# IMMUNOMODULATION BY DIETARY MUSHROOM COMPOUNDS

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## SUMMARY

Mushrooms are long used food supplements that display profound immunomodulatory activities, due to the presence of both polysaccharides (β-glucans) and proteins (FIP). These compounds work either alone or in concert and both target different aspects of the immune system. While β-glucans are considered to bind to innate receptors and activate innate cells (dendritic cells and monocytes/macrophages), immunomodulatory proteins will be taken up, processed and presented on antigen-presenting cells to CD4$^+$ Th cells in the context of MHC class II molecules. The outcome is different and results in the activation of both innate and antigen-specific adaptive immune reactivity. The combined presence in a whole extracts is therefore expected to be of more use than either active compound alone. Here we will describe some of the immunomodulatory activities present in such extracts from edible mushrooms.

## INTRODUCTION

**Immunomodulation**

Immunomodulation is the manipulation of the immune system by augmenting or decreasing the magnitude of the immune responsiveness. The augmentation of the immune response is known as immunostimulation or immunopotentiation, while suppression of immune responsiveness is called immunosuppression. The necessity of suppression of the function of the immune system is well recognized in the areas of transplantation and immunopathological disorders like autoimmunity. Conversely, augmentation of the immune response has been a target for increasing the host's resistance to infections and diseases. Specific immunomodulation is limited to a single antigen such as a vaccine and thus immunopotentiation is used for the development of resistance against particular diseases. Non-specific immunomodulation implies for a more generalized change in the immune responsiveness leading to altered host reactivity to many different antigens. Prevention and treatment of diseases are generally achieved by a wide range of antibacterial, antiviral, antiparasitic and antifungal agents and vaccines. The impact of chemotherapy and vaccination on many complex diseases, however, has reached a plateau and if further progress is to be made, different strategies have to be developed. Immunomodulation is one of the most important alternatives in order to control

diseases associated with the environment with additional advantages of amplifying specific responses to vaccines still needed to control severe, life-threatening diseases. The immunomodulation offers the additional benefit of reversing immunosuppression caused by various cancers, stress, infection, food-related problems, reproductive problems, and chronic inflammatory conditions.

**Diet**

A well-balanced diet is beneficial for a good immune function. The minerals zinc, copper, iron and selenium and the vitamins A, C and E have been established to be essential for a normal immune response (*Cunningham-Rundles* et al., 2005). Recently, food ingredients, such as fish oil, are being studied for potential immunomodulatory properties, and functional foods that influence the immune function are being developed, including pre- and probiotics (*Field* and *Schley*, 2004; *Salminen* et al., 1998).

It may be less well known that mushrooms have immunomodulating properties as well (*Wasser*, 2002). Some 140,000 species exist on earth of which some 14,000 species are described. Generally, mushrooms contain by weight approximately 90% water, 10-40% protein, 3-28% carbohydrate, 2-8% fat, 3-32% fibre and 8-10% ash (*Breene*, 1990). For millennia, they have been valued as tasteful foods and as medicinal substances. The knowledge and practice of the medicinal use of mushrooms originates from traditional eastern medicine and in Japan, China, Korea, and other Asian countries modern clinical practice still utilises mushroom-derived preparations. Over the past three decades scientific research has been performed on the medicinal properties of a growing number of mushroom species. Mushrooms

are claimed to exhibit antiviral, antibacterial, cholesterol-lowering, blood pressure-lowering and hypoglycaemic effects. Mushroom products are commercially available to contribute to the treatment of infectious diseases, cardiovascular disorders, diabetics, and to prevent various diseases (*Wasser* and *Weis*, 1999; *Zaidman* et al., 2005).

Several major substances with immunomodulatory and/or anti-tumour activity have been isolated from mushrooms. These include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides (PSP), polysaccharide proteins, and proteins. Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterized in mushrooms with proven medicinal properties. The major immunomodulating effects of these active substances derived from mushrooms include mitogenicity and activation of immune cells, such as haematopoietic stem cells, lymphocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, resulting in the production of cytokines. The therapeutic effects of mushrooms, such as anticancer activity, suppression of autoimmune diseases, and allergy have been associated in many cases with their immunomodulating effects.

The most extensively studied application of medicinal mushrooms is their anti-tumour activity. Polysaccharides, mostly β-glucans, have been established to be one of the most potent anti-tumour-active compounds (*Borchers* et al., 1999, 2004). While most regular chemotherapeutic agents are based on direct cytotoxicity to cells, β-glucans mediate their anti-tumour activity by stimulating the immune system (*Brown* and *Gordon*, 2003). Unfortunately, regular cancer therapy often has many serious side effects and can be unsuccessful. In addition, infectious diseases

become increasingly difficult to treat, as pathogens are becoming resistant to many antibiotics (*Sheldon*, 2005). β-glucans may contribute to the solution of these problems by stimulating the immune system to enhance anti-tumour and anti-infective responses.

## IMMUNOREGULATION AND CHRONIC INFLAMMATORY IMMUNE-MEDIATED DISEASES

Properties of many diseases, particularly systemic auto-immune diseases characterised by persistent inflammation, strongly support the involvement of helper T-lymphocytes (*Abbas* and *Lichtman*, 2005). For example, pathogenic auto-antibody responses generally are of high affinity IgG class, after having undergone affinity maturation, which requires helper T cells. The protein antigens to which many auto-antibodies are directed generally require T cell help. Many of the successful therapies, e.g. cyclosporin A, act primary on T cells. Besides roles as helpers, T cells may directly provoke cellular injury during inflammatory phases of the disease process. T cells, and in particular CD4$^+$ helper T cells produces effector molecules, called cytokines, upon activation. A multiplicity of cytokine abnormalities has been associated with various auto-immune and immune-mediated diseases. It is thus becoming common practice to analyse the role of helper T cells and the cytokines they produce in studying the immunpathological basis of particular diseases, to aid in the unambiguous diagnosis of the disease, to rationally design T cell and/or cytokine based immunotherapy protocols, and to provide parameters to monitor the efficacy of treatment.

Naive CD4$^+$ helper T cells (Th) develop into functionally mature effector cells upon stimulation with relevant antigenic peptides presented by major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APC). Based on the characteristic set of cytokines produced, Th cells are commonly segregated into at least two different subpopulations: Th1 cells producing exclusively interleukin-2 (IL-2), interferon-gamma (IFN-γ) and lymphotoxin (O'Garra and Arai, 2000). Th2 cells on the other hand, produce IL-4, IL-5, IL-6, IL-10 and IL-13. These Th1 and Th2 subsets appear to be extremes in cytokine production profiles and within these polarised subsets, individual Th cells exhibit differential rather than co-ordinated cytokine gene expression. The Th-1 and Th-2 subsets appear to cross-regulate each other's cytokine production profiles, mainly through IFN-γ and IL-10. From this concept it was rationalised that disturbances in the balance of between these two subsets may result in different clinical manifestations. IL-12 is a dominant factor promoting Th1 differentiation, and is produced by dendritic cells and macrophages. Moreover, IL-12 induces IFN-γ production by T cells and NK cells. It was reported that IL-18 acts as synergistically with IL-12 to induce Th1 development while polarisation of Th2 cells is critically dependent on the presence of IL-4 produced by Th cells, basophiles and mast cells. APC-derived IL-6 has also been shown to induce small amounts of IL-4 in developing Th cells. IL-10 and APC-derived prostaglandin E$_2$ (PGE$_2$) inhibit IL-12 production and Th1 priming (see Figure 1).
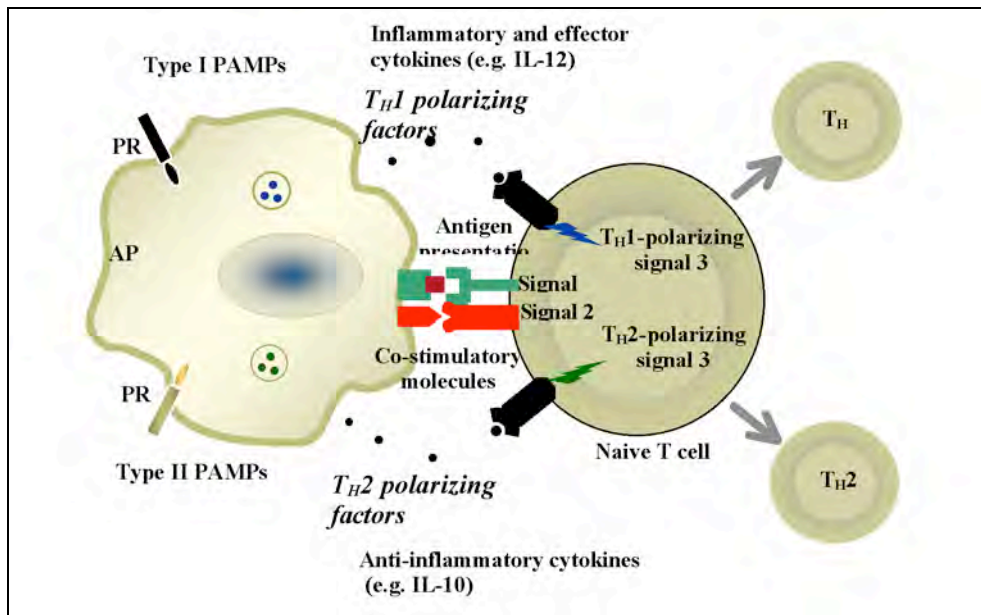
**Figure 1**: Cells and cytokines involved in Th1 or Th2 responses (Adapted from *O'Garra* and *Arai*, 2000).

Self-tolerance is induced in the T cell recognition repertoire by clonal deletion, anergy or silencing in the thymus. These processes are, however, not complete and thus potentially auto-reactive T cell can escape into the periphery where such T cells are continuously suppressed by cells that act functionally as suppressor cells. These cells, able to suppress other cells by cell surface mediated activity and/or production of suppressive cytokines, like IL-10 and (transforming growth factor) TGF-ß are now called regulatory cells. Regulatory T cells mediate active suppression of various immune responses. These T cells comprise classical Th2 cells, capable of inhibiting Th1 responses, but also alternative T cell populations. Two main populations are distinguished based on molecular and cellular differences: naturally occurring $CD4^+CD25^+$ naturally occurring regulatory T cells (Treg) that activate the Foxp3 transcription factor and that primarily detectable in the periphery. Alternatively, antigen-induced regulatory T cell populations (Tr, Th3) were identified based on their high secrewtion of IL-10 and TFG-ß and their relation with tolerance induction on mucosal surfaces in the gastro-intestinal and the respiratory system. The mechanism of peripheral tolerance has been focused mainly on the suppression of classical cell-mediated (IFN-γ producing) Th1 responses and in animal (models of) diseases based on excessive activity of Th1 cells. It is now clear that such tolerance induction is also active in humoral type 2 responses. One of the primary mechanisms of tolerance induction is via secretion of immuno-suppressive cytokines, like IL-10, IL-4 and TGF-ß. As mentioned before, regulatory T cells have been isolated from *in vitro* cultures, which appeared to produce low levels of IL-2, no IL-4, but high levels of IL-10 and TGF-ß. This demonstrates the importance of cytokines in regulating and dampening

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the immune response. It will thus be of crucial importance to determine whether the immunomodulating capacity of herbal and fungal polysaccharides in many diseases act via induction of these regulatory T cell subsets.

