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## Review

# Medicinal importance of fungal $\beta$ -(1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-glucans

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### ABSTRACT

Non-cellulosic  $\beta$ -glucans are now recognized as potent immunological activators, and some are used clinically in China and Japan. These  $\beta$ -glucans consist of a backbone of glucose residues linked by  $\beta$ -(1  $\rightarrow$  3)-glycosidic bonds, often with attached side-chain glucose residues joined by  $\beta$ -(1  $\rightarrow$  6) linkages. The frequency of branching varies. The literature suggests  $\beta$ -glucans are effective in treating diseases like cancer, a range of microbial infections, hypercholesterolaemia, and diabetes. Their mechanisms of action involve them being recognized as non-self molecules, so the immune system is stimulated by their presence. Several receptors have been identified, which include: dectin-1, located on macrophages, which mediates  $\beta$ -glucan activation of phagocytosis and production of cytokines, a response co-ordinated by the toll-like receptor-2. Activated complement receptors on natural killer cells, neutrophils, and lymphocytes, may also be associated with tumour cytotoxicity. Two other receptors, scavenger and lactosylceramide, bind  $\beta$ -glucans and mediate a series of signal pathways leading to immunological activation. Structurally different  $\beta$ -glucans appear to have different affinities toward these receptors and thus generate markedly different host responses. However, the published data are not always easy to interpret as many of the earlier studies used crude  $\beta$ -glucan preparations with, for the most part, unknown chemical structures. Careful choice of  $\beta$ -glucan products is essential if their benefits are to be optimized, and a better understanding of how  $\beta$ -glucans bind to receptors should enable more efficient use of their biological activities.

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## Introduction

Non-cellulosic  $\beta$ -glucans are recognized as potent immunological stimulators in humans and some are now used clinically in China and Japan. These  $\beta$ -glucans appear to possess potential for treating several diseases. About half the mass of the fungal cell wall consists of  $\beta$ -glucans (Seviour *et al.*

1992; Klis *et al.* 2001; McIntosh *et al.* 2005), but many are also excreted into the growth medium, making their recovery, purification and chemical characterization much simpler (Seviour *et al.* 1992; Schmid *et al.* 2001). Their homeostasis is maintained by  $\beta$ -glucan synthases and  $\beta$ -(1  $\rightarrow$  3) or  $\beta$ -(1  $\rightarrow$  6)-glucanase activities (Pitson *et al.* 1996a, 1996b; Jayus *et al.* 2004; Martin *et al.* 2006). Inhibition of  $\beta$ -glucan synthases by

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compounds like caspofungin, although expensive, has been successful in treating *Aspergillus* spp. and *Candida* spp. infections in humans (Walsh et al. 2004).

Non-cellulosic  $\beta$ -glucans consist of a backbone of glucose residues usually joined by  $\beta$ -(1 $\rightarrow$ 3) linkages, to which glucose side-chain residues are often attached, as shown in examples in Fig 1 (Schmid et al. 2001; McIntosh et al. 2005). In some  $\beta$ -glucans no side-chain substitution occurs, as with the bacterial  $\beta$ -glucan, curdlan, which contains only  $\beta$ -(1 $\rightarrow$ 3)-glucosidic linkages (McIntosh et al. 2005). No unbranched  $\beta$ -(1 $\rightarrow$ 3) or  $\beta$ -(1 $\rightarrow$ 6) fungal  $\beta$ -glucans are known, although the extent of the side-chain substitutions can vary considerably (Schmid et al. 2001). For example, many including schizophyllan (also named sizofiran) from *Schizophyllum commune*, and scleroglucan from *Sclerotium glaucanicum* both have a  $\beta$ -(1 $\rightarrow$ 3)-linked backbone, with on average one  $\beta$ -(1 $\rightarrow$ 6)-glucose substitution every three backbone residues (Johnson et al. 1963; Kikumoto & Kimura 1971). Epiglucan from *Epicoccum nigrum* also has a backbone of  $\beta$ -(1 $\rightarrow$ 3)-linked glucose residues, but now with two  $\beta$ -((1 $\rightarrow$ 6) substitutions on average every three residues, while the  $\beta$ -(1 $\rightarrow$ 3)-linked backbone of lentinan (also called SPG) from *Lentinus edodes* has two  $\beta$ -(1 $\rightarrow$ 6) side chains every five residues (Sasaki & Takasuka 1976; Schmid et al. 2001). Pestalotan produced extracellularly by *Pestalotia* sp. 815 has a  $\beta$ -(1 $\rightarrow$ 3)-linked backbone, but with three  $\beta$ -(1 $\rightarrow$ 6) side chains every five residues (Misaki et al. 1984). Many other fungal  $\beta$ -glucans have been described, but their detailed structures and branching frequencies are still mostly unclear (Seviour et al. 1992). It appears that to be effective these glucans must contain  $\beta$ -(1 $\rightarrow$ 3) or  $\beta$ -(1 $\rightarrow$ 6) linkages, but little else is known about how frequencies of branching and other chemical and physical properties determine their effectiveness, as discussed later (Demleitner et al. 1992). Examples of some non-cellulosic fungal  $\beta$ -glucans of possible medical importance are listed in Table 1.

Walls of pathogenic fungi also contain  $\beta$ -(1 $\rightarrow$ 3) or  $\beta$ -(1 $\rightarrow$ 6)-glucans (Taylor et al. 2007; Saijo et al. 2007). Although (for practical reasons) these are not applied clinically, they are probably important in eliciting anti-fungal host responses. However, which wall component is the stimulator/s is not always clear, and it is also not clear whether all fungi behave in the same mechanistic way. For example, *A. fumigatus* synthesizes a  $\beta$ -(1 $\rightarrow$ 3)-glucan with 4%  $\beta$ -(1 $\rightarrow$ 6) branches and a linear  $\beta$ -(1 $\rightarrow$ 3),(1 $\rightarrow$ 4)-glucan (Fontaine et al. 2000) and both stimulate immune responses in mice (Hohl et al. 2005). Yet the role of *Candida albicans* cell wall  $\beta$ -glucans appears uncertain. Evidence suggest some competition with laminarin (Netea et al. 2006), which, although a  $\beta$ -glucan and able preferentially to bind to the cell receptors, fails to induce any subsequent host immune responses. However, walls of *Pneumocystis carinii* f. sp. *muris* also contain a  $\beta$ -(1 $\rightarrow$ 3)-glucan, and digestion by a  $\beta$ -(1 $\rightarrow$ 3)-glucanase greatly reduces its immunostimulatory effect (Vassallo et al. 2000). In the living organism, wall  $\beta$ -glucans are apparently shielded by mannan layers, but  $\beta$ -glucans become exposed after heat treatment, and induce stronger host immunoresponses (Gantner et al. 2005). Further work with well-characterized  $\beta$ -glucans from the walls of other pathogenic fungi is needed to clarify their roles, if any, in host defence mechanisms.

Natural products containing fungal  $\beta$ -glucans have been consumed for probably thousands of years, and anecdotally, especially in China and Japan (Mayell 2001; Lindequist et al. 2005), basidiocarps have long been considered to improve general health.  $\beta$ -Glucans were only recently recognized as the effective ingredients (Lucas et al. 1958) (Williams & Di Luzio 1980). Subsequently, detailed investigations into their influence on health have been reported, mainly using animal models (Vetvicka & Yvin 2004). Several fungal  $\beta$ -glucans appear to be effective immunomodulators, and they appear to impact positively on cancers and several bacterial infections (Brown & Gordon 2001; Vetvicka & Yvin 2004). Although not yet medically prescribed in most countries, their administration is now increasing. For example, in Japan lentinan is approved for clinical treatment of gastric and colorectal cancers, and encouraging survival rates have been reported (Nakano et al. 1999; Munemoto et al. 2002). Some of their possible mechanisms of action have also been elucidated (Brown & Gordon 2001, 2005). In this review, we discuss the potential of fungal  $\beta$ -glucans in medicine, and the known mechanisms underlying their biological effects. We suggest why structurally different  $\beta$ -glucans may have different efficiencies of action, and how they might be better applied clinically, safely and effectively.

## Effects of $\beta$ -glucans on the immune system

Some fungal  $\beta$ -glucans markedly stimulating our immune system protect us from attack by pathogenic microbes and from harmful effects of environmental toxins and carcinogens (Vetvicka & Yvin 2004; Brown & Gordon 2005).  $\beta$ -Glucans are not synthesized by humans, so these compounds are recognized by our immune systems as non-self molecules, inducing both innate and adaptive immune responses (Fig 2) (Brown & Gordon 2005).

