MINIREVIEW

The Immunobiology of Mushrooms

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There has been enormous interest in the biologic activity of mushrooms and innumerable claims have been made that mushrooms have beneficial effects on immune function with subsequent implications for inhibition of tumor growth. The majority of these observations are anecdotal and often lack standardization. However, there remains considerable data on both in vitro and in vivo effects that reflect on the potential of mushroom compounds to influence human immunity. A number of these effects are beneficial but, unfortunately, many responses are still characterized based on phenomenology and there is more speculation than substance. With respect to tumor biology, although many neoplastic lesions are immunogenic, tumor antigens frequently are self antigens and induce tolerance and many patients with cancer exhibit suppressed immune responses, including defective antigen presentation. Therefore, if and when mushroom extracts are effective, they more likely function as a result of improved antigen presentation by dendritic cells than by a direct cytopathic effect. In this review we attempt to place these data in perspective, with a particular focus on dendritic cell populations and the ability of mushroom extracts to modulate immunity. There is, at present, no scientific basis for the use of either mushrooms or mushroom extracts in the treatment of human patients but there is significant potential for rigorous research to understand the potential of mushrooms in human disease and thence to focus on appropriate clinical trials to demonstrate effectiveness and/ or potential toxicity. Exp Biol Med 233:259–276, 2008

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DOI: 10.3181/0708-MR-227
1535-3702/08/2333-0259$15.00
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Key words: cytotoxicity; tumor biology; tolerance; cytokines; mushrooms; self-antigens

Introduction

The ability of mushrooms to inhibit tumor growth and modulate immune functions has been studied for about 50 years (38, 39). After early studies were conducted with whole mushrooms, it soon became obvious that the tumor-inhibiting activity was contained in the polysaccharide fraction. Some of the most effective anti-tumor agents were subsequently purified and identified as large 1,6-branched 1,3-β-glucans. The isolated compounds included lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune*, grifolan from *Grifola frondosa*, and SSG from *Sclerotinia sclerotiorum*. They were found to inhibit tumor growth mostly by stimulating the immune system, particularly the innate branch, such a macrophages and natural killer (NK) cells, but also T cells and their cytokine production. The various glucans differed in their molecular mass, degree of branching, solubility, viscosity, and three-dimensional configuration (e.g., triple helix). These characteristics were all identified as factors influencing certain bioactivities of β-glucans, indicating that these compounds inhibited tumor growth by different, though probably somewhat overlapping, mechanisms.

Since then, numerous other fractions and compounds with immunomodulatory activity have been partially purified or isolated from mushrooms (See Table 1 for some of the compounds frequently discussed in this review, their abbreviations, characteristics, and the method used for their extraction). Many of them are or contain polysaccharides with varying sugar composition and some are α- rather than

Table 1. Extraction Procedures for Some of the Fractions or Compounds Used in Some of the Studies Under Discussion

Mushroom	Fraction name	Procedure	Method reference cited	Used in
Ganoderma lucidum	PS-G, a protein-bound polysaccharide	Fruiting bodies extracted with boiling water for 8–12 h, followed by 2 steps of EtOH precipitation, gel filtration on Sephadex G50 column, and anion exchange chromatography with a DEAE-cellulose column	(97)	(19, 28)
Ganoderma lucidum	*Gl*-PS, a polysaccharide peptide with a MW of 584,900 Da	Only a perfunctory description of the method is provided: "isolated from boiling water extract of GL followed by EtOH precipitation, dialysis, and protein depletion"		(50, 72)
Ganoderma lucidum	Ganopoly, polysaccharide with average MW of 4.85×10^5 Da	Fruiting bodies extracted with 70° C water for 3 hours, precipitated with 75% EtOH, purified by high-performance anion exchange and gel filtration chromatography		(57–59)
Ganoderma lucidum (Reishi)	EORP (extract of reishi polysaccharides), a glycoprotein fraction	Crude *G. lucidum* polysaccharide extract prepared via alkaline extraction with 0.1 N of NaOH, followed by neutralization and EtOH precipitation was obtained commercially. Extraction of crude extract with water at 4° C for 24 hours, concentration of supernatant and lyophilization. Fractionation of the extract with Sephacryl S-500 column. EORP was one of 5 fraction obtained	(98, 99)	(62, 75, 100, 101)
Grifola frondosa	D-fraction, a glycoprotein fraction, but generally referred to as a polysaccharide	Powdered fruit bodies extracted with H_2O at 121° C for 30 min, centrifuged, supernatant precipitated with 50% EtOH at 4°C for 24 hours, then with 80% EtOH at 4° C for 24 hours, the precipitate (crude D-fraction) was purified by DEAE-cellulofine ion exchange chromatography to yield the D-fraction	(102)	(23, 25–27, 48, 49, 56, 103, 104)
Agaricus blazei	AbM Gold label, a semi-particulate extract	No method is provided for this extract that is commercially available in Japan		(63–66)
A. blazei	ABH, fraction H of *A. blazei* mycelia extract	Mycelia were digested with hemicellulase for 1 h at 45° C, inactivated and freeze-dried, then mixed with distilled water, centrifuged, and the supernatant filtered.		(13, 14)
A. blazei	ABPC	A commercial product using ABH as source material		(43)[a]
A. blazei	ABM, a 170 kDa α-1,6-branched α-1,4-glucan[b]	Dried fruiting body was extracted 6 times with 80% EtOH at 80° C for 6 h, residues were extracted with hot water for 4 h, filtered, and the solution lyophilized. After precipitation of proteins, the supernatant was submitted to anion exchange chromatography on DEAE-Sepharose CL-B6 and further separated on Sepharose 6B	(105)	(55, 79, 80)