Recent progress has provoked a breakthrough in our understanding of basic mechanisms underlying the development of chronic inflammatory immune-mediated diseases by showing that Tr-cells are important to sustain T-cell tolerance. This is achieved via the production of cytokines, like IL-10. Tr cells are involved in the suppression of both Th1 (auto-immune) and Th2 (allergic) diseases. The role of Tr-cells is currently a focus in allergic disease model systems, as in mice. This research will tell much on the immunological mechanisms that are at the basis of the development of allergies, and provide opportunities for effective immune therapy based on induction of Tr-cells (*Boonstra* et al., 2000; *Van der Velden* et al., 2001).

## DEVELOPMENT OF ORAL TOLERANCE

Induction and/or maintenance of oral tolerance to orally ingested antigens also require microbial colonization of the alimentary tract in early life. As the intestine is the first line of defence from the environment, and must integrate complex interactions between diet, external pathogens, and local immunological and non-immunological processes, it is critical that protective immune responses are made to potential pathogens yet it is equally important that hypersensitivity reactions to dietary antigens are minimised. Thus, the gut immune system must distinguish not only between self and non-self, but also between potentially dangerous foreign antigens and common harmless foodstuffs to which it is constantly exposed. Such suppressive mechanisms to avoid local and peripheral overreaction (hypersensitivity) against innocuous substances bombarding the mucosal surfaces are referred to as 'oral tolerance' when induced via the gut against dietary antigens (*Brandtzaeg*, 2006). Similar down-regulatory mechanisms apparently operate against antigens from the commensal micro flora.

In physiological circumstances tolerance towards the indigenous intestinal microbiota is established and maintained. Different doses of orally administered antigens may induce anergy in antigen-specific T cells or may stimulate the production of cytokines, like TGF-ß, that inhibit lymphocyte proliferation, resulting in suppression of the immune response. Moreover, since TGF-ß also induces isotype switching to IgA antibody production, the mucosal immune system is further protected (*Abbas* and *Lichtman*, 2005). It is unclear why soluble proteins in large doses induce systemic T cell tolerance, whereas oral immunisation with attenuated poliovirus vaccines induces protective T-cell dependent antibody responses and long-lived memory.

Permeability of the intestinal barrier is greatly enhanced during foetal life, but gut closure starts before birth and is considered more or less complete by 33 weeks of gestation. Paracellular permeability can thereafter be increased, resulting in sensitisation to dietary antigens, large enough to be presented by DC to T-cells and resulting in a pro-inflammatory response. Pro-inflammatory cytokines, like IFN-γ and TNF-α induce paracellular leakiness, while IL-10 and TGF-ß promote tight junc-

tion formation and particularly these cytokines play a central role in oral tolerance (*Adams* et al., 1993; *Planchon* et al., 1994).

Besides, there is evidence indicating that IL-10, a tolerogenic cytokine, is produced in response to microbial stimuli: Mice with defective IL-10 production infected with *Helicobacter hepaticus* developed Th1-type intestinal inflammation, whereas normal mice produced IL-10 and remained health. Interestingly, IL-10 deficient mice have decreased levels of resident lactobacilli in the neonatal period, and normalising the amount of these bacteria in the colon prevented the development of intestinal inflammation. In humans, carriers of *H. pylori* infected individuals posses Treg that can *in vitro* suppress responses of antigen-specifically stimulated T cells. *In vivo* it was suggested that the induction of *H. pylori* specific Treg cells suppress protective antigen-specific responses and contribute to the persistence of the infection (*Penner* et al., 2005).


# IMMUNOMODULATION BY MUSHROOM-DERIVED ß-GLUCANS

## Structure of β-glucans

The interest in β-glucans as anti-infective and anti-tumour agents originates from the early 1900s, when an insoluble yeast (*Saccharomyces cerevisiae*) cell wall particle, named zymosan, was developed. Experiments showed that intravenous injection of zymosan could stimulate the immune system. Later β-glucan was identified as the biologically active constituent. β-glucans are a heterogeneous group of glucose polymers, mostly consisting of a backbone of $\beta(1\rightarrow3)$-linked β-D-glucopyranosyl units with $\beta(1\rightarrow6)$-linked side chains of varying distribution and length (see Figure 2).

These polysaccharides are major cell wall structural components in fungi and are found in plants and some bacteria as well. As they are not present in animals, they are recognized by the innate immune system and are considered to be classic pathogen-associated molecular patterns (PAMP). β-glucans mostly show a triple-strand right winding helix structure. Various β-glucans have been isolated from diverse mushroom species showing different immunological activity, which could be correlated with their solubility in water, molecular weight, conformation (tertiary structure) and degree of branching. Lentinan (*Chihara* et al., 1970; *Yap* et al., 2001) is isolated from the fruiting body of *Lentinus edodes* (shiitake mushroom) and consists of five $\beta(1\rightarrow3)$-β-D-glucopyranosyl units in a linear linkage and two $\beta(1\rightarrow6)$-linked side chains (degree of branching: 0.4). The molecular weight is about $4\text{-}8 \times 10^5$ g/mol. Grifolan has a degree of branching of 0.33 and a molecular weight of approximately $5 \times 10^6$ g/mol. The compound is isolated from the liquid-cultured mycelium of *Grifola frondosa* (maitake mushroom). Another β-glucan has been extracted from this mushroom: Maitake D-fraction (*Kodama* et al., 2003). This is a high molecular weight polysaccharide. In contrast with other β-glucans, which have a $\beta(1\rightarrow3)$ main chain, D-fraction consists of a $\beta(1\rightarrow6)$ main chain with $\beta(1\rightarrow3)$ branches. Schizophyllan is obtained from a culture medium of *Schizophyllum commune* (split gill fungus) (*Hashimoto* et al., 1991). Its branching rate is 0.33 and the molecular weight is approximately $4.5 \times 10^5$ g/mol. SSG is a compound isolated from the culture filtrate of *Sclerotinia*
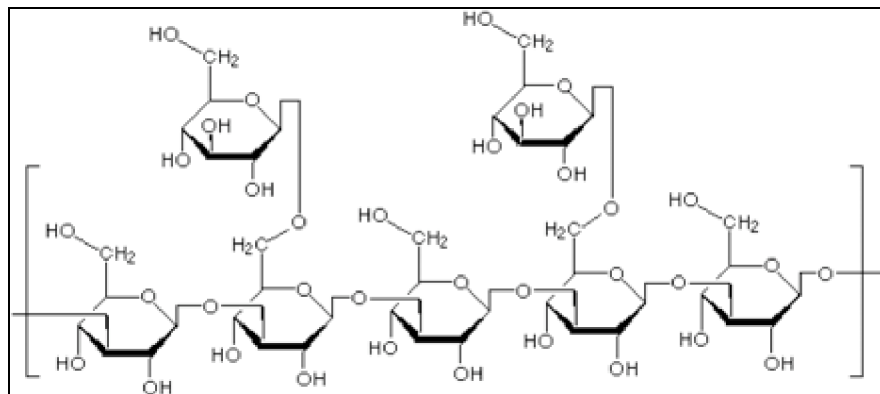
**Figure 2**: Chemical structure of the branched β-glucan lentinan.

*sclerotiorum* (white mould) and has a branching rate of 0.5 and a molecular weight of approximately 2 x $10^6$ g/mol. As this field of research is growing new mushroom species are being investigated for their immunostimulating glucans, including *Agaricus blazei*, *Sparassis crispa*, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Sclerotium glucanicum* (*Brown* and *Gordon*, 2003; *De Ruiter* et al., 1992; *Karacsonyi* and *Kuniak*, 1994; *Kulicke* and *Lettau*, 1997; *Dong* et al., 2002). Several animal studies were performed on the use of β-glucans in foods to modulate natural disease resistance (*Charampopoulos* et al., 2002; *Chesterman* et al., 1981; *Gibson* and *Roberfroid*, 1995) and to study the influence of β-glucans to reproductive and pregnancy-related problems (*Cozens* et al., 1981a; 1981b; 1981c).

**Effects of β-glucans on the immune system**

Particular mushroom β-glucans have an immunomodulatory and anti-tumour effects (*Bohn* and *BeMiller*, 1995). These substances are regarded as biological response modifiers. This basically means that they cause no harm and place no additional stress on the body; they help the body to adapt to various environmental and biological stresses; and they exert a non-specific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune systems, as well as regulatory functions.

Three β-glucans are used as biological response modifiers: Lentinan from *Lentinus edodes,* D-fraction from *Grifola frondosa,* and schizophyllan from *Schizophyllum commune.* Lentinan and schizophyllan are approved in Japan for clinical use to improve the immunity of cancer patients. β-glucans are used as a mild and non-invasive form of treatment of cancer and other diseases, and in the prevention of metastasis spread of tumours. In cancer treatment they can be used as a co-treatment in conjunction with other forms of therapy, such as chemotherapy and surgery.

The anti-tumour action of β-glucans is largely mediated via activation of the immune response; the polysaccharides do not attack cancer cells directly (*Arinaga* et al., 1992a; 1992b; *Baba* et al., 1986; *Chihara* et al., 1970; *Maeda* et al., 1988; *Minato* et al., 1999; *Ng* et al., 2002; *Oka* et al., 1992, 1996; *Ooi* et al., 2000; *Sudate* al., 1996; *Taguchi* et al., 1980, 1987; *Usui* et al., 1983; *Wada* et al., 1987; *Wasser*, 2002; *Zaidman* et al., 2005; *Zhang* et al., 2002, 2005). Experiments showed that the anti-tu-

mour effect was lost in neonatal thymectomised mice or after administration of anti-lymphocyte serum. These results suggest that the anti-tumour action is T-lymphocyte dependent. In addition, macrophages play an important role, because the anti-tumour activity can be inhibited by pre-treatment with anti-macrophage agents. The production of various cytokines is induced by β-glucans, which results in the proliferation, maturation, and differentiation of immune cells, such as NK cells and T-lymphocytes.