### Effects on the innate immune system

Innate immunity is present when we are born, and is a relatively non-specific system, responding to many, but not all, structurally related antigens (Brown & Gordon 2001; Munz et al. 2005). Certain  $\beta$ -glucans, including zymosan, grifolan (GRN) and lentinan (Table 1), appear to activate phagocytes, thus leading to elimination of pathogens by phagocytosis (Ladanyi et al. 1993; Kurashige et al. 1997; Brown et al. 2002). Among these, the macrophages preferentially attack dead cells and intracellular pathogens (Munz et al. 2005). Natural killer cells (NKs) circulate in blood to lyse cancer and virus-infected cells, whereas neutrophils are effective against pyogenic bacteria. Krestin (PSK), the protein-bound polysaccharide K containing  $\beta$ -(1 $\rightarrow$ 3)-glucans from *Coriolous versicolor* increases NK cytotoxic activity *in vitro* (Garcia-Lora et al. 2001; Garcia-Lora et al. 2003), whereas lentinan also activates type 3 complement receptors (CR3), which coat bacterial cells to facilitate their engulfment by phagocytes (Vetvicka et al. 1996; Vetvicka & Yvin 2004). In addition, zymosan (the cell wall particulate glucan from *Saccharomyces cerevisiae*), and MG [the microparticulate form of  $\beta$ -(1 $\rightarrow$ 3)-glucans from *S. cerevisiae*] both stimulate macrophages to produce cytokines, local immunomodulators, and

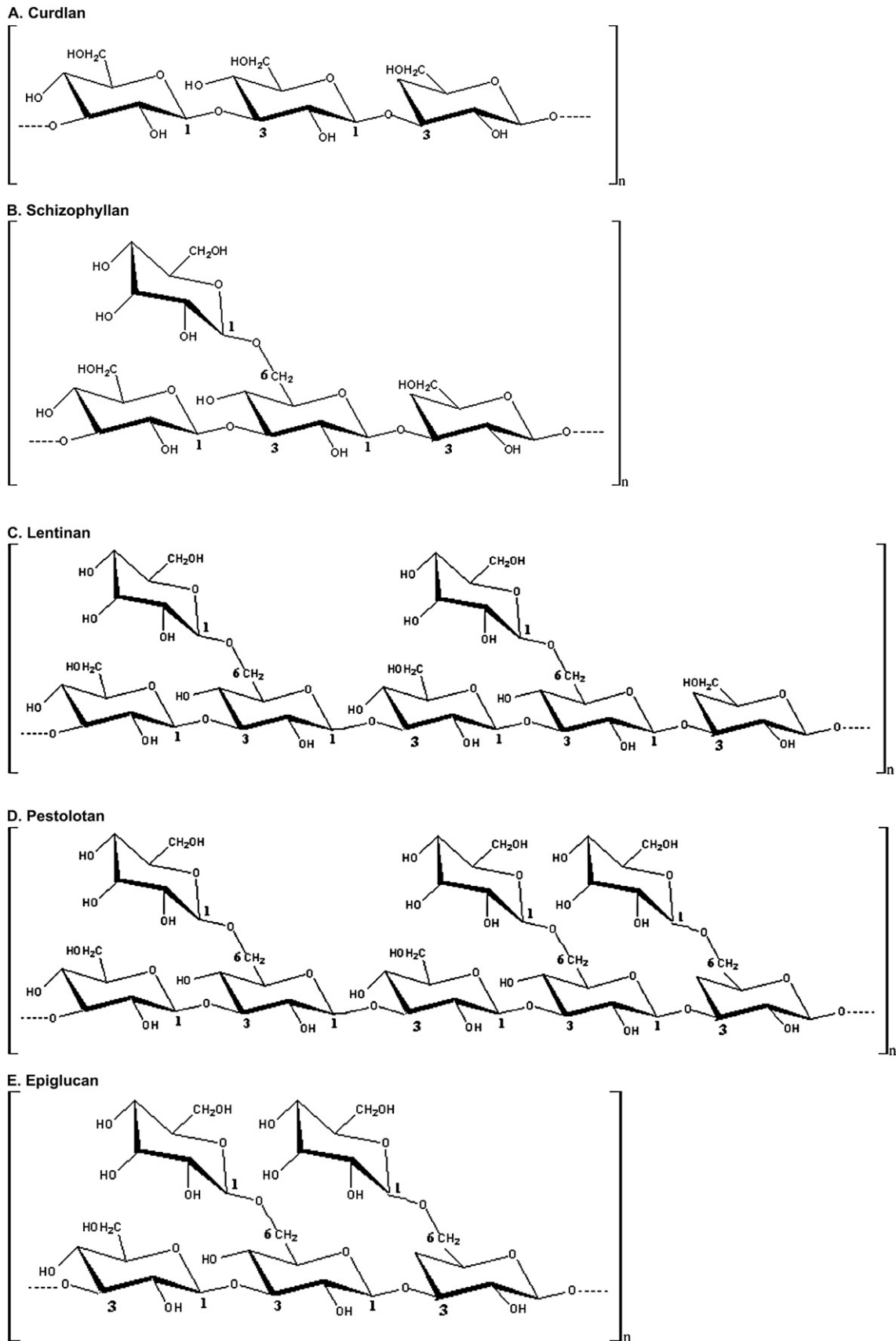
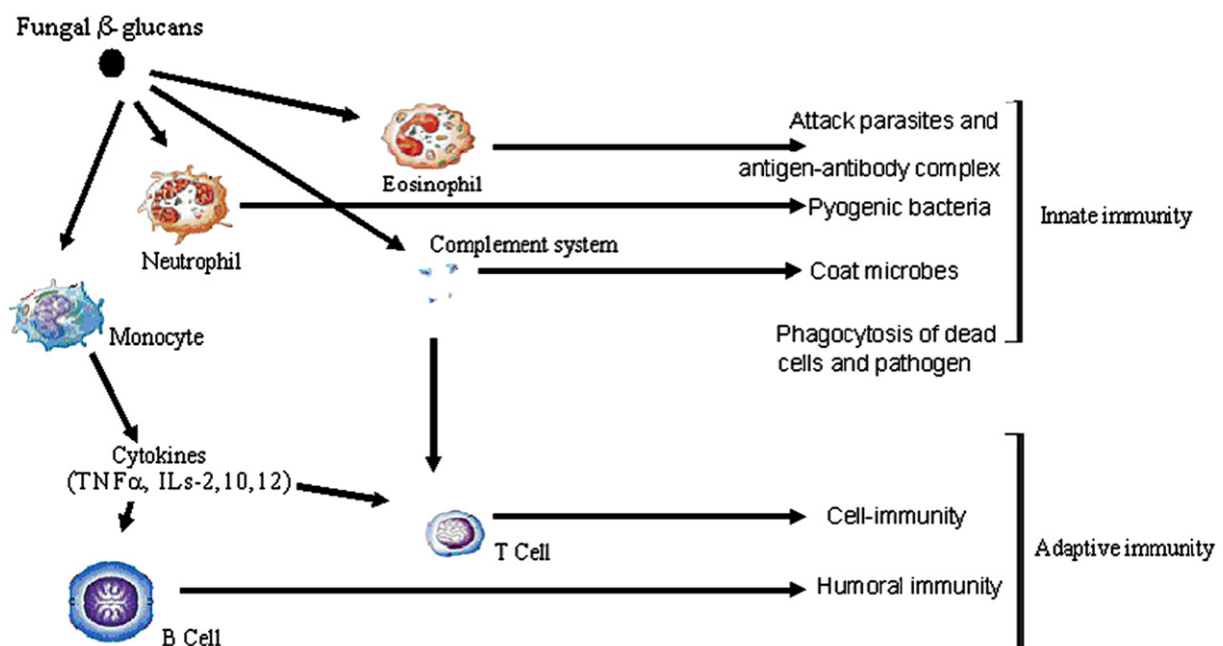


Fig 1 - Examples of structures of  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6) glucans showing branching patterns of their repeating units.

