-CSF production in splenocytes from CY-treated mice was higher than that in normal mice regardless of SCG stimulation. Neutralization of GM-CSF significantly inhibited the induction of IFN-γ, TNF-α, and IL-12p70 by SCG in CY-treated mice. These results suggest that GM-CSF plays an important role in cytokine induction by SCG in CY-treated mice as well as DBA/2 mice. On the other hand, the expression of CR3 and dectin-1 was up-regulated by CY treatment. Blocking dectin-1 significantly inhibited the induction of TNF-α and IL-12p70 by SCG. Taken together, these results suggest that enhancement of the levels of endogenous GM-CSF production and dectin-1 expression were involved in the high sensitivity to SCG in CY-treated mice.

CY displays a range of contrasting immunomodulatory properties, which vary mainly according to the treatment dose. We attempted to control the reactivity to SCG by modulating the endogenous GM-CSF level by administering various doses of CY. Administration of CY (50 or 100 mg/kg) induced the splenocytes to produce GM-CSF regardless of SCG stimulation, and to produce elevated levels of IFN-γ, TNF-α, and IL-12p70 in response to SCG stimulation. On the other hand, treatment with a high dose of CY (200 mg/kg) resulted in neither endogenous GM-CSF production nor cytokine induction by SCG. These results show that the level of cytokine induction by SCG is regulated by the amount of endogenous GM-CSF produced in response to CY treatment. This finding suggests that changing the dose of CY could enable modulation of the reactivity to SCG and therefore of cytokine induction by SCG. The pattern of induction was different for each cytokine; for example, the production of TNF-α and IL-6 was significantly increased by SCG from day 4 to day 7, while that of IFN-γ and IL-12p70 was significantly increased from day 5 to day 7 (Fig. 6). Endogenously produced GM-CSF was detected from day 4 to day 7. TNF-α and IL-12p70 were induced by SCG in adherent splenocytes alone. On the other hand, IFN-γ induction by SCG required adherent cell–T cell interaction. Leukocyte recovery in CY-treated mice was different among cell populations, such as granulocytes, macrophages, natural killer cells, B cells, and T cells. These results suggest that cell population changes in CY-treated mice may have accounted in part for the differences of the pattern of induction among cytokines from the kinetic point of view. The thymus is known to atrophy in response to CY-administration, due to the apoptosis of thymocytes. Hiramine et al. [68] and our group [15] showed a marked decrease in the proportion of the $CD4^+CD8^+$ subset in the thymus of mice on day 2 after CY treatment, while this subset rebounded on day 7. These results suggest that the suppression of the supply of mature T cells from the thymus to the spleen could inhibit the IFN-γ induction by SCG in CY-treated mice on day 4. On the other hand, the addition of rmGM-CSF to a splenocyte culture system had no influence on the induction of IL-6 by SCG [23]. 1,3-β-Glucan induced macrophages to produce IL-6 [37]. The numbers of macrophages and granulocytes were significantly augmented in CY-treated mice [15]. In addition, IL-6 production in PECs was increased by SCG when rmGM-CSF was added to the culture medium (Fig. 7). These findings suggest

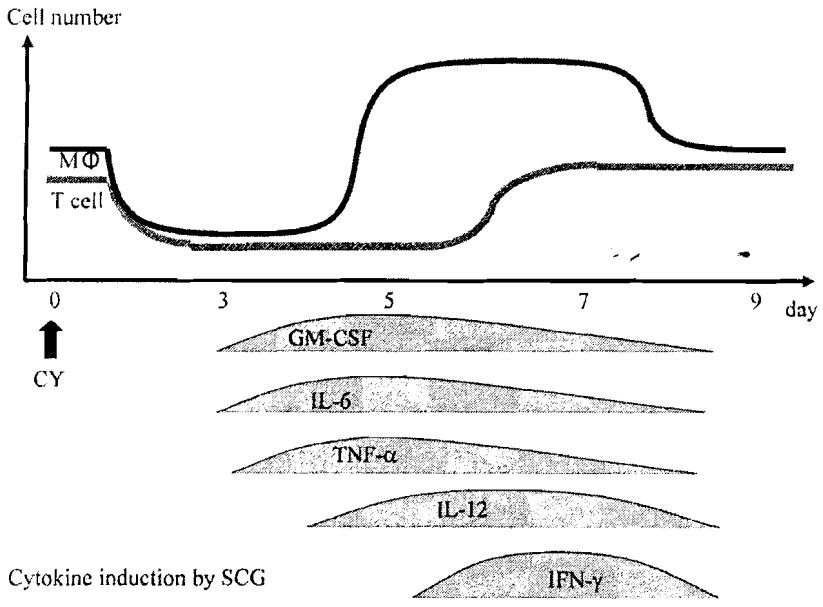

Figure 6 Kinetics of cell population recovery of macrophages (MΦ) and T cells after CY treatment and cytokine induction by SCG. The populations of macrophages (MΦ) and T cells were markedly decreased in splenocytes of mice after CY treatment. The number of MΦ recovered from day 4. The recovery of the T cell population was delayed. Endogenously produced GM-CSF was detected from day 4 to day 7. The production of TNF-α and IL-6 was significantly increased by SCG from day 4 to day 7, while that of IFN-γ and IL-12p70 was significantly increased from day 5 to day 7.