Besides anti-tumour activity, many β-glucans are thought to possess anti-infective activity against various bacterial, viral, and parasitic infections (*Irinoda* et al., 1992). For example, schizophyllan demonstrated protective effects against *Staphylococcus aureus,* and *Escherichia coli* infections in mice. SSG reduced the number of live intracellular *Mycobacterium tuberculosis* in macrophage cultures when it was incubated together with the bacteria (*Markova* et al., 2003; *Hetland* et al., 2002).

Little is known about the biological effects of β-glucans after oral administration (*Rice* et al, 2005). Oral administration of glucan phosphate led to a significant, but modest, increase in the serum level of the pro-inflammatory cytokine IL-12. No changes were observed in serum levels of other tested cytokines. Oral administration of glucan phosphate to mice one day before challenge with *Staphylococcus aureus* or *Candida albicans* led to increased long-term survival.

The precise mechanism which mediates the effects of β-glucans on the immune system is not totally clarified yet. However, research is starting to shed some light on the cellular receptors and molecular mechanisms involved (*Brown* et al., 2003; *Falch* et al., 2000; *Hamano* et al., 1999; *Hamuro* et al., 1974; *Kataoka* et al., 2002;

*Kerekgyarto* et al., 1996; *Kodama* et al., 2003; *Liu* et al., 1999; *Masihi* et al., 1997; *Murata* et al., 2002; *Oka* et al., 1992, 1996; *Ooi* and *Liu*, 2000). The immune response triggered by β-glucans was primarily designed for the control of fungal pathogens. The immune response to pathogens relies upon the cooperation between the innate and the adaptive immune system. Innate immunity is the first line of defence against infections. Cells of the innate immune system recognise structures that are characteristic for microbes, such as lipopolysaccharides in Gram-negative bacteria and β-glucans in fungi. The receptors for these structures, called pattern recognition receptors (PRR), include scavenger, lipopolysaccharide, mannose, β-glucan, and Toll-like receptors (TLR). Innate immunity to fungi is mainly mediated by neutrophils and macrophages. They liberate fungicidal substances, like reactive oxygen intermediates and lysosomal enzymes, and phagocytose fungi for intracellular killing. Helper T cells and cytotoxic T cells cooperate in the adaptive immune response against fungal infections.

**Role of β-glucan receptors**

To date, four different β-glucan receptors have been identified: Complement receptor type 3 (CR3), lactosylceramide, scavenger receptors and Dectin-1. They have been reported on monocytes, macrophages, neutrophils, eosinophils, natural killer (NK) cells, certain lymphocytes, as well as on non-immune cells including endothelial cells, alveolar epithelial cells, and fibroblasts.

CR3 is a heterodimeric integrin receptor, consisting of CD11b and CD18 chains and is expressed on monocytes, neutrophils, NK cells, and selected lymphocytes. It functions as a phagocytic receptor for iC3b-opsonized par-

ticles (iC3b is an inactive complement protein), including opsonised particulate glucans, and it possesses a lectin domain, which recognises a variety of β-glucans directly. As leukocytes lacking CR3 can still bind and respond normally to β-glucans, the receptor does not seem to be indispensable. On the other hand, CR3-dependent cytotoxicity to tumour cells is stimulated by β-glucan priming: Experiments have shown that incubation of NK cells or neutrophils with small soluble β-glucans primed the CR3-receptor to enhance the cytotoxicity against iC3b-opsonised target cells that were otherwise resistant to CR3-mediated cytotoxicity. In addition, it plays a role in the recruitment of leukocytes to sites of inflammation by binding to adhesion molecules on endothelial cells.

Lactosylceramide is a glycosphingolipid present in the plasma membranes of many cells. It has been suggested that the interaction of β-glucans with this receptor can induce macrophage inflammatory protein-2 and the activation of the nuclear transcription factor NF-κB and can enhance the neutrophil oxidative burst and antimicrobial functions. The mechanisms are still unknown. There are indications that macrophage scavenger receptors can recognise β-glucans as well.

Dectin-1, a receptor first discovered in mice, seems to have an important role in mediating the biological response to β-glucans (*Gantner* et al., 2003; *Herre* et al., 2004). It is a transmembrane receptor with an immunoreceptor tyrosine-based activation (ITAM) motif in its cytoplasmic tail. Upon β-glucan binding to the extracellular side of Dectin-1 at the cell surface, the ITAM motif becomes phosphorylated, generating a signal which induces phagocytosis and the respiratory burst (the production of reactive oxygen intermediates). It is

present on monocytes, macrophages, and neutrophils, and at lower levels on dendritic cells and a subpopulation of splenic T lymphocytes. Human Dectin-1 differs from its murine counterpart in that it is alternatively spliced, in a cell-specific manner, giving rise to several isoforms of which only two are functional. They are sometimes referred to as the β-glucan receptor and are similar in structure and function to murine Dectin-1. Dectin-1 also recognises an endogenous ligand on activated T cells in a β-glucan independent manner and may act as a T cell co-stimulatory molecule. Zymosan, a particle from yeast cell wall which consists of a variety of compounds, including β-glucans, mannans, mannoproteins and chitin, was found to interact with Toll-like receptors (TLR). Both TLR2 and TLR6 are required for activation of NF-κB in macrophages and dendritic cells, leading to the production of the pro-inflammatory cytokines TNF-α and IL-12 in response to zymosan. The adaptor protein MyD88 mediates the intracellular signalling to NF-κB. The binding of β-glucans to Dectin-1 alone does not stimulate the production of TNF-α and IL-12. However, Dectin-1 enhances the production of these pro-inflammatory cytokines when TLR2 is stimulated. TLR2 was found not to recognise β-glucans, but some other component of zymosan. This suggests that the strong pro-inflammatory activities reported for β-glucans in many studies, might be partly caused by unidentified TLR2 triggering contaminants in impure extracts. This hypothesis is supported by the results of a study performed by *Kataoka* et al. (2002) who found that branched (1→3)β-glucans, like lentinan and schizophyllan, could not stimulate NF-κB activity in macrophages. Taken together, for the activation of NFκB and the induction of a pro-inflammatory

response Dectin-1 needs to cooperate with TLR-2. The activation of Dectin-1 alone by β-glucans is sufficient to stimulate the respiratory burst and phagocytosis (see Figure 2). It seems that TLR2 does not recognise β-glucans but other compounds present in zymosan

Recently, new insights in the role of Dectin-1 in the response to β-glucans were provided by *Rogers* et al. (2005) and by *Palma* et al. (2006). Mouse B cell hybridoma cells (which normally cannot bind zymosan) were transduced with Dectin-1. These Dectin-1 transduced cells were now able to bind β-glucans of zymosan and produced IL-2 and IL-10. This shows that cytokine production is also occurring independent of TLR signalling. The same research group clarified part of the signalling pathway of Dectin-1. They discovered that a kinase called Syk was recruited upon β-glucan binding to Dectin-1. They also found that single phosphorylation of the membrane-proximal tyrosine (position 15) in the ITAM-motif is sufficient to recruit Syk and to couple to downstream IL-2 and IL-10 responses. Experiments were performed with Syk-deficient dendritic cells from chimaeric mice and it was found that these cells were completely unable to produce IL-2 or IL-10 in response to zymosan but could still bind zymosan and produce normal levels of IL-12 and IL-6. It can be concluded that Syk is required for zymosan-induced IL-2 and IL-10 production, but not for IL-12 synthesis, which could be signalled via the TLR2 pathway. IL-10 induction can occur independently of TLR signalling; IL-2 production is enhanced by TLR signalling (see Figure 3).

The importance of the Dectin-1/Syk pathway lies in the biological function of IL-2 and IL-10 during an infection. IL-2 and IL-10 stimulate the development of regulatory T cells thereby limiting immunopathology to a local infection, allowing persistence of immunity and resistance to re-infection, and maintaining immunological memory. In addition, IL-10 acts on activated macrophages to terminate their inflammatory responses and returning the immune system to its resting state. The Decin-1/Syk pathway could therefore be exploited therapeutically in allergy, autoimmunity, and graft rejection. Normally, resistance to yeast infections is characterised by a strong T helper1-type response, in which IL-12 and TNF-α play an important role. In this way the infection can be cleared effectively.

In summary, it seems that β-glucans induce the production of IL-2 and IL-10 and the development of regulatory T cells via the Dectin-1/Syk pathway, while other compounds present in zymosan (and probably other fungal compounds) stimulate a pro-inflammatory T helper1-type response with production of IL-12 and TNF-α. Dectin-1 is responsible for phagocytosis and induction of the respiratory burst as well. Furthermore, CR3, lactosylceramide, and scavenger receptors mediate the stimulatory effects of β-glucans on the immune system. It should be noted that the amount or influence of the cytokines produced via the Dectin-1/Syk pathway might be small, because in animal and *in vitro* experiments a pro-inflammatory cytokine pattern is usually found, in which IL-12 and TNF-α predominate, at least for lentinan. This may be explained by the influence of other β-glucan receptors or by contaminants binding to Toll-like receptors.