**Table 1 – Examples of fungal  $\beta$ -glucans and their chemical diversity**

| Glucan                                 | Abbreviation | Fungal Source                              | Structure  | Reference                           |
|--|--------------|--|--|-------------------------------------|
| Curdlan                                |              | <i>Alcaligenes faecalis</i><br>(bacterial) | Linear $\beta$ -(1 $\rightarrow$ 3)-glucan   | (McIntosh, Stone & Stanisich, 2005) |
| Grifolan                               | GRN          | <i>Grifola frondosa</i>                    | Two $\beta$ -(1 $\rightarrow$ 6) branched glucose residues for every five $\beta$ -(1 $\rightarrow$ 3) glucose residues                              | (Munz, Steinman & Fujii, 2005)      |
| Lentinan                               |              | <i>Lentinula edodes</i>                    | One $\beta$ -(1 $\rightarrow$ 6) branched glucose residue for every three $\beta$ -(1 $\rightarrow$ 3) glucose residues                              | (Sasaki & Takasuka, 1976)           |
| Schizophyllan(Sizofiran)               | SPG          | <i>Schizophyllum commune</i>               | One $\beta$ -(1 $\rightarrow$ 6) branched glucose residue for every three $\beta$ -(1 $\rightarrow$ 3) glucose residues                              | (Kikumoto S, 1971)                  |
| (Sonifilan)                            | SPG          |  | Ultrasonic partial degradation of schizophyllan  | (Johnson et al. 1963)               |
| Scleroglucan                           | SSG          | <i>Sclerotinia sclerotiorum</i>            | One $\beta$ -(1 $\rightarrow$ 6) branched glucose residue for every three $\beta$ -(1 $\rightarrow$ 3) glucose residues                              | (Garcia-Lora et al., 2003)          |
| Zymosan                                |              | <i>Saccharomyces cerevisiae</i>            | Crude cell wall extract of genetically engineering yeast containing a mixture of $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6) glucans and mannose | (Sato et al., 2003)                 |
|  | MG           | <i>Saccharomyces cerevisiae</i>            | Microparticulate form of the $\beta$ -(1 $\rightarrow$ 3) glucan   |                                     |
| Betafectin                             | PGG          | <i>Saccharomyces cerevisiae</i>            | $\beta$ -(1 $\rightarrow$ 6) branched glucose residues of $\beta$ -(1 $\rightarrow$ 3) glucan  | (Cheung & Modak, 2002)              |
| Krestin                                | PSK          | <i>Trametes versicolor</i>                 | Protein-bound $\beta$ -(1 $\rightarrow$ 3) glucan  | (Kurashige, Akuzawa & Endo, 1997)   |
| Yeast whole $\beta$ -glucan particules | WPG          | <i>Saccaromyces cerevisiae</i>             | Crude preparation $\beta$ -(1 $\rightarrow$ 6) branched glucose residues of $\beta$ -(1 $\rightarrow$ 3) glucans                                     | (Lebron et al., 2003)               |
| Pestolotan                             |              | <i>Pestalotia sp. 815</i>                  | Three $\beta$ -(1 $\rightarrow$ 6) branched glucose residues every five $\beta$ -(1 $\rightarrow$ 3) residues  | (Misaki et al., 1984)               |
| Epiglucan                              |              | <i>Epicoccum nigrum</i>                    | Two $\beta$ -(1 $\rightarrow$ 6) branched glucose residues every three $\beta$ -(1 $\rightarrow$ 3) glucose residues                                 | Schmid et al., 2001                 |

**Fig 2 – Immunostimulation by fungal  $\beta$ -glucans.**

these in turn activate adaptive immunity (Fig 2), (Ladanyi *et al.* 1993; Kurashige *et al.* 1997) (Brown *et al.* 2002; Sato *et al.* 2003; Young *et al.* 2004). Zymosan can stimulate the production and activity of cytokines such as interleukin (IL)-2, IL-10 and IL-12 (Du *et al.* 2006; Brown 2006), and *in vitro* studies show that MG is taken up rapidly by peritoneal macrophages (Berner *et al.* 2005). Synthesis of mRNAs of cytokines, e.g. tumour necrosis factor alpha (TNF- $\alpha$ ), IL-6 and IL-1, is then up-regulated and their secretion increased (Berner *et al.* 2005). However, neither zymosan, PSK nor MG are chemically pure  $\beta$ -(1 $\rightarrow$ 3)-glucans, so it is possible that other chemical components in these preparations may be responsible. However, when chemically characterized zymosan, treated to produce the particulate form containing only  $\beta$ -(1 $\rightarrow$ 3)-glucan was added to cultured macrophage cells, the production of IL-10, reactive oxygen species (ROS) and TNF increased in a dose-dependent way (Saijo *et al.* 2007). Similarly, the addition of a pure glucan (SCG) from *Sparassis cripa* increased the production of IL-12, TNF and interferon gamma. Thus, increasing evidence is now available from studies with high purity  $\beta$ -glucans and a range of host cells that would seem to indicate that these are the effective immunomodulators.

### Effects on adaptive immunity

The adaptive immune system responds to introduced foreign antigens (Munz *et al.* 2005). It involves both B and T cells. The former produce antibodies to mediate humoral immunity, whereas T cells induce cell-mediated immunity (Munz *et al.* 2005). Cytokines promote T cell differentiation to helper T cells 1 (Th1) and 2 (Th2), which mediate cell and humoral immunities, respectively (Trinchieri 2003). The adaptive immune response also involves dendritic cells (DC), derived from monocytes, and these present antigens to T-cells for activation of immune responses (Munz *et al.* 2005). These DC are activated by PSK, thus facilitating the expression of adaptive immunity (Kanazawa *et al.* 2004; Munz *et al.* 2005).

Fungal  $\beta$ -glucan-induced immune responses are different in their actions to immune therapies based on supplementation of elements of the immune system, e.g. exposure to IL2 and interferon gamma. Instead, they appear to act by stimulating the whole immune system. Consequently, these  $\beta$ -glucans may have an advantage in treating diseases. Furthermore, many can be administered orally, and combinational application with other immune therapies may generate a more potent end result. For example, such a synergistic effect is believed to result from combining  $\beta$ -glucan application with either BCG or interferon gamma (Berner *et al.* 2005; Drandarska *et al.* 2005).

### Possible mechanisms of fungal $\beta$ -glucan action

Multicellular organisms possess receptors called 'pattern recognition receptors' (PRRS), to detect innately non-self structures (including pathogen-associated molecular patterns, or PAMPs) (Brown & Gordon 2005). Thus, fungal  $\beta$ -glucans probably act as PAMPs and are recognized by appropriate cell-surface receptors, initiating immune responses. In humans, a number of such receptors have been identified. These are dectin-1, complement receptor 3 (CR3), scavenger receptors,

lactosylceramide (LacCer), and the toll-like receptor (TLR). Their roles and consequences of their interactions with  $\beta$ -glucans are summarized in Table 2 and Fig 3 (Brown & Gordon 2005). Evidence suggests that dectin-1 is most important in the activation innate immune responses in macrophages (Herre *et al.* 2004a; Willment *et al.* 2005), as blocking with an anti-dectin-1 antibody and knockout of the dectin-1 gene resulted in the abolition of all macrophage-mediated responses (Steele *et al.* 2003; Taylor *et al.* 2007). Other receptors may also be involved. They include those detected in the U937 cell line, where two receptors other than CR3 could bind with scleroglucan, schizophyllan, laminarin, a glucan phosphate and a glucan sulphate (Mueller *et al.* 2000). However, their roles are much less clearly defined than that of dectin-1.

### Dectin-1

Dectin-1 is a lectin consisting of four components: an extracellular carbohydrate-recognition domain (CRD), a stalk, a trans-membrane region, and an intracellular cytoplasmic tail (Ariizumi *et al.* 2000; Brown & Gordon 2001; Marshall *et al.* 2004). Several human dectin-1 isoforms have been cloned and characterized (Ross 2000; Hermanz-Falcon *et al.* 2001; Willment *et al.* 2001; Yokota *et al.* 2001). Of these, BGRA and BGRB are the two major isoforms, with BGRB lacking the stalk (Willment *et al.* 2001). Dectin-1 is commonly expressed in macrophages, neutrophil lineages, DC, and some T-cells, but not in NK cells (Ross 2000; Herre *et al.* 2004a; Underhill *et al.* 2005; Willment *et al.* 2005).

The function of dectin-1 in binding to zymosan is achieved by the CRD, which also binds to intact fungal cells and other soluble fungal  $\beta$ -(1 $\rightarrow$ 3) and/or  $\beta$ -(1 $\rightarrow$ 6)-glucans (Ariizumi *et al.* 2000; Marshall *et al.* 2004). Dectin-1 consists of 244 amino acids, and has six cysteine residues, all of which are highly conserved (Ariizumi *et al.* 2000). Two amino acids (Trp221 and His223) located after the fourth cysteine residue appear to be critical in its binding function (Adachi *et al.* 2004). Zymosan binding may also require other compounds, like the CD63 tetraspanin receptor and pentraxin3, a tumour necrosis factor (Diniz *et al.* 2004; Mantegazza *et al.* 2004). Evidence has shown that dectin-1 binds specifically to  $\beta$ -(1 $\rightarrow$ 3)-glucans, but only those consisting of at least 10-mer oligosaccharides. However, the mechanism for this binding selectivity is not known (Palma *et al.* 2006). There is a need to identify more precisely the structural features of  $\beta$ -glucans that determine the subsequent level of activation of immune responses, and more studies with those whose chemical structures have been fully characterized are required.

Binding of dectin-1 with the ligand activates several signalling pathways to promote innate immune responses through activation of phagocytosis, ROS production, and induction of inflammatory cytokines (Willment *et al.* 2001; Grunebach *et al.* 2002). The cytoplasmic domain of dectin-1 has an immunoreceptor tyrosine-based activation motif (ITAM) to activate a tyrosine kinase, which in turn stimulates ROS production but not phagocytosis (Underhill *et al.* 2005; Rogers *et al.* 2005). Activation of this tyrosine kinase also induces synthesis of TNF- $\alpha$ , and IL-2, IL-10, IL-12.