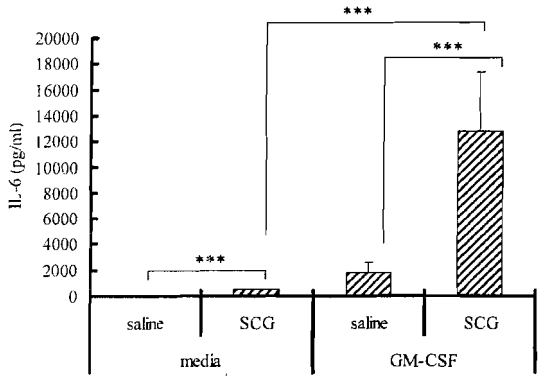

Figure 7 Cytokine production by PECs stimulated with SCG in C57BL/6 mice in the presence of rmGM-CSF. PECs were isolated from C57BL/6 mice. Cell suspensions were adjusted to 1×10^6 cells/ml in 10% FCS/RPMI medium and incubated for 48 h with SCG (100 μg/ml) in the presence of rmGM-CSF (BD Biosciences) (1 ng/ml). After incubation, the supernatant was collected, and the IL-6 concentration in the supernatant was determined by ELISA. The data represent the mean of six samples. Significant difference from the control, ***$P<0.001$.

that the number and/or the characteristics of macrophage lineage cells could be related to IL-6 induction by SCG. These findings also confirm that the pattern of cytokine induction by SCG in the CY-treated mice is remarkably influenced both qualitatively and quantitatively by the appearance of SCG-responsive cells according to the amount of endogenous GM-CSF and the cell population changed by CY treatment. Thus, external regulation using agents that modulate GM-CSF production or dectin-1 expression, such as CY, could control the reactivity to β-glucan and consequently the expression of various biological activities.

6. Going back to mushrooms/yeasts as functional foods

Many animal studies have demonstrated the antitumor activities of β-glucans derived from certain mushrooms/yeasts. As an immunostimulation agent, which acts through the activation of macrophage and NK cell cytotoxicity, β-glucan can inhibit tumor growth at the promotion stage. Lentinan from *L. edodes* [5] and Sonifilan (SPG) from *S. commune* [6] are approved in Japan for clinical use in human cancer treatment. There have been randomized controlled studies on the use of β-glucan in cancer treatment. These studies mainly used SPG and lentinan on stomach cancer. Glucan administered to cancer patients can enhance the effect of anticancer chemotherapy or radiation therapy and has positive effects on the survival and quality of life of cancer patients. In mice, SPG-mediated antitumor activity was inhibited by blocking of dectin-1 by using anti-dectin-1 antibodies [69]. This result indicated that dectin-1 effects β-glucan-mediated antitumor activity in mice. Immunotherapy using monoclonal antibodies is a novel strategy for cancer treatment. In mice with established subcutaneous non-Hodgkin's lymphoma xenografts, a combination of intravenous complement-activating antibody, rituximab, and WPG from yeast had a higher therapeutic efficacy than treatment with any of these alone [70]. WPG potentiated the activity of antitumor monoclonal antibodies, leading to enhanced tumor regression and survival, but not in CR3 knockout mice [50].

There have been numerous reports of immune modulation associated with oral ingestion of mushroom β-glucans. Orally administered WPG showed antitumor activity in the combination with antitumor monoclonal antibodies [50]. Tsukada et al. [71] reported that the number of intraepithelial lymphocytes (IEL) in the intestine was increased by oral administration of β-glucan. In combination with CY, oral administration of β-glucan extracted from *S. crispa* with hot water enhanced survival longer than CY alone (in preparation). It must be assumed that orally ingested β-glucans interact with either intestinal epithelial cells and/or intestinal DC, ultimately resulting in the priming or activation of other immune cells. However, the mechanisms of the antitumor activities of β-glucan through mucosal immunity appear to be complex. In addition, individual β-glucans differ in their effectiveness as immunomodulators. These aspects of β-glucan encourage us to make extensive investigations on the mechanisms of the antitumor activities of β-glucan.

7. Conclusion

Comparative genome analysis is a powerful tool for understanding biological function. Approximately 99% of mouse genes have a homologue in the human genome. Thus, the mouse system for genetic research can provide a model for human physiology and disease, and has led to major discoveries in such fields as immunology. The study of mouse strain differences of reactivity to β-glucan is important for the clarification of individual variations in humans. We believe that further analysis of the homologous genes between DBA/1 and DBA/2 mice will help identify the genes that control the susceptibility to β-glucan.

In this review, we demonstrate that GM-CSF and dectin-1 are very important factors for the response to β-glucan. In the highly responsive strains of mice (DBA/1 and DBA/2), high levels of GM-CSF and dectin-1 expression are observed. While the β-glucan response is influenced by genetic factors, it can also be regulated by external conditions. In fact, control of the levels of GM-CSF and dectin-1 could modulate β-glucan reactivity. Approaches to control the levels of GM-CSF and dectin-1 might improve the therapeutic efficacy of cancer immunotherapies using β-glucan. The mechanism of the response to β-glucan could also provide clues about the role of β-glucan in fungal infection.

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