**Oral delivery and gastro-intestinal absorption**

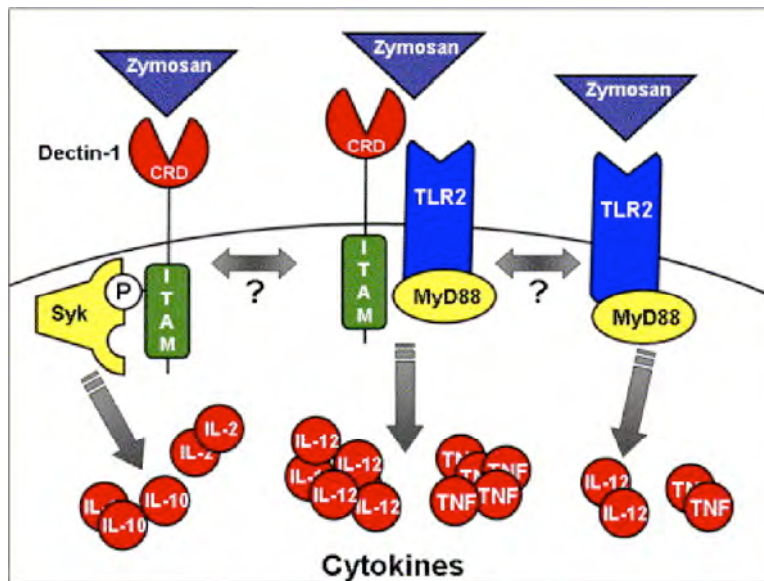To understand if and how oral delivery of β-glucans can have a biologi

**Figure 3**: Zymosan can interact with Dectin-1 leading to production of IL-2 and IL-10. Triggering of TLR2 induces IL-12 and TNF-□ production and cooperative signalling of Dectin-1 with TLR2 augments this (From *Rogers* et al., 2005).

cal effect it is important to know if they are absorbed in the gastrointestinal tract, as humans cannot digest them. *Rice* et al. (2004, 2005) examined the absorption, pharmacokinetics, and biological effects of the three water-soluble β-glucans glucan phosphate, laminarin and scleroglucan that were administered by oral gavages to rodents. Laminarin (molecular weight: $7.7 \times 10^3$; degree of branching (DB): 1/10; single helix) and scleroglucan (MW: $1.02 \times 10^6$; DB: 1/3; triple helix) both have a backbone of $(1\rightarrow3)$-β-D-glucopyranosyl units and $\beta(1\rightarrow6)$-linked side chains and mainly scleroglucan is comparable to the mushroom β-glucans described above. Oral administration produced measurable plasma levels of the three β-glucans. Laminarin showed two peak plasma levels: one at 3 hours and one at 12 hours. Scleroglucan plasma levels peaked twice as well: at 15 minutes and at 3 hours. It is interesting that scleroglucan, the largest β-glucan, was ab-

sorbed most rapidly. The maximum plasma level was 115 ng/ml for laminarin and 355 ng/ml for scleroglucan when rats were given an oral dose of 1 mg/kg. It is noteworthy that most *in vitro* studies are performed with higher concentrations of β-glucans (approximately 5-500 μg/ml), so it is questionable if the low plasma levels after oral administration are sufficient for immunostimulation. In contrast, *Tani* et al. (1992) performed an *in vitro* study with macrophages and NK cells and stated that 25 to100 ng/ml was the optimal concentration of lentinan to improve cytotoxicity. The bioavailability of laminarin and scleroglucan was 4.9 and 4.0%, respectively. A water-insoluble, particulate glucan preparation was not detected in plasma. A possible explanation is that particles are phagocytosed and transported by macrophages.

Fluorescently labelled β-glucans were used to determine which cells in the gastrointestinal tract could bind them. It was demonstrated that gut-as-

sociated lymphoid tissue (GALT) cells, isolated from Peyer's patches, can bind β-glucans. Macrophages showed an increase in Dectin-1 expression and dendritic cells increased their TLR2 expression. In an experiment with intestinal epithelial cells it appeared that only a subpopulation (10%) of these cells incorporated β-glucan. These cells were found not to express Dectin-1 receptor, so uptake of β-glucans by this cell type could not have been mediated by this receptor in contrast to GALT cells. *Hashimoto* et al. (1991) suggested that high molecular weight β-glucans may be taken up by microfold cells in the intestine, where they interact with the GALT. It is possible that the subpopulation of epithelial cells in Rice's experiment consists of microfold cells. The data support an active uptake mechanism for β-glucans.

A different explanation might be a prebiotic effect of β-glucans on the gut flora. A prebiotic is a food ingredient that is not hydrolysed by the human digestive enzymes in the upper gastro-intestinal tract and beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that can improve host health. Oat β-glucan has been reported to selectively support the growth of lactobacilli and bifidobacteria in rat experiments and in *in vitro* studies. To date, it is not known if mushroom β-glucans could have a similar effect. In addition, there could be an effect of contaminating substances like proteins or fats in impure lentinan extracts.

It should be noted here, that the choice to use laminarin and glucan phosphate for these experiment by *Rice* et al. (2004,2005) is unusual, because laminarin is generally considered inactive and the biological effects of glucan phosphate are not clear. Taken together, these experiments show that orally administered β-glucans interact with a variety of gastrointestinal cells, enter the systemic circulation, and persist in the plasma up to 24 hours.

**Distribution, metabolism, and excretion**

*Suda* et al. (1996) studied the distribution and metabolism of i.p. administered, labelled SSG (MW: $2x10^6$) in mice. Following administration, the concentration of SSG was first high in peritoneal exudate and blood, but these concentrations lowered sharply in 48 hours. In contrast, concentrations rose mainly in liver and spleen, and a slight increase was found in the kidney, intestine and faeces. After one month about 30% of administered SSG was present in the liver and about 10% in the spleen.

Because of the absence of (1→3)-β-D-glucanase in mammals, these glucans are thought to be metabolised by oxidative degradation by macrophages (*Miura* et al., 1995, 1996). However, the majority of SSG in liver and spleen was recovered from the non-cellular fraction and not from macrophages. These results suggest that β-glucans are not easily taken up by macrophages to degrade and exclude them from the body. Even five weeks after administration, the metabolites of SSG extracted from the liver of the mice retained significant anti-tumour effects. Nevertheless, the molecular weight of SSG in the liver was lowered with time, and it became about $1x10^5$ g/mol. It was suggested that the degree of branching decreased as well. In contrast, no significant change was observed in the molecular weight and degree of branching of SSG in the spleen.

The elimination of β-glucans from the blood is quite slow (*Sortwell* et al., 1981). After a single dose (intravenous: 1 mg/kg) administered to rats, the elimination half-life was 2.6 hours for

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laminarin and 3.1 hours for scleroglucan. The administration (i.p.) of multiple doses of β-glucans (0.25 mg/week) to mice led to saturation of the liver and spleen and circulation of glucan in the blood, as blood glucan concentrations were high all the time.

Not much is known about the clearance of β-glucans from the whole body. Lower molecular weight β-glucans (such as glucan phosphate, MW: $1.3 \times 10^5$) are possibly cleared by glomerular filtration in the kidney. High molecular weight glucans (such as lentinan) are retained mainly in the liver and degraded by liver macrophages, the Kupffer cells, which may take several weeks.

## EFFECTS OF LENTINAN ON THE IMMUNE SYSTEM

The immunomodulatory effects and the applications of lentinan are various. Lentinan is well known for its anti-tumour effect, and recently the anti-infective properties are being appreciated as well. Lentinan influences many components of the immune system: Several cells as well as molecules. *Chihara* et al., (1969) reported the anti-tumour activity of lentinan after conducting an experiment with Sarcoma 180 transplanted in CD-1/ICD mice. These results were confirmed by other research groups, like *Baba* et al (1986), who found a rapid decrease in the number of tumour cells from sarcoma 180 tumours in ICR mice and an accumulation of polymorphonuclear leukocytes (now called neutrophils) in the tumour after i.p. lentinan injection. Later, lentinan also showed anti-tumour activity to syngeneic and autochthonous tumours. Tumour regressions of up to 100% were reported in experimental animals in different studies. Clinical studies with lentinan in cancer therapy followed, although very few are placebo-controlled and double-blind, and showed that lentinan was particularly effective in patients with gastric and colorectal cancer. A follow-up, randomised controlled study in patients with advanced and recurrent stomach cancers showed a prolongation of life. The results of clinical studies also gave indications that lenti-

nan treatment increased NK and LAK cell activity and induced IL-1 and TNF-α production by human monocytes/macrophages. LAK cells (lymphokine activated killer cells) are IL-2 activated NK cells. There are also indications that lentinan could prevent chemical and viral oncogenesis. Furthermore, i.p. injection of lentinan in mice induced vascular dilatation and haemorrhage dose-dependently. This response is probably mediated by T cells and macrophages and correlates well with anti-tumour activity. Recently, many other possible applications for lentinan besides tumour suppression have been investigated. Lentinan may be useful in boosting the immune response against various infections. Lentinan has shown antiviral (e.g. human immunodeficiency virus, *Herpes simplex*), antibacterial (e.g. *Mycobacterium tuberculosis, Listeria monocytogenes*), antiparasitic (*Schistosoma* spp.), and antifungal effects (*Candida albicans*). *Irinoda* et al. (1992) treated mice with lentinan (intranasal or intravenous) before giving them an aerosol of virulent influenza virus. Significant protection was achieved by administrating 200 µg of lentinan intranasally.

The protective effects of lentinan against *Mycobacterium tuberculosis* infection were studied by *Markova* et al. (2003) in *in vitro* and *in vivo* mouse

models. The administration of lentinan before infection at a dose of 1 mg/kg three times at 2-day intervals could reduce mycobacterial infection. Peritoneal macrophages from animals treated with lentinan were greatly stimulated. *Wierzbicki* et al. (2002) studied the effect of the addition of lentinan to an orally administered vaccine against human immunodeficiency virus (HIV) envelope glycoprotein. Lentinan was found to increase envelope glycoprotein-specific T-helper 1 type cytokine production (IL-2 and IFN-γ) and cytotoxic T-lymphocyte activities but had no effect on humoral responses.

**Influence at the cellular level**

Lentinan exerts its influence on different cells of the immune system. An *in vitro* assay showed that phagocytosis by mouse macrophages was enhanced. In addition, *in vivo* lentinan administration to mice led to a higher number of macrophages, a higher percentage of activated macrophages, and enhanced antibody-dependent macrophage-mediated cytotoxicity compared with controls. In tumour-bearing mice the cells responsible for the anti-tumour activity of lentinan were studied. T cells played a role in the specific cytotoxicity and NK cells contributed to the a-specific cytotoxicity to tumours (*Borchers* et al., 1999).

In an *in vitro* experiment human peripheral blood mononuclear cells were cultured with lentinan (25 to 1000 ng/ml). Cytotoxicity of macrophages and NK cells was increased. The optimal concentration of lentinan was from 25 to 100 ng/ml, which is equivalent to the plasma concentration obtained after clinical doses of this agent (*Tani* et al., 1992).

In 15 patients with gastric carcinoma, peripheral blood mononuclear cells were obtained before and 3, 5, and 7 days after lentinan administration (2 mg i.v.). The ability to generate lymphokine activated killer (LAK) cell activity, tested by *in vitro* activation of blood cells with IL-2 was significantly augmented 5 days after lentinan injection, when compared with before administration. LAK cells are IL-2 activated NK cells. NK cell activity was significantly enhanced after seven days. The conclusions of this study can be questioned, because it was performed without control patients receiving a placebo, and the data of only 9 of 15 patients are presented without an explanation for the missing data (*Arinaga* et al., 1992).