**Table 2 – Nature of the known receptors for  $\beta$ -glucans**

| Receptor type | Chemical nature  | Affected cells   | Effects on immune response  | Other ligands                           |
|---------------|--|--|---|---|
| Dectin-1      | Glycoprotein   | Monocytes<br>Macrophages<br>Neutrophils<br>DC cells<br>T-cells | Phagocytosis<br>Endocytosis<br>ROS production<br>Cytokines (TNF- $\alpha$ , IL-2, 10, 12)<br>MIP-2, | Intact fungi, T-cells                   |
| CR3           | A heterodimer<br>Consist of CD11b and<br>CD18 chains                                       | Myeloid<br>NK<br>Neutrophils<br>Lymphocytes                    | Tumour cytotoxicity   | A variety of pathogens                  |
| Scavenger     | A heterogeneous<br>group of molecules  | Myeloid<br>Endothelial   | Immunity  | LDL<br>HDL<br>Polyanionic<br>Microbes   |
| LacCer        | Glycosphingolipid containing<br>a hydrophobic<br>ceramide lipid and a<br>hydrophilic sugar | Neutrophils<br>Epithelial                                      | ROS<br>Anti-microbial Cytokines<br>(NF- $\kappa$ B) Lyn kinase                                      | A variety of microbes                   |
| TLR           | A novel<br>protein family with<br>associated protein MyD88                                 | Macrophages<br>DC<br>Lymphocytes<br>Epithelial                 | Cytokines (NF- $\kappa$ B, TNF- $\alpha$ , IL12)  | A wide<br>spectrum of<br>microorganisms |

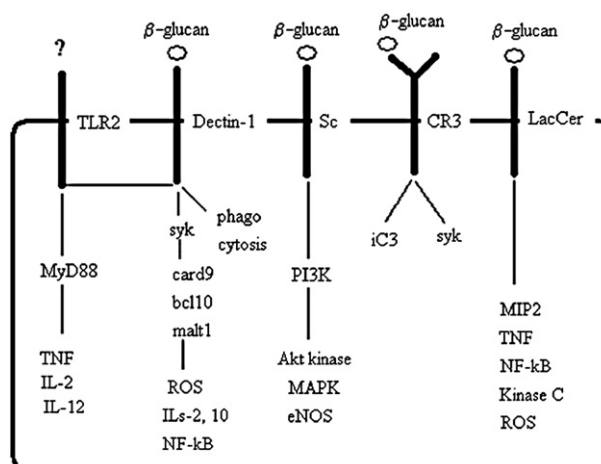
CR3, complement receptor 3; CRD, carbohydrate-recognition domain; DC, dendritic cell; eNOS, endothelial nitric oxide synthase; LacCer, lactosylceramide; IL, interleukine; MIP, macrophage inflammatory protein-2; NK, natural killer cell; NF $\kappa$ B, nuclear factor-kappaB; ROS, reactive oxygen species; TNF- $\alpha$ , tumour necrosis factor-alpha.

Several pathways have now been identified as being involved in dectin-1 downstream signalling (Fig 3). First, some evidence suggests it might act synergistically with TLR to produce strong inflammatory responses by stimulating cytokines such as TNF- $\alpha$ , IL-2 and IL-12 (Gantner et al. 2003; Brown 2006). Although both dectin-1 and TLRs are activated by zymosan (Rogers et al. 2005), the TLR ligand is not yet known. The suggestion is that it may not be a  $\beta$ -glucan as laminarin had no effect on its signalling pathway, and hot alkali treated zymosan only bound to dectin-1 (Gantner et al. 2003). Recent evidence also showed that SCG and NaClO-oxidized zymosan (which contained only  $\beta$ -glucan) induced production of TNF and IL-12 and interferon gamma was not affected by a deficiency of MyD88, indicating its effect is independent of the TLR pathway (Saijo et al. 2007). Whether other fungal  $\beta$ -glucans can bind to TLR is not known. This needs to be determined, and it could be that this synergistic effect arises because phosphorylated dectin-1 can form a complex with TLR (Brown 2006).

In addition, another pathway independent of TLR is mediated via spleen tyrosine kinase (Syk) to produce other cytokines, including the macrophage inflammatory protein-2 (MIP2, CXCL2) and IL-2 and IL-10 in mice DC cells (Rogers et al. 2005). After binding to the ligand, dectin-1 is phosphorylated by a non-receptor tyrosine kinase Src. Syk is then activated, which in turn activates the card9-bcl10-Malt1 complex (caspase recruitment domain 9 - B-cell chronic lymphocytic leukaemia/lymphoma 10-mucosa-associated lymphoid tissue lymphoma translocation gene 1). This complex mediates the induction of cytokines like nuclear factor (NF)- $\kappa$ B and IL production. Zymosan failed to activate NF- $\kappa$ B, TNF- $\alpha$ , IL-2 and

IL-6 production in card9-/- and Malt1-/- mouse dendritic cells indicating the importance of the card9-bcl10-Malt1 complex in this pathway (Gross et al. 2006). Consequently, animals with Card9-/- were much more susceptible to *Candida albicans* infections (Gross et al. 2006). Surprisingly, dectin-1-deficient mice had a similar capability to counteract *C. albicans* infections, indicating that another pathway to activate card9 by  $\beta$ -glucan must exist (Saijo et al. 2007). There is now evidence that CR3 also activates Syk (Li et al. 2006).

Furthermore, phagocytosis in macrophages is another signalling pathway activated by the dectin-1 receptor (Underhill

**Fig 3 – Possible fungal  $\beta$ -glucan mediated signal pathways.**

et al. 2005; Brown 2006) which seems to be independent of any involvement by either TLR or Syk. The dectin-1 cytoplasmic domain has three consecutive acidic amino acids signalling phagocytosis and activation of phagocyte lysosomal endosomes (Engering et al. 2002; Serrano-Gomez et al. 2004; de la Rosa et al. 2005). However, its detailed mode of action is less clear. Dectin-1 has also been shown to mediate zymosan-induced arachidonic acid production and cyclooxygenase 2 expression through its cytoplasmic domain. Both promote acute tissue inflammation (Suram et al. 2006).

### CR3 receptor

The CR3 receptor, consisting of CD11b and CD18 domains, recognizes a wide range of microbial cells, and functions as an adhesion molecule. It is expressed mainly on neutrophils, monocytes and NK cells, but not macrophages (Ross 2000). Two binding sites exist in CD11b. One is for  $\beta$ -glucans, and is located within the C terminus, while the other for iC3b (cleaved component 3 fragment of serum complement system), is located within the N-terminus (Thornton et al. 1996; Xia & Ross 1999).

Binding of  $\beta$ -glucans to CR3 increases adhesion to microbial cells and activates the iC3b pathway causing tumour cytotoxicity (Xia & Ross 1999; Xia et al. 2002) (Tsikitis et al. 2004; Kanse et al. 2004). Such activation requires occupation of both binding sites as cytotoxicity is blocked by an anti-CR3 antibody (Vetvicka et al. 1996; Li et al. 2006). Zymosan increased NK cell-mediated killing of tumour cells via activation of CR3 (Vetvicka & Yvin 2004; Diniz et al. 2004). However, the trials on which this conclusion was based were only conducted in neutrophils and NK cells, and other cells may possess different receptors. NK cells have no dectin-1 receptors, so CR3 may be the major receptor. Neutrophil cells have both CR3 and dectin-1 receptors, but it is not known what happens when CR3 receptors are blocked, or what the specific role of dectin-1 is. Zymosan also binds to dectin-1 in neutrophil cells to activate immune responses (Willment et al. 2005), and if an anti-CR3 antibody inhibits these, then it is possible that dectin-1 and CR3 are both needed for zymosan-induced activation.

### Scavenger receptors

Scavenger receptors located in myeloid and endothelial cells comprise a heterogeneous group of proteins with two transmembrane domains, two intracellular domains and one extracellular domain (Acton et al. 1994; Rice et al. 2002; Assanasen et al. 2005). These recognize a range of foreign cells, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and selected polyanionic ligands (Assanasen et al. 2005). Although lentinan can bind to these receptors, no specific  $\beta$ -glucan scavenger receptors have yet been identified (Rice et al. 2002). Multiple signalling pathways appear to be activated by the Src receptor, including those involving Src family kinase(s), phosphatidylinositol-3 kinase (PI3K), Akt kinase, and p38 mitogen-activated protein kinase (MAPK), and an endothelial nitric oxide synthase (eNOS) (Mineo et al. 2003; Assanasen et al. 2005). However, their possible role, if any, in

the biological effects mediated by fungal  $\beta$ -glucans is still unknown, and no evidence is available that they are important.

### LacCer

LacCer located in neutrophil and endothelial cells is a glycolipid containing a hydrophobic ceramide lipid and a hydrophilic sugar moiety. It recognizes both microbial cells and fungal  $\beta$ -(1 $\rightarrow$ 3)-glucans (Zimmerman et al. 1998). In alveolar epithelial cells, the  $\beta$ -(1 $\rightarrow$ 3)-glucan cell wall component of *Pneumocystis carinii* increased synthesis of NF- $\kappa$ B, MIP-2 and TNF- $\alpha$  through a protein kinase C signalling pathways (Wang et al. 2005; Evans et al. 2005), while in neutrophil cells, LacCer increased ROS production to kill microbes through activation of PI-3K, MAPK, and protein kinase C mediated by the Src family kinase Lyn (Iwabuchi & Nagaoka 2002). The role played by  $\beta$ -(1 $\rightarrow$ 3)-glucans in mediating these immune responses again requires further study, preferably in experiments using purified preparations of known chemical structure.

### TLRs

TLRs are transmembrane receptors of a novel protein family. At least 11 members of this family exist in humans (Roeder et al. 2004). They respond to the presence of a diverse group of microbes including fungi, bacteria, viruses and protozoa (Roeder et al. 2004). Fungal cells and zymosan bind to TLR 2 and 4, and both activate innate immune responses (Takeda et al. 2003). TLR2 causes an increase in the levels of NF- $\kappa$ B and cytokine production, including TNF- $\alpha$  and IL-12, which is mediated by the adaptor protein MyD88 (Ozinsky et al. 2000; Kataoka et al. 2002; Lebron et al. 2003). However, most of these data were again generated in studies with chemically crude  $\beta$ -glucans. For example, the zymosan used probably contained the lipopolysaccharide (LPS) endotoxin from Gram-negative bacteria, which is also a strong inducer of TLRs (Kawai et al. 2001; Duenas et al. 2006; Lapaque et al. 2006).

In summary, what seems clear is that recognition of  $\beta$ -glucans by these receptors leads to elimination of bacterial pathogens, and a single soluble  $\beta$ -glucan preparation may activate several receptors, as seen with zymosan which binds to dectin-1 and CR3 (Ariizumi et al. 2000; Diniz et al. 2004; Takeda et al. 2003). Fungal  $\beta$ -glucans may activate different immune cell populations through specific receptors, although sometimes several receptors may exist in a single cell. Dectin-1 seems to be most important in macrophages, and while CR3 has a role in NK cells, different receptors are present in neutrophils. Although co-ordinated effects between dectin-1 and TLR are well documented, those involving other receptors have not been well studied.

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## Importance of $\beta$ -glucans in the treatment of cancer

Since their anti-tumour activity was first demonstrated nearly 50 y ago, many animal experiments have demonstrated the remarkable effects of certain fungal  $\beta$ -glucans on a range of tumours (Vetvicka & Yvin 2004). Several human clinical trials have also shown possible treatment benefits, although these data are still preliminary and controversial. Currently,

lentinan, schizophyllan, and PSK are approved in Japan for clinical use in human cancer treatment (Mizuno *et al.* 1999).

Cancer is uncontrolled cell proliferation induced by many factors including environmental chemicals, viruses, bacteria, hormones, and chronic inflammation (Nam *et al.* 2005). Three developmental stages are recognized (Borchers *et al.* 2004; Nam *et al.* 2005). The first is initiation, in which a mutagen binds to the cell DNA and causes damage, which by itself is usually insufficient to induce tumour production. The second stage is activation of a tumour promoter that leads to the formation of small benign tumours. Finally in the third stage, progression, the normal tight control over the cell cycle is lost, resulting in uncontrolled cell proliferation (Borchers *et al.* 2004). Studies with oligonucleotide microarrays in hepatocellular carcinoma have revealed that multiple gene mutations are involved in tumour development (Nam *et al.* 2005). Thus, as many as 3084 altered genes correlated with tumour progression from the formation of dysplastic nodules eventually to overt hepatocellular carcinoma. Among these, 240 genes were associated with different stages in tumour development.

Although surgical resection remains the most effective early treatment of solid cancers, chemotherapies and immunotherapies are often used (Nilsson *et al.* 2004). Progress in understanding the molecular mechanisms of carcinogenesis has allowed these non-surgical therapies to become more effective. Some fungal  $\beta$ -glucans appear to beneficially influence both cancer promotion and progression (Takaku *et al.* 2001; Nilsson *et al.* 2004; Nam *et al.* 2005), and such treatment of rats has led to formation of much smaller tumours than those seen in controls (Vetvicka & Yvin 2004).

Fungal  $\beta$ -glucans also have synergistic effects with monoclonal antibodies used in cancer treatment. Monoclonal antibody therapy targets key components of the biological pathways involved in carcinogenesis. Furthermore, yeast  $\beta$ -glucans given orally with monoclonal antibody therapy increased neuroblastoma tumour regression and long-term survival in mice (Cheung *et al.* 2002; Cheung & Modak 2002; Yan *et al.* 2005). In mice with established subcutaneous non-Hodgkin's lymphoma xenografts, a combination of intravenous complement-activating antibody, rituximab, and WGP from yeast had a higher therapeutic efficacy than treatment with either alone (Modak *et al.* 2005). WGP induced the killing ability of Mab G250, an antibody against the enzyme carbonic anhydrase, and present only in renal carcinoma cells, (Sier *et al.* 2004), by assisting granulocytes to kill iC3b-coated cancer cells. Intravenously and orally administered  $\beta$ -glucans (e.g. WPG) promoted tumour regression by stimulating granulocytes and macrophages, as well as triggering cytotoxicity of tumour cells, but not in CR3<sup>-/-</sup> mice, indicating a critical role for CR3 (Allendorf *et al.* 2005; Yan *et al.* 2005). Two domains of the CR3 receptor must both be occupied by iC3b and the  $\beta$ -glucan to exert this killing effect (Sier *et al.* 2004; Li *et al.* 2006). As no  $\beta$ -glucans are synthesized by humans, monoclonal antibodies enhance treatment capabilities only after their administration (Sier *et al.* 2004).

Although these data are impressive, they are not easy to interpret mechanistically, as none of the  $\beta$ -glucans used in the studies were confirmed as being chemically pure. In addition, some chemical variation in the structure of most natural

products is to be expected. Explanations for their mechanism of action are also limited to considering a role for the CR3 receptor alone.

Thus, concomitant use of the anticancer drug S-1 and lentinan prolonged survival times of colon-26-bearing mice (Mushiake *et al.* 2005), but not in athymic mice, which suggests some role for DCs. These mice showed increased CD86<sup>+</sup> DCs infiltration into colon-26 and more potent activities for T-cell proliferation and T-lymphocyte cytotoxicity. Consequently, DC may play a key role in stimulating anti-tumour immunity in combination with lentinan. Orally administered WGP may be taken up by macrophages in the gastrointestinal tract and shuttled to the bone marrow where it is degraded and secreted as smaller soluble  $\beta$ -(1 $\rightarrow$ 3)-glucan molecules, which then bind to the receptor CR3 (Hong *et al.* 2004). Further consideration will be given to the possible metabolic fate of ingested  $\beta$ -glucans later.

Certain fungal  $\beta$ -glucans may also ameliorate chemotherapy and radiation treatment by increasing patient tolerance, and speed recovery from toxic effects (Harada *et al.* 2002; Gu *et al.* 2005), the most severe of which is leukopenia, associated with an increased risk of infection. In a mouse model with cyclophosphamide-induced leukopenia, oral administration of the  $\beta$ -glucans from *Sparassis crispa* markedly increased the rate of leukocyte recovery (Harada *et al.* 2002). Furthermore, intraperitoneal injection of a yeast derived  $\beta$ -glucan decreased mortality in tumour-bearing mice exposed to whole-body X-ray radiation, and increased their leukocyte and lymphocyte numbers, including both NK and lymphokine-activated killer (LAK) cells (Gu *et al.* 2005).

PSK has been successful in postoperative treatment of resectable cancer in humans, increasing survival rates (Munemoto *et al.* 2002; Ito *et al.* 2004). A literature search has revealed 48 published clinical trials using PSK (Sakamoto *et al.* 2006). Three involved 1094 patients, and results showed that adjuvant immunochemotherapy with PSK increased five-year overall survival rates from 72.2% to 79%, and five-year disease-free rates from 65.9% to 72.2% (Nakazato *et al.* 1994; Ito *et al.* 2004; Ohwada *et al.* 2004). Although both sets of data are statistically significant, in the trial of Ito *et al.* no difference was recorded for seven-year overall survival and disease-free rates (Ito *et al.* 2004). Thus, these data are still very preliminary; again the major weakness in interpreting these data is that PSK is not a chemically pure  $\beta$ -glucan, but a protein (25%) bound polysaccharide extracted from mycelium of *C. versicolor* (Kobayashi *et al.* 1995). It is thought to consist of a main chain of  $\beta$ -((1 $\rightarrow$ 4)-linked glucose residues with a side-chain of  $\beta$ -(1 $\rightarrow$ 3) as well as  $\beta$ -(1 $\rightarrow$ 6) linked glucose residues. There is also the possibility that PSK, like WSP, may be degraded to smaller molecules in the body, which then bind to receptors.