In an *in vivo* study tumour-bearing mice received chemotherapy with or without lentinan (0.1 mg/day i.p.). Additional lentinan treatment induced increased intra tumour CD86$^+$ dendritic cell (DC) infiltration and splenic DCs were more potent stimulators of T cell proliferation. In addition, the activity of splenic cytotoxic T cells was increased. Furthermore, the survival period of the mice treated with lentinan was significantly longer than that of mice treated with chemotherapy alone. It should be noted here, that the immunohistochemical staining for CD86 is not very specific for DCs, as other cells also possess CD86 (*Musiake* et al., 2005).

The effect of lentinan on B cells, neutrophils, eosinophils, basophils and mast cells is not clarified yet. However, it is known that neutrophils can bind lentinan. Monocytes bind lentinan stronger, but lymphocytes bind lentinan only minimally. This suggests that the effect of lentinan on lymphocytes is mediated by the stimuli of DCs and monocytes/macrophages, such as cytokine production. Lentinan is able to restore the suppressed activity of helper T-cells to their normal state in tumour-bearing hosts, leading to restoration of humoral immune responses. In addi-

tion, lentinan promotes a skewing of the Th1/Th2 balance towards Th1 (*Oka* et al., 1996).

**Influence on Th1/Th2 balance**

A very important feature of lentinan is that it affects cytokine production. Lentinan influences the cytokine production of different cells, including macrophages and T helper cells. Activated macrophages produce cytokines which have a role in inducing inflammatory reactions and in stimulating T cells. In this way T helper cells are stimulated to differentiate, and their cytokines activate macrophages and B cells.

$CD4^+$ T cells can differentiate into different subsets, of which T helper 1 (Th1) and T helper 2 (Th2) cells are very important (see Figure 1). The two subsets are distinguished by the cytokines they produce: IFN-$\gamma$ is the signature of Th1 cells; and IL-4, IL-5, IL-10, and IL-13 are produced by Th2 cells. These cytokines determine the effector functions and promote growth or differentiation of their own respective subset. The development of Th1 cells is induced by IL-12 produced by activated macrophages and dendritic cells and is antagonised by IL-4 and by IL-10. IL-4 favours induction of Th2 cells. Th1 cells are important for the intracellular destruction of phagocytosed pathogens, including bacteria, parasites, yeasts and viruses and the elimination of cancer cells. IFN-$\gamma$ acts on macrophages to stimulate phagocytosis and killing of pathogens and on B-lymphocytes to produce opsonising antibodies. Th1 cells also produce TNF-$\alpha$, which activates neutrophils and stimulates inflammation, and IL-2 which acts as an autocrine growth factor. The principal function of Th2 cells is in eradicating helminths and other extracellular parasites by activating mast cells and eosinophils, and stimu-lating IgE-production. If uncontrolled, Th1 cells can mediate immunopathology and autoimmune diseases. Over-activation of Th2 cells can lead to allergic manifestations.

*Murata* et al. (2002) found that lentinan administered i.p. to C57BL/6 and DBA/2 mice could skew the T-helper response toward Th1. Peritoneal macrophages isolated from lentinan-treated animals could produce more nitric oxide and IL-12 in response to stimulation, while the production of IL-6, IL-10, and Prostaglandin E2 ($PGE_2$) was decreased. $PGE_2$ is known to function as an endogenous immuno-suppressive mediator. Nitric oxide is considered an effector molecule of cytotoxic macrophages against tumours. The lowered amount of IL-6 contributes to the inhibition of Th2 induction. IL-12 strongly stimulates Th1 development and the lowering of IL-10 neutralises the inhibition of activation of Th1 cells by IL-10. To study the effect on the Th cells, lentinan (5 mg/kg) or saline (0.5 ml) was injected i.p. on days 1, 3 and 5 and spleens were harvested the day after the final injection. The culture supernatants of purified splenic $CD4^+$T cells stimulated for 24 h with coated anti-CD3 antibody were analysed for IFN-$\gamma$ and IL-4. IFN-$\gamma$ was significantly increased, but IL-4 levels did not change significantly. This indicates a polarisation toward Th1, which can be related to the cytokine pattern induced in macrophages. Skewing of Th1/Th2 balance to Th1 favours cellular immune responses against tumours and intracellular pathogens.

Experiments performed with other cell types also give indications for a Th1 polarisation. *Liu* et al. (1999) administered lentinan i.p. to mice and the cytokine gene expression levels of IL-1$\alpha$, IL-1$\beta$, TNF-$\alpha$, IFN-$\gamma$, and monocyte colony stimulating factor (M-CSF) were analysed in peritoneal exudate

cells (PECs) and splenocytes. The biological effects of IL-1 are similar to those of TNF: they both induce inflammation. The expression of all five cytokines was up-regulated in PECs of treated mice; in splenocytes only IL-1α expression was not up-regulated. This cytokine pattern was also found in a study by *Arinaga* et al. (1992a,b), in which patients with gastric carcinoma received intravenous administration of lentinan. The ability of monocytes to produce IL-1α, IL-1β, and TNF-α *in vitro* in response to LPS stimulation was significantly augmented as compared with before treatment. It should be noted that this study was not placebo controlled.

Although most studies show a similar cytokine profile, the production of these molecules seems to be dependent on the genotype of the host and the specific health/disease status. *Kerekgyarto* et al. (1996) found that the cytotoxic activity and TNF secretion of murine macrophages was elevated by lentinan when applied *in vitro* or *in vivo*. The effectiveness of lentinan to induce these responses was highly influenced by the genotype of the host. In the experiment by Irinoda with mice

infected with influenza virus (described above), TNF production could not be detected. The amount of IL-6 produced differed between uninfected and infected mice: in uninfected mice, IL-6 was higher in lentinan treated mice than in untreated mice; in infected mice, lentinan treated mice first showed higher IL-6 levels, later much lower levels than untreated mice. IL-6 has diverse actions including the stimulation of the synthesis of acute-phase proteins by the liver and the proliferation of antibody-producing cells.

A remarkable cytokine pattern was observed by *Masihi* et al. (1997) in their experiment with bacillus Calmette-Guerin (BCG)-primed mice. BCG is widely used as a vaccine against tuberculosis. It has also been recognized as an immune modulator and it induces local inflammation. Mice were pre-treated with lentinan and LPS was used to stimulate cytokine production. Lentinan induced an inhibition of up to 82% of TNF, a moderate reduction of 25% of IL-lβ, and no significant differences in IL-6 or IL-10 levels, and a marked depression of chemiluminescence (respiratory burst) activity.

## IMMUNE ACTIVITIES OF FUNGAL POLYSACCHARIDES

Mushrooms are abundant sources of a wide range of useful natural products (*Zaidman* et al., 2005; *Borchers* et al., 2004; *Wasser*, 2002; *Ooi* and *Lui*, 2000). Medicinal properties have been attributed to mushrooms for thousands of years, particularly in traditional Chinese and Japanese medicine.

Mushrooms have recently attracted much attention on account of their *in vivo* and *in vitro* immunomodulatory activity (*Shamtsyan* et al., 2004), which has been demonstrated for many mushrooms, including extracts and isolated compounds from the fruiting body,

spores, mycelia, and culture medium of various mushrooms (*Kodama* et al., 2003, 2005). The major immuno-modulating effects of these active substances include mitogenicity, stimulation of haematopoietic stem cells and activation of immune effector cells, such as helper T cells, cytotoxic T cells, macrophages, dendritic cells, endothelial cells, neutrophils, monocytes, and NK cells (*Lull Nogiera* et al., 2005).

One of the approaches to evaluate potential immunomodulating activity is the assessment of the capacity of ex-

tracts or pure compounds to influence the production of cytokines by immune cells. Cytokines are soluble glycoproteins which are critically involved in the immune response. The functions of these proteins are diverse and include roles in normal humoral and T cell-mediated immune response. Various pathologic conditions are accompanied by changes in cytokine levels and by disturbances in the cytokine-mediated interplay between innate and acquired immune responses (*Stanilova* et al., 2005).

For the last years a few studies have reported the immunomodulating activity of mushrooms in a human peripheral blood mononuclear cells (PBMC) assay. PBMC represent a heterogeneous population of immune cells (B cells, T cells, and various granulocytes) that arise from pluripotent haematopoietic stem cells in the bone marrow. PBMC account for cellular and humoral immune responses; some PBMC (B and T cells) have the inherent ability to proliferate rapidly after antigenic and mitogenic stimulation (*Zempleni* and *Mock*, 2000).

The immune activities of mushroom derived polysaccharides are well documented and are considered to have function of promoting activities of antigen non-specific immune NK cells that are able to rapidly and effectively kill cancerous cells; placing a premium on the production of interferons that effectively prevent virus reproduction; increasing the activities of complement $C_3$ that enhance animals' disease resistance; increasing the number and activities of the phagocytes (neutrophilic granulocytes) that release $H_2O_2$ dissolving cancerous cells; protecting the normal cells and preventing the reduction of leucocytes (*Wargovich* et al., 2001). Therefore, mushroom polysaccharides can play an important role in the health care of human.

*Berovič* et al. (2003) studied the effects of extra- and intra-cellular polysaccharides isolated from mycelia of *Ganoderma lucidum* on the induction of IFN-γ and TNF-α, synthesis in primary cultures of human peripheral blood mononuclear cells (PBMC) isolated from a buffycoat. They found that the TNF-α inducing activity was comparable with romurtide, which has been used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy. *Jin* et al. (2003) treated PBMC with PG101, a water-soluble extract from cultured mycelia of *Lentinus lepideus*. PG101 increased levels of TNF-α, IL-1β, IL-10, and IL-12 by 100- to 1000-fold, whereas GM-CSF and IL-18 were activated by an order of magnitude. On the contrary, IFN-γ and IL-4 were not affected. Considering the type of affected cytokines, it is possible that PG101 could be used to enhance the immune system in immunosuppressed or immunocompromised individuals or to control the haematopoiesis of specific cell types or lineages.

In brief, as immunodulaters, polysaccharides affect the growth of immune organs (bursa, thymus, and spleen), activities of immune cells (granulocyte, monocyte and macrophage), functions of both cellular immunity and humoral immunity as well as cytokines and complement system (*Yuan* and *Shi*, 2000). The effects of herb polysaccharides on immune system are summarized below according to *Xue* and *Meng* (1996):
- Stimulating growth of immune organs: The organs of the immune system are concerned with the growth, development, and deployment of lymphocytes. Herb polysaccharides, e.g. *Isatidis radix, Ligustri fructus, Polypori scierotium, Astragali membranacea radix, Tremella fuciformis, Cistanchea*

*herba* and *Cordyceps* polysaccharide increase the weights of immune organs.