Lentinan has also been used in similar clinical trials and been shown to prolong survival times of humans with gastric cancer from 199 to 297 d of the median survival period (Nakano *et al.* 1999). A crude  $\beta$ -(1 $\rightarrow$ 6)-glucan extracted from *Agaricus blazei* basidiocarps caused apoptosis or programmed cell death in human ovarian cancer HRA cells. As the apoptotic effect of this  $\beta$ -glucan could be abolished by applying the p38 MAPK-specific inhibitor, SB203580 (Kobayashi *et al.* 2005), this would seem to involve activation of the p38 MAPK



pathway by translocation of an apoptosis activator Bax from the cytosol to the mitochondria, cytochrome c release and caspase 9 activation. In our opinion, these clinical trials are still very preliminary, and the data from them should be treated cautiously.

In addition, lentinan, GRN, SSG, and SPG also reduce preventatively the overall risks of cancer (Greenwald *et al.* 2001). As these are caused by mutations, oxidative stress, and inflammation, and develop in several stages from multiple gene mutations, they are preventable by blocking the appropriate regulatory molecules involved in these processes. These are known to include Nrf2 (nuclear factor erythroid 2-related factor 2), epidermal growth factor receptor kinases, phosphatidylinositol 3-kinase, components of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway, NF- $\kappa$ B and cyclin D (Sporn & Liby 2005). Cancers examined include melanoma, adenocarcinoma, mammary carcinoma, lymphocytic leukaemia, and Lewis lung carcinoma (Suzuki *et al.* 2001). The most impressive data come with lentinan, which in animal models prevented carcinogenesis in chronic ulcerative colitis by inhibiting expression of P450 1A2, which catalyses pre-carcinogenic compounds (Mitamura *et al.* 2000; Okamoto *et al.* 2004). This inhibition was mediated by an increase in TNF- $\alpha$  levels and DNA-binding activity of the NF- $\kappa$ B. Other fungal  $\beta$ -glucans of known chemical composition should be screened to see whether common mechanisms of action might exist.

In summary, fungal  $\beta$ -glucans may exert multiple effects on cancer cells and prevent cancer. Their mechanisms of action appear to be complex. The regulation by  $\beta$ -glucans of the signalling pathways of tumour production involves many key elements (Sporn & Liby 2005). Most studies so far demonstrate that  $\beta$ -glucans cause regression in tumour size, but not total tumour eradication. These are different because regressed tumours can enlarge rapidly when treatment stops.

### Importance of fungal $\beta$ -glucans in the treatment of infectious diseases

The major current problem in controlling infectious diseases is antibiotic resistance (Suzuki *et al.* 2001). Some fungal  $\beta$ -glucans are effective against almost all known pathogenic microbes, by acting through several different immunomodulatory mechanisms.

#### Anti-viral activity of fungal $\beta$ -glucans

SPG, lentinan, zymosan, and bacterial curdlan all appear effective against several viral agents (Mayell 2001), although most data available are only descriptive. SPG controls chronic hepatitis B infections, by modulating both cellular and humoral immune responses (Kakumu *et al.* 1991). In mice injected with hepatitis virus strain MHV-A 59, a  $\beta$ -glucan prepared from *Saccharomyces cerevisiae* cells increased infected cell survival by inhibiting cell necrosis and activating phagocytic activity (Vetvicka & Yvin 2004).

Zymosan from *S. cerevisiae* enhanced the immune response in humans administered the human immunodeficiency virus (HIV) vaccine by stimulating Th cell-mediated

immunity through activation of the complement system and interferon gamma (Ara *et al.* 2001). The same  $\beta$ -glucan also decreased viral nucleic acid levels in cells of pigs infected with swine influenza virus, and led to increases in their interferon gamma and nitric oxide levels (Jung *et al.* 2004).

Some preliminary clinical trials have been conducted with lentinan in the treatment of HIV patients suffering no accompanying opportunistic infections. In a placebo-controlled trial in San Francisco, patients (ten per group) with HIV were administered 2, 5, and 10 mg lentinan or the placebo intravenously. Results showed increased CD4 cell numbers in those administered lentinan (Gordon *et al.* 1998). When lentinan was applied with didanosine in HIV-positive patients with CD4 levels of 200–500 cells mm<sup>-3</sup>, CD4 cell numbers increased by about 142 CD4 cells mm<sup>-3</sup> over 12 m, whereas didanosine alone led to decreased CD4 numbers (Gordon *et al.* 1995). These trials suggest that lentinan was more effective in combination with other antiviral agents, although the patient numbers used were small.

#### Anti-bacterial activities of fungal glucans

Some  $\beta$ -glucans, including lentinan, WGP, PPG, and SSG, are also effective against bacterial infections. Thus, lentinan reduced *Mycobacterium tuberculosis* infections by increasing macrophage levels *in vivo* in a rat model and *in vitro* examinations showed these macrophages had increased killing ability toward *M. tuberculosis* cells (Markova *et al.* 2003; Markova *et al.* 2005). Both PPG and WGP have effectively treated mice against *Bacillus anthrax* infections (Kournikakis *et al.* 2003), correlating with increases in levels of cytokines IL-2 and IL-10, although experiments to clarify their modes of action by blocking cytokines were not conducted. When SSG was tested against *Streptococcus pneumoniae* types 4 and 6B in mice infected experimentally intraperitoneally (Hetland *et al.* 2000), it protected animals against both strains when applied 3 d before challenge. However, it was only effective against the type 4 strain when administered after infection.

PPG from *Saccharomyces cerevisiae* also effectively treated *Staphylococcus aureus* infections involving strains resistant to several  $\beta$ -lactam antibiotics including methicillin, by improving patient survival by 80 % (Liang *et al.* 1998). It also successfully treated methicillin-resistant strains of *S. aureus* and *S. epidermidis* in guinea pigs (Kernodle *et al.* 1998). As with other examples given here, only a restricted range of  $\beta$ -glucans have been examined in this way and further work with others (Table 1) seems warranted. PPG can also prevent wound infections, and was more effective in combination with the antibiotic cefazolin than alone (Kaiser & Kernodle 1998). However, no explanation was provided.

When SPG and zymosan were examined for their effects on tissue injury induced by LPS, the outcomes were controversial. The claim that SPG reduced LPS-induced toxicity both in rats and perfused liver (Kukan *et al.* 2004) by reducing the basis for LPS toxicity that requires induction of TNF- $\alpha$  at a transcriptional level (Harrison *et al.* 2004), has been questioned. Zymosan may also act by stimulating hepatic macrophages (Kupffer cells) and increasing their sensitivity to LPS, leading to increased TNF- $\alpha$ -induced hepatic lesion (Beutler & Cerami 1989; Yamaguchi *et al.* 2001).

Overall, the evidence seems to suggest that  $\beta$ -glucan therapy should be better based on disease prevention than its cure, where the immune system can be stimulated first to cope better with subsequent infections. No studies have been performed yet to examine the possible effect of dectin-1 and other receptors in this antibacterial role of fungal  $\beta$ -glucans, although it seems unlikely that different receptors would be involved.

### Anti-fungal activity of fungal $\beta$ -glucans

Currently, opportunistic fungal infections are problematic, especially in immunocompromised patients with HIV and cancer, so any stimulation of the immune system may be critical in patient survival. As mentioned earlier,  $\beta$ -glucans are major components of walls of pathogenic fungi and probably act as important inducers of immune responses against them by binding to the dectin-1 receptor (Netea et al. 2006; Saijo et al. 2007; Taylor et al. 2007). Some edible  $\beta$ -glucans can activate phagocytic cells including macrophages, neutrophils and DC to enhance the host's innate response to fungal infections (Herre et al. 2004b). For example, MG up-regulated synthesis of the cytokines TNF- $\alpha$ , IL-6 and IL-1 mRNA in macrophages, neutrophils, and DCs (Berner et al. 2005). Several  $\beta$ -glucan receptors appear to be involved. Cell wall  $\beta$ -glucans of *Pseudomonas carinii* bind to dectin-1 and Lac receptors on alveolar epithelial cells to promote innate immune responses (Hahn et al. 2003). The signalling protein MIP2 is activated and cytokines then released. A synergy exists between interferon-gamma and these wall  $\beta$ -glucans, as pre-treatment with interferon-gamma increases the sensitivity of response. Other  $\beta$ -glucans like zymosan can protect host cells against *Candida albicans* and *P. carinii* infections, involving their recognition by dectin-1 (Hobson et al. 2004; Gantner et al. 2005; Steele et al. 2003; Viriyakosol et al. 2005). TLR has also been shown to be involved in host cell immune responses to some fungal infections (Roeder et al. 2004).