- Promoting activities of immune cells: Herb polysaccharides, e.g. *Isatidis radix, Codongopsis radix,, Tremella fuciformis, Bupleuri radix, Angelicae sinensis radix, Astragali membranacea radix* and *Polypori scierotium* polysaccharide, increase the number and activities of many interdependent cell types such as T, B lymphocytes, macrophage, NK cells that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumour cells.
- Enhancing functions of cellular immunity: Herb polysaccharides, e.g. *Isatidis radix, Tremella fuciformis, Astragali membranacea radix, Lycii fructus* and *Angelicae sinensis radix* polysaccharide, increased spleen and thymus index, rate of T-lymphocyte transformation and proliferation as well as production of IL-2, while decreased prohibition effects of serum, macrophage and suppressor T cell populations on T-cells' function and against deformation and necrosis of lymphocytes in spleen, thymus and lymph nodes.
- Promoting humoral immunity: Herb polysaccharides, e.g. *Eucommiae cortex, Cordyceps, Codongopsis radix, Astragali membranacea radix, Tremella fuciformis* and *Atractylodis macrocephalae rhizoma* polysaccharide, enhanced humoral immune response by increasing spleen and serum antibody production, antibody titres and plague forming cells (PFC).
- Inducing cytokine production and complement: Cytokines are central molecules that control host immune response to infectious agents. Cytokines, which are produced and secreted by activated T-cells and NK cells activated by antigens, are responsible for clonal T-cell proliferation and antibody production of B-cells, proliferation and activity of macrophages, and NK cells. Herb polysaccharides (e.g. *Phytolaccae radix, Ginseng radix, Tremella fuciformis, Poriae albae sclerotium* and *Eleutherococci radix* polysaccharide) increased cytokine production such as interferons (IFN-$\alpha$, IFN-$\beta$ and IFN-$\gamma$) and interleukin (IL-2), TGF-$\beta$, and TNF-$\alpha$.

Cell wall fragments of higher plants and yeasts, such as from the brewing industry, are known to have an impact on the immune system and to be able to stimulate innate immunity. Such preparations are already applied in the feed industry, for instance in shrimp and broiler feed (*Thanardkit* et al., 2002; *Von Wettstein* et al., 2000).

From preliminary immuno-assays, it has become clear that mushroom extracts show superior immune-modulating properties when compared to for instance $\beta$-glucans form beer yeast or from herbs. Striking differences between fungal and yeast cell walls may exist, as fungi are reputated for their high content in amino-glucans (*Hamuro* et al., 1974; *de Ruiter* et al., 1992; *Usui* et al., 1983; *Karácsonyi* and *Kuniak*, 1994; *Dong* et al., 2002; *Wasser*, 2002). This phenomenon is unexplained yet, i.e. it is not known what e.g. the differences in chemical structure between cell wall components from fungi resp. yeast are in relation to immune modulation, or whether fungi produce additional biologically active immune-modulating substances. Modification of polysaccharide fragments may increase their activity (*Zhan* and *Cheung*, 2002).

# FUNGAL IMMUNOMODULATORY PEPTIDES

Proteins and peptides from mushrooms are also known to activate macrophages. An ubiquitin-like peptide isolated from fruiting bodies of the mushroom *Agrocybe cylindracea* enhanced NO production in murine peritoneal macrophages with potency comparable to that of LPS. Two lectins isolated from the mushroom *Tricholoma mongolicum* (TML-1 and TML-2) stimulated the production of nitrite ions and TNF-$\alpha$ by macrophages in normal and tumour-bearing mice.

Vvo, a fungal immunomodulatory protein (FIP) purified from the edible mushroom, *Volvariella volvacea*, induced most Th1-specific cytokines (IL-2, IFN-$\gamma$, and LT) and one TH2-specific cytokine (IL-4) within 4 hours in mouse spleen cells. This result indicates that Vvo principally acts on Th1 cells and to a lesser extent on Th2 cells in the early event of activation. It is known that IL-4 acts on B cells to induce activation and differentiation, leading in particular to the production of IgE. The lower effect of Vvo compared with other FIPs on the prevention of systemic anaphylaxis may be attributed to the elevated expression of IL-4. Fve, a FIP isolated from the fruiting body of *Flammulina velutipes*, selectively stimulates a Th1 response in hPBMCs (*Hsu* et al., 1997, 2003). Recently *Hsieh* et al. (2003) have characterized the immunomodulatory effects of Fve in more detail and investigated the prophylactic use of Fve via the oral route in a murine model of food allergy. They have demonstrated that oral administration of Fve during allergen sensitization could induce a Th1-predominant allergen-specific immune response in mice and protect the mice from systemic anaphylaxis-like symptoms after subsequent oral challenge with the same allergen. It is worth pointing out that Fve could be administered orally and retain its activity, while most protein drugs cannot. This characteristic greatly promotes the potential of immunoprophylactic use of Fve. *Liu* et al (1999) have demonstrated the efficacy of local nasal immunotherapy (LNIT) for group 2 allergen of house dust mite *Dermatophagoides pteronyssinus-* (Dp2-) induced airway inflammation in mice, using Dp2 peptide and Fve or LZ-8, a FIP isolated from *G. lucidum*.

In contrast to the polysaccharide metabolites of edible mushrooms, only little is known about the effect of their proteins on the immune system. *Hsieh* et al. (2003) studied at the possibilities of therapy for food allergy using an edible-mushroom derived protein. They used a Fungal Immunomodulatory Protein (FIP) isolated from the commonly eaten mushroom *Flammulina velutipes*, called FIP-*fve*. It was demonstrated that oral administration of FIP-*fve* during allergen sensitization could induce a Th1- predominant allergen specific immune response in mice which protected the mice from systemic anaphylaxis-like symptoms after subsequent oral challenge with the same allergen. They concluded that the FIP-*fve* could activate T cells and selectively stimulate a Th1 response. In addition they demonstrated that the suppression of allergen-specific IgE response may play a crucial role in this protection. FIP-*fve* might either activate primed type-1 T cells or drive naive T cells to a type-1 phenotype. This strategy is convenient in practice and may have the potential to be used clinically in young children for the prevention of allergic diseases, but the optimal dosage, efficiency, and adverse effects in humans should be determined. It is well established that many

mushrooms-extracted compounds are commonly used as immunomodulators or as biological response modifiers. The basic strategy underlying immunomodulation is to identify aspects of the responses that can be enhanced or suppressed in such a way as to augment or complement a desired immune response (*Wasser*, 2002; *Zaidman* et al., 2005). Indications for the latter are for instance the identification of an immune-modulating protein (Fip-*vvo*) from *Volvariella volvacea* (*Hsu* et al., 1997).

Utilization of a T cell polarization pulse, followed by comparison of relative cytokine production by intracellular cytokine staining under polarized conditions allows *ex vivo* assessment of the T cell polarization state *in vivo* (*Cameron* et al., 2005). Since strong and clear T cell polarization typically takes place in chronic disease states or exposure to (potentially toxic) agents, this technique provides an assessment of the current direction of polarization the T cells are heading towards during acute responses, on a population basis via relative cytokine production, and on a cell-by-cell basis via intracellular cytokine staining (ICS). Most importantly, the comparison of cytokine pro-

duction by enzyme-linked immunosorbent assay must be made on a relative scale, as outlined since the potency of the key Th1 and Th2 cytokines, IFN-γ and IL-4 respectively, may not necessarily be equal. Similar relative comparisons have been made during micro-array analysis of Th1 and Th2 gene transcripts. Additionally, all polarizations must be compared to the results obtained from CD4+ T cells isolated from healthy controls polarised under identical conditions. The rationale for this is apparent when looking at the data, because some individuals are naturally Th2 biased in their cytokine profile, while others are naturally Th1 biased. The addition of ICS to this method most importantly allows for further assessment of the T helper population in experimental groups, to determine if a mixed cytokine profile detected via ELISA and comparison of relative cytokine production truly represents an undifferentiated Th0 response of naive cells, or rather if it is indicative of a heterogeneous population of simultaneously differentiating Th1 and Th2 cells. Additionally, it can allow for further characterization of the cytokine-producing subsets.

## CONCLUSIONS

A wide variety of immune interactions can be identified by which components present in mushroom extracts can exert their immunomodulatory activity. Some of these interactions are preferred by β-glucan, particularly those associated with TLR interactions on innate immune cells and resulting in the induction of pro-inflammatory cytokine production. Others, like FIP will be presented by APC to T-cells and (in)directly modulate Th cell differentiation. By integrating the various

mechanisms exploited by these mushrooms predictions can be made for their immunomodulating activity *in vivo*. Figure 4 summarizes these known and suggested activities of the various mushrooms used for dietary immune intervention in humans. From these known interactions *in vitro* extrapolations to the potential use of such extracts for the treatment of human diseases can be inferred and increasing research is performed according to this paradigm.
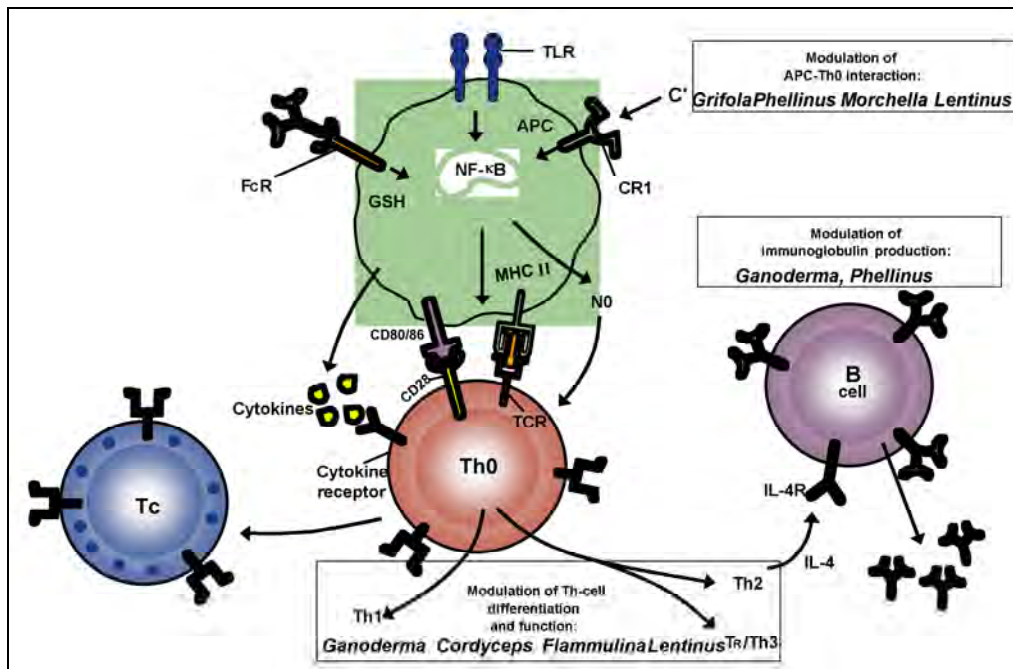
56

**Figure 4**: Summary of published interactions of isolated components or extracts of various edible mushrooms on the induction of immune responses. From: *Lull Nogiera* et al., 2005.

## LITERATURE

Abbas, A.K. and Lichtman A.H.: Cellular and molecular immunology. Elsevier Saunders, Philadelphia (2005).

Adams, R.B, Planchon, S.M., and Roche, J.K.: IFN-γ modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. J. Immunol. 150, 2356-2363 (1993).

Arinaga, S., Karimine, N., Takamuku, K., Nanbara, S., Nagamatsu, M., Ueo, H., and Akiyoshi, T.: Enhanced production of interleukin 1 and tumor necrosis factor by peripheral monocytes after lentinan administration in patients with gastric carcinoma. Int. J. Immunopharmacol. 14, 43-47 (1992a).

Arinaga, S., Karimine, N., Takamuku, K., Nanbara, S., Inoue, H., Nagamatsu, M., Ueo, H., and Akiyoshi, T.: Enhanced induction of lymphokine-activated killer activity after lentinan administration in patients with gastric carcinoma. Int. J. Immunopharmacol. 14, 535-539 (1992b).

Baba, H., Kunimoto, T., Nitta, K., Sato, K., Hashimoto, S., Kohno, M., Kita, Y., and Ogawa, H.: Rapid tumor regression and induction of tumor-regressing activity in serum by various immune-modulating agents. Int. J. Immunopharmacol. 8, 569-572 (1986).

Berovic, M., Habijanic, J., Zore, I., Wraber, B., Hodzar, D., Boh, B., and Pohleven, F.: Submerged cultivation of *Ganoderma lucidum* biomass and immunostimulatory effects of fungal polysaccharides. J. Biotechnol. 12, 77-86 (2003).

Bohn, J.A., and BeMiller, J.N.: (1→3)-β-D-Glucans as biological response modifiers: A review of structure-functional activity relationships. Carbohydr. Polym. 28, 3-14 (1995).

Boonstra, A., van Oudenaren, A., Barendregt, B., An, L., Leenen, P.J., and Savelkoul, H.F.J.: UVB irradiation modulates systemic

immune responses by affecting cytokine production of antigen-presenting cells. Int. Immunol. 12, 1531-1538 (2000).

Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L., and Gershwin, M.E.: Mushrooms, tumors and immunity. Proc. Soc. Exp. Biol. Med. 221, 281–293 (1999).

Borchers, A.T., Keen, C.L., and Gershwin, M.E.: Mushrooms, tumors and immunity: An update. Exp. Biol. Med. 229, 393-406 (2004).

Brandtzaeg, P.: The innate and adaptive immune system of the intestinal epithelium. In: Defence mechanisms of the innate system: Influence of microbes (Eds.: Heidt, P.J., Bienenstock, J., Midtvedt, T., Rusch, V., and van der Waaij, D.). Herborn litterae, Herborn, 55-88 (2006).

Breene, W.M.: Nutritional and medicinal value of speciality mushrooms. J. Food Production 53, 883-894 (1990).

Brown, G.D. and Gordon, S. Fungal β-glucans and mammalian immunity. Immunity 19, 311-315 (2003).

Charalampopoulos, D., Wang, R., Pandiella, S.S., and Webb, C.: Application of cereals and cereal components in functional foods: A review. Int. J. Immunopharmacol. 79, 131-141 (2002).

Chesterman, H., Heywood, R., Allen, T.R., Street, A.E., Edmondson, N.A., and Prentice, D.E.: The intravenous toxicity of lentinan to the beagle dog. Toxicol. Lett. 9, 87-90 (1981).

Chihara, G., Hamuro, J., Maeda, Y.Y., Arai, Y., and Fukuoka, F.: Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). Cancer Res. 30, 2776-2781 (1970).

Cozens, D.D., Masters, R.E., Clark, R., and Offer, J.M.: The effect of lentinan on fertility and general reproductive performance of the rat. Toxicol. Lett. 9, 55-64 (1981a).

Cozens, D.D., Masters, R.E., and Clark, R.: The effect of lentinan on pregnancy of the New Zealand white rabbit. Toxicol. Lett. 9, 65-70 (1981b).

Cozens, D.D., Hughes, E.W., Clark, R., and Offer, J.M.: The effect of lentinan on the in utero foetal development of the rat and on postnatal development of the F1 offspring. Toxicol. Lett. 9, 77-80 (1981c).

Cunningham-Rundles, S., McNeeley, D.F., and Moon, A.: Mechanisms of nutrient modulation of the immune response. J. Allergy Clin. Immunol. 115, 1119-1128 (2005).

De Ruiter, G.A., Josso, S.L., Colquhoun, I.J., Voragen, A.G.J., and Rombouts, F.M.: Isolation and characterization of β(1-4)-D-glucuronans from extracellular polysaccharides of moulds belonging to Mucorales. Carbohyd. Polym. 18, 1-7 (1992).

Dong, Q., Yao, J., Yang, X., and Fang, J.: Structural characterisation of a water-soluble β-D-glucan from fruiitmg bodies of Agaricus blazei Murr. Carbohyd. Res. 337, 1417-1421 (2002).

Falch, B.H., Espevik, T., Ryan, L., and Stokke, B.T.: The cytokine stimulating activity of (1→3)-β-D-glucans is dependent on the triple helix conformation. Carbohydr. Res. 329, 587-596 (2000).

Field, C.J. and Schley, P.D.: Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumor metabolism and immune function: Lessons from n-3 fatty acids. Am. J. Clin. Nutr. 79, 1190S-1198S (2004).

Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., and Underhill, D.M.: Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J. Exp. Med. 197, 1107-1117 (2003).

Gibson, G.R. and Roberfroid, M.B.: Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. 125, 1401–1412 (1995).

Hamano, K., Gohra, H., Katoh, T., Fujimura, Y., Zempo, N., and Esato, K.: The preoperative administration of lentinan ameliorated the impairment of natural killer activity after cardiopulmonary bypass. Int. J. Immunopharmacol. 21, 531-540 (1999).

Hamuro, J., Maeda, Y., Fukuoka, F., and Chihara, G.: Antitumour polysaccharides,

lentinan and pachymaran as immunopotentiators. Mushroom Sci. IX, 477-487 (1974).

Hashimoto, K., Suzuki, I., and Yadomae, T.: Oral administration of SSG, a β -glucan obtained from *Sclerotinia sclerotiorum*, affects the function of Peyer's patch cells. Int. J. Immunopharmacol. 13, 437-442 (1991).

Herre, J., Gordon, S., and Brown, G.D.: Dectin-1 and its role in the recognition of β-glucans by macrophages. Mol. Immunol. 40, 869-76 (2004).

Hetland, G. and Sandven, P.: β-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures. FEMS Immunol. Med. Microbiol. 33, 41-45 (2002).

Hsieh, K.Y., Hsu, C.I., Lin, J.Y., Tsai, C.C., and Lin, R.H.: Oral administration of an edible mushroom-derived protein inhibitis the development of food-related allergic reactions in mice. Clin. Exp. Allergy 33, 1595-1602 (2003).

Hsu, C.I., Lin, J.Y., Tsai, C.C., and Lin, R.H.: Oof an edible-mushroom-derived protein inhibitis the development of food-related allergic reactions in mice. Clin. Exp. Allergy. 33, 1595-1602 (2003).

Hsu, H.C., Hsu, C.I., Lin, R.H., Kao, C.L., and Lin, J.Y.: Fip-*vvo*, a new fungal immunomodulatory protein isolated from *Volvariella volvacea*. Biochem. J. 323, 557-565 (1997).

Irinoda, K., Masihi, K.N., Chihara, G., Kaneko, Y., and Katoriv, T.: Stimulation of microbicidal host defence mechanisms against aerosol influenza virus infection by lentinan. Int. J. Immunopharmacol. 14, 971-977 (1992).

Jin, M., Jeon, H., Jung, H.J., Kim, B., Shin, S.S., Choi, J.J., Lee, J.K., Kang, C.Y., and Kim, S.: Enhancement of repopulation and hematopoiesis of bone marrow cells in irradiated mice by oral administration of PG101, a water-soluble extract from *Lentinus lepideus*. Exp. Biol. Med. (Maywood) 228, 759-766 (2003).

Karácsonyi, S., and Kuniak, L.: Polysaccharides of Pleurotus ostreatus: Isolation and structure of pleuran, an alkali-

insoluble β-D-glucan. Carbohyd. Polym. 24, 107-111 (1994).

Kataoka, K., Muta, T., Yamazaki, S., and Takeshige, K.: Activation of macrophages by linear (1→3)-β-D-glucans. J. Biol. Chem. 277, 36825-36831 (2002).

Kerekgyarto, C., Virag, L., Tanko, L., Chihara, G., and Fachet, J.: Strain differences in the cytotoxic activity and TNF production of murine macrophages stimulated by lentinan. Int. J. Immunopharmacol. 18, 347-353 (1996).

Kodama, N., Kakuno, T., and Nana, H.: Stimulation of the natural immune system in normal mice by polysaccharide from maitake mushroom. Mycoscience 44, 257-26 (2003).

Kodama, N., Murata, Y., Asakawa, A., Inui, A., Hayashi, M., Sakai, N, and Nanba, H.: Maitake D-fraction enhances antitumor effects and reduces immunosuppression by mitomycin-C in tumor-bearing mice. Nutrition 21, 624-629 (2005).

Kulicke, W.M., Lettau, I., and Thielking, H.: Correlation between immunological activity, molar mass, and molecular structure of different (1→3)-β-D-glucans. Carbohydr. Res. 297, 135-143 (1997).

Liu, F., Ooi, V.E.C., and Fung, M.C.: Analysis of immunomodulating cytokine mRNAs in the mouse induced by mushroom polysaccharides. Life Sci. 64, 1005-1011 (1999).

Lull Nogiera, C., Wichers, H.J., and Savelkoul, H.F.J.: Anti-inflammatory and immunomodulating properties of fungal metabolites. Mediators Inflammation 14, 63-80 (2005).

Maeda, Y.Y., Watanabe, S.T., Chihara, C., and Rokutanda, M.: Denaturation and renaturation of a beta-1,6;1,3-glucan, lentinan, associated with expression of T-cell-mediated responses. Cancer Res. 48, 671-675 (1988).