From *in vitro* experiments, peritoneal macrophages from mice treated with the particulate  $\beta$ -glucan from *Saccharomyces cerevisiae* showed enhanced activity against *P. brasiliensis* infections (Pelizon et al. 2005). Their spleen cells had higher synthetic abilities for TNF- $\alpha$  and IL-12, and IL-12 treatment can inhibit spread of *P. brasiliensis* cells to the liver and spleen (Calich et al. 1998; Arruda et al. 2002). In a monocyte monolayer cell culture, both mRNA and cytokine TNF- $\alpha$  and IL-1  $\beta$  were induced by purified yeast glucan particles (Abel & Czop 1992), an event that was partially preventable by trypsin exposure, indicating a possible involvement of trypsin-sensitive  $\beta$ -glucan receptors, which were not identified.

Treatment of opportunistic fungal infection by traditional chemotherapy is not very effective. However, combinational therapy with antifungal agents and the immune stimulator granulocyte colony-stimulating factor (G-CSF) has successfully treated immunocompromised mice infected with *Aspergillus fumigatus* (Sionov et al. 2005). Fungal  $\beta$ -glucans have not yet been incorporated into such treatment regimes, although theoretically there seems no reason why they should not act synergistically with other anti-fungal agents and immune stimulators.

## Role of fungal $\beta$ -glucans in the treatment of hypercholesterolaemia, diabetes, high blood pressure, and wound healing

### Hypercholesterolaemia

Cardiovascular disease related to elevated blood cholesterol levels is still the most common cause of death in humans in western countries (Strong et al. 2005). Cholesterol contains LDLs and HDLs (Strong et al. 2005). LDL has been associated with an increased risk of coronary artery disease. Yeast  $\beta$ -glucans appear to be effective in lowering blood cholesterol concentrations, but the mechanism by which this occurs is still unclear (Nicolosi et al. 1999); there are few studies that have tested other fungal  $\beta$ -glucans for this activity.

### Diabetes

Several fungal  $\beta$ -glucans may reduce blood glucose concentrations after eating, possibly by delaying stomach emptying so that dietary glucose is absorbed more gradually (Lo et al. 2006). Orally ingested fruiting bodies and the acidic polysaccharide of both *Tremella mesenterica* and *T. aurantia* both reduced blood glucose concentrations in induced diabetic rats (Kiho et al. 1995). However, the structure of the active polysaccharide component was later shown to have  $\alpha$ -(1 $\rightarrow$ 3)-linked D-mannopyranosyl residues as the backbone and (1 $\rightarrow$ 3)-linked  $\beta$ -D-xylopyranose side chains at position 2, and  $\beta$ -D-glucopyransyluronic acid at position 4 (Kiho et al. 2000), so it was not a  $\beta$ -glucan. This emphasizes the risks associated with interpreting data from experiments where crude fungal preparations are used. A crude exopolysaccharide produced from submerged mycelial cultures of *Phellinus baumii* also exhibited hypoglycaemic effects in streptozotocin-induced diabetic rats (Kiho et al. 2000), but again the active component was not convincingly verified as a  $\beta$ -glucan. In genetically diabetic mice fed 20% whole mushroom maitake powder and its chemically derived fractions prevented an increase in blood glucose levels by increasing insulin sensitivity (Mayell 2001). A  $\beta$ -glucan prepared by hot water extraction of *Agaricus blazei* basidiocarps showed anti-hyperglycaemic, anti-hypertriglyceridaemic, anti-hypercholesterolaemic and anti-arteriosclerotic activity in diabetic rats, although the active component was not identified (Kim et al. 2005). When these preparations were digested by an endo  $\beta$ -(1 $\rightarrow$ 6)-glucanase from *Bacillus megaterium*, the resulting di- and tri-saccharides had double the anti-diabetic activities shown by the parent  $\beta$ -glucans, again raising question as to whether  $\beta$ -glucans or their derived oligosaccharides are the effective agents. No purified fungal  $\beta$ -glucan whose chemical structure has been fully characterized has yet been tested against diabetes. Consequently, the need is to clarify the structural features required and to identify the nature of the  $\beta$ -glucan binding receptors related to such activities if its promise is to be realized.

### Blood pressure

Some fungal  $\beta$ -glucans may also control blood pressure. An intravenous infusion of an aqueous extract of *Pleurotus sajor-caju*

mycelium caused a decrease in the mean systemic blood pressure in rats, although the biologically active component was not identified (Tam *et al.* 1986). However, in genetically deficient rats with spontaneous hypertension (SHR), a diet containing 5% maitake from *Grifola frondosa* had the same effect (Kabir *et al.* 1987; Kabir & Kimura 1989). Because of the complex chemistry of these basidiocarps, attempts have been made to elucidate which chemical components are responsible, and data again suggest that these may not always be  $\beta$ -glucans. Thus, in one trial a lectin from *Tricholoma mongolicum* basidiocarps appeared to be the effective agent (Wang *et al.* 1996). Responses with whole maitake basidiomes have also been compared with those from their ether-soluble and water-soluble extracts (Talpur *et al.* 2002), and as only consumption of the ether-soluble extract decreased systolic blood pressure in SHR, any role for  $\beta$ -glucans seems improbable. Furthermore, the component from Tamogi-take mushroom (*Pleurotus cornucopiae*) that inhibited angiotensin I and so decreased blood pressure in SHR was shown to be D-mannitol, (Hagiwara *et al.* 2005). Yet consumption of whole maitake basidiocarps and the water-soluble extract also led to a decrease in blood pressure in Zucker fatty rats, a diabetes rat model (Talpur *et al.* 2002). Thus, the possible involvement of fungal  $\beta$ -glucans in reducing hypertension is not yet clear, and as with many of the biological effects discussed here, now seems the time to direct our efforts to assessing more carefully purified, and structurally characterized individual fungal  $\beta$ -glucans.

### Wound healing

Macrophage activities stimulated by  $\beta$ -glucans from *Saccharomyces cerevisiae* may also benefit wound healing and reduce scar tissue levels after surgery or trauma, as revealed by both animal and human studies (Mayell 2001; Portera *et al.* 1997). In normal human dermal fibroblasts this  $\beta$ -glucan preparation stimulated procollagen mRNA and collagen biosynthesis, together with increased NF-1 (Wei *et al.* 2002b). Inhibition of NF-1 by pentifylline blocked induction of procollagen mRNA by the same  $\beta$ -glucan, which also induced mRNA synthesis of many other wound growth factors including activator protein-1, specificity protein-1, neurotrophin 3, platelet-derived growth factor A, B, fibroblast growth factor acidic, fibroblast growth factor basic, transforming growth factor  $\alpha$ ,  $\beta$ , and vascular endothelial growth factor (Wei *et al.* 2002a). However, how the synthesis of these factors is regulated in the signal transduction pathway is unclear.

### Immunomodulation by fungal $\beta$ -glucans: structure–function relationships

Traditionally the beneficial effects described here were reported in humans eating fungal basidiocarps, and not from consumption of single pure  $\beta$ -glucans, so most of the available information on relationships between structure and function is confused by this. However, it seems clear that individual fungal  $\beta$ -glucans differ in their effectiveness as immunomodulators. Even  $\beta$ -glucans with similar reported structures, molecular weights and solution conformations

can differ markedly. This may reflect our inability to acquire sufficient detail from their structural analyses to allow us to recognize possible subtle structural differences between them, using the present methodologies of nuclear magnetic resonance (NMR) and methylation analyses to determine branching patterns. Yet these response differences are much more noticeable when structurally quite different glucans are compared. The most popular  $\beta$ -glucans, lentinan, schizophyllan, grifolan, and SSG from *Lentinus edodes*, *Schizophyllum commune*, *Grifola frondosa*, and *Sclerotinia sclerotiorum*, are all  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6)-glucans, but with different reported branching frequencies (Table 1). All are effective against the same tumour model, but at quite different doses. Some papers have reported correlations between  $\beta$ -glucan effectiveness and molecular structure, size, branching frequency, structural modification, conformation and their solubility, but it is still difficult to confidently make generalizations because of the often contradictory data available. Furthermore, as mentioned earlier in this review we do not understand well the metabolic fate of ingested  $\beta$ -glucans, and whether they require modification and possibly conversion to smaller oligosaccharides, to have an effect. These are difficult questions to address experimentally, but the acidity of the stomach may readily generate partial acid hydrolysis products from them.