Markova, N., Kussovski, V., Drandarska, I., Nikolaeva, S., Georgieva, N., and Radoucheva, T.: Protective activity of Lentinan in experimental tuberculosis. Int. Immunopharmacol. 3, 1557-1562 (2003).

Masihi, K.N., Madaj, K., Hintelmann, H., Gast, G., and Kaneko, Y.: Down-regulation of

tumor necrosis factor-α, moderate reduction of interleukin-1β, but not interleukin-6 or interleukin-10, by glucan immunomodulators curdlan sulfate and lentinan. Int. J. Immunopharmacol. 19, 463-468 (1997).

Minato, K., Mizuno, M., Terai, H., and Tsuchida, H.: Autolysis of lentinan, an antitumor polysaccharide, during storage of *Lentinus edodes*, shiitake mushroom. J. Agric. Food Chem. 47, 1530-1532 (1999).

Miura, T., Ohno, N., Suda, M., Miura, N.N., Shimada, S., and Yadomae, T.: Inactivation of (1→3)-β-D-glucan in mice. Biol. Pharm. Bull. 18, 1797-1801 (1995).

Miura, N.N., Ohno, N., Aketagawa, J., Tamura, H., Tanaka, S., and Yadomae, T.: Blood clearance of (1→3)-β-D-glucan in MRL lpr/lpr mice. FEMS Immunol. Med. Microbiol. 13, 51-57 (1996).

Murata, Y., Shimamura, T., Tagami, T., Takatsuki, F., and Hamuro, J.: The skewing to Th1 induced by lentinan is directed through the distinctive cytokine production by macrophages with elevated intracellular glutathione content. Int. Immunopharmacol. 2, 673-689 (2002).

Mushiake, H., Tsunoda, T., Nukatsuka, M., Shimao, K., Fukushima, M., and Tahara, M.: Dendritic cells might be one of key factors for eliciting antitumor effect by chemoimmunotherapy *in vivo*. Cancer Immunol. Immunother. 54, 120-128 (2005).

Ng, M.L. and Yap, A.T.: Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes)*. J. Altern. Complement Med. 8, 581-589 (2002).

O'Garra, A. and Arai, N.: The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell. Biol. 10, 542-550 (2000).

Oka, M., Yoshino, S., Hazama, S., Shimoda, K., and Suzuki, T.: Immunological analysis and clinical effects of intraabdominal and intrapleural injection of lentinan for malignant ascites and pleural effusion. Biotherapy 5, 107-112 (1992).

Oka, M., Hazama, S., Suzuki, M., Wang, F., Wadamori, K., Iizuka, N., Takeda, S.,

Akitomi, Y., Ohba, Y., Kajiwara, K., Suga, T., and Suzuki, T.: *In vitro* and *in vivo* analysis of human leukocyte binding by the antitumor polysaccharide, lentinan. Int. J. Immunopharmacol. 18, 211-216 (1996).

Ooi, V.E.C. and Liu, F.: Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr. Med. Chem. 7, 715-729 (2000).

Palma, A.S., Feizi, T., Zhang, Y., Stoll, M.S., Lawson, A.M., Diaz-Rodriguez, E., Companero-Rhodes, M.A., Costa, J., Gordon, S., Brown, G.D., and Chaiu, W.: Ligands for the β-glucan receptor, Dectin-1, assigned using "designer" microarrays of oligosaccvharide probes (neoglycolipids) generated from glucan polysaccharides. J. Biol. Chem. 281, 5771-779 (2006).

Penner, R., Fedorak, R.N., and Madsen, K.L.: Putraceuticals: Non-medicinal treatments of gastro-intestinal diseases. Curr. Op. Pharmacol. 5, 596-603 (2005).

Planchon, S.M., Martins, C.A., Guerrant, R.L., and Roche, J.K. : Regulation of epithelial baqrrier function by TGF- β1. Evidence for its role in abrogating the effect of a T cell cytokine. J. Immunol. 153, 5370-5379 (1994).

Rice, P.J., Adams, E.L., Ozment-Skelton, T., Gonzalez, A.J., Goldman, M.P., Lockhart, B.E., Barker, L.A., Breuel, K.F., DePonti, W.K., Kalbfleisch, J.H., Ensley, H.E., Brown, G.D., Gordon, S., and Williams, D.L.: Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. J. Pharmacol. Exp. Ther. 314, 1079-1086 (2005).

Rice, P.J., Lockhart, B.E., Barker, L.A., Adams, E.L., Ensley, H.E., and Williams, D.L.: Pharmacokinetics of fungal (1−3)-β-D-glucans following intravenous administration in rats. Int. Immunopharmacol. 4, 1209–1215 (2004).

Rogers, N.C., Slack, E.C., Edwards, A.D., Nolte, M.A., Schulz, O., Schweighoffer, E., Williams, D.L., Gordon, S., Tybulewicz, V.L., Brown, G.D., and Reis e Sousa, C.: Syk-dependent cytokine induction by

Dectin-1 reveals a novel pattern recognition pathway for C type lectins. Immunity 22, 507-517 (2005).

Salminen, S., Bouley, C., Boutron-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M., and Rowland, I.: Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80, S147-S171 (1998).

Sheldon, A.T.Jr.: Antibiotic resistance: A survival strategy. Clin. Lab. Sci. 18, 170-180 (2005).

Shamtsyan, M., Konusova, V., Maksimova, Y., Goloshchev, A., Panchenko, A., Simbirtsev, A., Petrishchev, N., and Denisova, N.: Immunomodulating and anti-tumor action of extracts of several mushrooms. J. Biotechnol. 30, 77-83 (2004).

Sortwell, R.J., Dawe, S., Allen, D.G., Street, A.E., Heywood, R., Edmondson, N.A., and Gopinath, C.: Chronic intravenous administration of lentinan to the rhesus monkey. Toxicol. Lett. 9, 81-85 (1981).

Stanilova, S.A., Dobreva, Z.G., Slavov, E.S., and Miteva, L.D.: C3 binding glycoprotein from *Cuscuta europea* induced different cytokine profiles from human PBMC compared to other plant and bacterial immunomodulators. Int. Immunopharmacol. 5, 723-734 (2005).

Suda, M., Ohno, N., Hashimoto, T., Koizumi, K., Adachi, Y., and Yadomae, T.: Kupffer cells play important roles in the metabolic degradation of a soluble anti-tumor (1→3)-β-D-glucan, SSG, in mice. FEMS Immunol. Med. Microbiol. 15, 93-100 (1996).

Taguchi, T.: Phase I, II trials of Lentinan in cancer patient. Int. J. Immunopharmacol. 2, 172 (1980).

Taguchi, T.: Clinical efficacy of lentinan on patients with stomach cancer: End point results of a four-year follow-up survey. Cancer Detect. Prev. Suppl. 1, 333-349 (1987).

Thanardkit, P., Khunrae, P., Suphanthakira, M., and Verduyn, C.: Glucan from spent brewer's yeast: preparation, analysis and use as a potential immunostimulant in shrimp feed. World J. Microbiol. Biotechnol. 18, 527-539 (2002).

Usui, T., Iwasaki, Y., Mizuno, T., Tanaka, M., Shinkai, K., and Arakawa, M.: Isolation and characterisation of antitumor active β-D-glucans from the fruit bodies of *Ganoderma applanatum*. Carbohyd. Res. 115, 273-280 (1983).

Van der Velden, V.H., Laan, M.P., Baert, M.R., de Waal Malefyt, R., Neijens, H.J., and Savelkoul, H.F.J.: Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: Risk factors and regulatory role of IFN-gamma, IL-4 and IL-10. Clin. Exp. Allergy 31, 997-1006 (2001).

Tani, M., Tanimura, H, Yamaue, H., Iwahashi, M., Tsunoda, T., Tamai, M., Noguchi, K., and Arii, K.: *In vitro* generation of activated natural killer cells and cytotoxic macrophages with lentinan. Eur. J. Clin. Pharmacol. 42, 623-627 (1992).

Von Wettstein, D., Mikhaylenko, G., Froseth, J.A., and Kannangaram, C.G.: Improved barley broiler feed with transgenic malt containing heat-stable (1,3-1,4)-beta-glucanase. Proc. Natl. Acad. Sci. USA 97, 13512-13517 (2000).

Wada, T., Nishide, T., Hatayama, K., Chang, S.W., Tatsuta, M., and Yasutomi, M.: A comparative clinical trial with tegafur plus lentinan treatment at two different doses in advanced cancer. Gan To Kagaku Ryoho. 14, 2509-2512 (1987).

Wargovich, M. J., Woods, C., Hollis, D.M., and Zander, M.E.: Herbals, cancer prevention and health. J. of Nutrition. 131 (11 Suppl.), 3034S-3036S (2001).

Wasser, S.P. and Weis, A.L.: Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: A modern perspective. Crit. Rev. Immunol. 19, 65-96 (1999).

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

Wierzbicki, A., Kiszka, I., Kaneko, H., Kmieciak, D., Wasik, T.J., Gzyl, J., Kaneko, Y., and Kozbor, D.: Immunization strategies to augment oral vaccination with

DNA and viral vectors expressing HIV envelope glycoprotein. Vaccine 20, 1295-1307 (2002).

Xue, M. and Meng, X.S.: Review on research progress and prosperous of immune activities of bio-active polysaccharides. J. Traditional Vet. Medicine. 3, 15-18 (1996).

Yap, A.T. and Ng, M.L.: An improved method for the isolation of lentinan from shiitake mushroom (*Lentinus edodes*). Int. J. Med. Mushroom 3, 9-19 (2001).

Yuan, W. S. and Shi, S. M.: Pharmacological functions of Chinese medicinal fungus *Cordyceps sinensis* and related species. J. Food Drug Analysis 8, 248-257 (2000).

Zaidman, B.Z., Yassin, M., Mahanja, J., and

Wasser, S.P.: Medicinal mushroom modulators of molecular targets as cancer therapeutics. Appl. Microbiol. Biotechnol. 67, 453-468 (2005).

Zempleni, J. and Mock, D.M.: Utilization of biotin in proliferating human lymphocytes. J. Nutr. 130, 335S-337S (2000).

Zhang, P., and Cheung, P.C.K.: Evaluation of sulfated *Lentinus edodes* β-(1→3)-D-glucan as a potential antitumor agent. Biosci. Biotechnol. Biochem. 66, 1052-1056 (2002).

Zhang, L., Li, X., Xu, X., and Zeng, F.: Correlation between antitumor activity, molecular weight, and conformation of lentinan. Carbohydr. Res. 340, 1515-1521 (2005).