### Molecular structure and size

The anti-tumour effects of fungal  $\beta$ -glucans seem to be related to their structural features. For example, a  $\beta$ -(1 $\rightarrow$ 3)-linked backbone seems essential (Seviour *et al.* 1992; Demleitner *et al.* 1992). So, although schizophyllan showed anti-tumour activity against Sarcoma 180 tumour cells, all glucans tested with  $\alpha$ -(1 $\rightarrow$ 3)-glucosidic links lacked any biological activity. Misaki *et al.* (1981) also showed that having  $\beta$ -(1 $\rightarrow$ 6)-linked side chains of glucose residues increased anti-tumour activity. Only a relatively small number of glucans have been examined, but most subsequent work has focused mainly on  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6)-glucans. However, factors other than their branching frequency may be important. Of these, molecular size seems to affect biological activity. Thus, for scleroglucan, only preparations of  $<50 \times 10^4 \text{ g mol}^{-1}$  or  $>110 \times 10^4 \text{ g mol}^{-1}$  were effective (Falch *et al.* 2000). Conversely, only low molecular weight lentinan had high anti-tumour activity (Zhang *et al.* 2005), so generalizations of this kind are risky.

### Solubility of $\beta$ -glucans

The solubility of  $\beta$ -glucans depend on their degree of polymerization and thus their physical organization (Zekovic *et al.* 2005). Unsurprisingly, soluble  $\beta$ -glucans appear to be stronger immunostimulators than insoluble ones (Di Luzio *et al.* 1979; Di Luzio *et al.* 1980; Xiao *et al.* 2004), although the reasons are not totally clear, and orally administered insoluble  $\beta$ -glucans may be subsequently degraded into smaller bioactive oligomers after ingestion (Hong *et al.* 2004).

### Branching frequency

The branching frequencies of fungal  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6)-glucans are thought to determine their biological activity (Seviour

et al. 1992), and a branching frequency of 0.2 (1 in 5 backbone residues) to 0.33 (1 in 3 backbone residues) was suggested as being optimal (Miyazaki et al. 1979). Thus, although both unbranched bacterial curdlan and several  $\beta$ -linked linear synthetic carbon 4 and 5 oligosaccharides (Jamois et al. 2005) showed some biological activities, chemical addition of  $\beta$ -(1 $\rightarrow$ 6)-linked glucose residues to the curdlan backbone led to increases in its activity against mice sarcoma 180 cells (Kiho et al. 1998). Binding studies with receptors in U937 cells suggested that the more highly branched scleroglucan, and schizophyllan had a higher affinity than that shown by the infrequently branched laminarin from the alga *Laminara digita* (one every ten), but this in turn had a higher affinity than either linear glucan phosphate or glucan sulphate preparations (Mueller et al. 2000). In addition, scleroglucan has a much higher receptor binding ability than schizophyllan, although they reportedly have the same branching frequencies (Mueller et al. 2000). In this study the chemical structures of both  $\beta$ -glucan preparations were meticulously confirmed. Yet the important question of how and why branching frequencies might influence the host immune responses is not clear. It is also possible that the reported branching frequencies of their repeating units represent only the molecular average and that a considerable variation in it might occur within individual long  $\beta$ -glucans chains.

#### Branch modification

Some published information is available on how modifying the glucan side chains might affect their biological activity, and in some, it may increase it. When insoluble scleroglucan and its sulphated, carboxymethylated, methylated, hydroxyethylated and hydroxypropylated derivatives were tested against sarcoma 180 tumour and gastric carcinoma cells (Misaki et al. 1984; Wang et al. 2004), the sulphated and carboxymethylated forms showed the highest activity. The parent form had no anti-tumour activity against these cancers. Some evidence suggests that increased anti-tumour activity can also be achieved by polyol modification of the side chains residues of pestalotan (Misaki et al. 1984); but until more similar studies are performed with a greater diversity of glucans, any potential benefits from this approach will remain unclear.

#### Helical conformation

$\beta$ -Glucans can adopt either a single or triple helical conformation in solution, where hydrogen bonds hold the individual polymer chains together. Those with a triple helical configuration have been regarded as being the more powerful immunomodulators (Falch et al. 2000). For example, in a study by Falch et al. scleroglucan was only biologically active in a linear triple helical arrangement, and its subsequent denaturation reduced its cytokine inducing activity in monocytes (Falch et al. 2000). However, with schizophyllan a helical conformation by itself is not decisive and other factors are more important. Thus, in a single helical conformation schizophyllan stimulated TNF- $\alpha$  activity in U937, Th1, and human PBMC cells, but the native triple helical form was ineffective. A  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6)-glucan from *Glomerella cingulata* was effective against tumour growth in mice, regardless of whether it

existed as a triple helix, or in a single-helical conformation (Gomaa et al. 1992). Furthermore, chemically synthesized carbon 4- and 5- $\beta$ -linked oligosaccharides possess a high biological activity in the absence of any triple helical structure (Jamois et al. 2005).

Further study is required before we fully understand possible structure–function relationships in  $\beta$ -glucans. As already mentioned, orally administered  $\beta$ -glucans may be chemically and/or enzymatically modified following their ingestion, to generate smaller oligosaccharides (Hong et al. 2004). Thus, the actual molecules binding to the cell receptors to produce the effects discussed here may in fact be these smaller oligosaccharides. If this is the case, it may be possible, by careful choice of  $\beta$ -(1 $\rightarrow$ 3)(1 $\rightarrow$ 6)-endoglucanases or partial acid hydrolysis, to deliberately generate oligosaccharides of desired properties from chemically pure  $\beta$ -glucans. Consequently, assessing their biological activities could be fruitful. Unfortunately when enzymatic digestion of the structurally heterogeneous  $\beta$ -glucan from *Volvariella volvacea* generated several oligosaccharides with different branching frequencies, their individual anti-tumour activities were not reported (Kishida et al. 1989). In addition, it may be possible by selecting different cultural conditions to persuade the fungus to synthesize glucans with different branching frequencies, as reported for *Botryosphaera* spp. (Steluti et al. 2004), which might then be used as substrates for possible enzymatic modification. It is not known if the same approach would succeed with other  $\beta$ -glucan-producing fungi, although there may eventually be other culture-dependent methods to generate tailor-made  $\beta$ -glucans for work of this kind, once their biosynthesis and methods of assembly are better understood.

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#### Adverse effects and efficient usage

When purified fungal  $\beta$ -glucans have been administered orally for treatment of human diseases, no adverse effects in humans have been recorded. However, co-administration of zymosan, sonifilan, grofolan, SSG, and non-steroidal anti-inflammatory drugs such as indomethacin has been reported to cause death (Yoshioka et al. 1998; Takahashi et al. 2001). Furthermore, particulate or slightly soluble fungal  $\beta$ -glucans given intravenously were reported to cause granuloma formation, microembolization, inflammation and pain (Di Luzio et al. 1979; Chesterman et al. 1981; Zekovic et al. 2005). Crude preparations from basidiocarps with high  $\beta$ -glucan levels may also have adverse side effects. Some wild mushrooms may contain high concentrations of toxic metals including arsenic, lead, cadmium and mercury (Borchers et al. 2004), and preparations with high protein contents may cause allergic responses including asthma (Borchers et al. 2004). Thus, it is important to restrict those used for human ingestion to approved and certified  $\beta$ -glucan products of known chemical composition.

In addition, although  $\beta$ -glucans are found in all fungi, they are complexed in cellular structures with other components, which can make them difficult to digest and be absorbed. Ideally those fungi producing exocellular  $\beta$ -glucans under carefully controlled laboratory conditions are more attractive sources and should be given specific attention, especially for

large-scale production. Furthermore the range of fungi reported to produce them continues to increase, and often the growth conditions known to stimulate their production are documented (Seviour et al. 1992; Selbmann et al. 2004; Steluti et al. 2004; Rezende et al. 2005; Leung et al. 2006).

## Conclusions

In summary, fungal  $\beta$ -glucans appear to be beneficial to humans with impaired immune systems, and those suffering from infectious diseases and cancer, as well as in helping patient recovery from chemotherapy and radiotherapy. They are also especially beneficial to middle-aged people, people with active and stressful lifestyles, and athletes. The mechanisms of action of these fungal  $\beta$ -glucans appear to depend on their capabilities to bind to cell receptors, which are known to include dectin-1, CR3, LacCer, and scavenger receptors. This event then leads to activation of multiple signal pathways which in turn promote immune responses in the affected cells. Data suggest that different fungal  $\beta$ -glucans have different effectiveness probably through their different binding affinity to each receptor, although the chemical purity of the glucans used in these studies is not always known. Correspondingly, the choice of which  $\beta$ -glucan to use may be important. Unfortunately, we still do not understand what structural features are best for inducing activities.

Future studies now need to characterize mechanistically how individual  $\beta$ -glucans of known structure bind to each receptor. Only then can we rationally improve this technology. This information may be obtained from 1) more precise identification of  $\beta$ -glucan structural diversity from the purification, characterization and screening of additional fungal  $\beta$ -glucans with structurally attractive overall features 2) synthesis of  $\beta$ -glucans with these optimal structural features by deliberate microbial engineering or by selection of appropriate culture conditions.

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