Table 1. (Continued)

Mushroom	Fraction name	Procedure	Method reference cited	Used in
Phellinus linteus (Berk. & M.A. Curtis) Teng	PG (also called PL), variously described as a 150 kDa[c] proteoglycan or acidic polysaccharides and consisting of 72% polysaccharides and 22% protein	Fruiting body was extracted with boiling water (100° C) for 6 hours, filtered, extracted with 3 volumes of 95% EtOH for 24 h, the precipitate was applied to a DEAE-cellulose column and eluted with 2 M NaCl	(106)	(11, 12, 73)
P. linteus	PL, described as an acidic polysaccharide with a MW of 153 kDa consisting of 13.2% protein and 82.5% carbohydrate	Extracted from mycelial culture in boiling water for 12 h, followed by EtOH precipitation, DEAE-cellulose and gel permeation chromatography	(107)	(77)

[a] From the description, it is not quite clear whether ABH or ABPC was used in this study.
[b] It is unclear whether ABM is the lyophilized solution or the isolated α-glucan.
[c] Several of the articles describe it as a 15 kDa proteoglycan, but cite the same paper for the method of extraction. This paper gives a molecular weight of 150 kDa (106).

β-glucans. In addition, some proteins, terpenes, furans, and various other small molecular weight mushroom compounds have been reported to modulate certain immune functions. The earlier focus on aspects of the innate immune system has shifted somewhat to include more investigations of the effects of mushroom compounds on adaptive immune responses.

Dendritic Cells. Dendritic cells (DCs) are central players in both innate and adaptive immunity. DCs are potent antigen-presenting cells and have the unique ability to activate naïve T cells (1). They arise from progenitors in the bone marrow, migrate to tissues and reside there as immature cells characterized by high capacity for antigen uptake. The capture of antigen in the presence of appropriate inflammatory stimuli triggers a maturation process that, in vivo, occurs simultaneously with migration to the lymphoid organs. Maturation involves a marked decline in the capacity for antigen uptake and upregulation of major histocompatibility complex (MHC) molecules for antigen presentation and of costimulatory molecules, such as CD40, CD54, CD83, B7.1 and B7.2 (CD80 and CD86) for effective T cell stimulation. In the lymph nodes, antigen-bearing mature DCs undergo a final maturation step, also termed licensing, in response to interactions with T cells, particularly the engagement of CD40 by the CD40 ligand expressed on cognate CD4+ T cells.

DCs not only have a critical role in priming naïve CD4+ T cells, but also in determining the polarization of the helper T cell responses (Th1 or Th2) (1). Th1 immune responses involve the production of interferon (IFN)γ and interleukin (IL)-2 and induce cell-mediated immunity, whereas Th2 immune responses involve IL-4, IL-5, IL-6, and IL-13 and induce humoral immunity. The generation of a Th1 immune response critically depends on the ability of DC to produce IL-12, a cytokine that in its biologically active form is a dimer (p70) consisting of a p35 and p40 subunit.

DC-primed CD4 T cells of the Th1 phenotype support the generation of potent antigen-specific CD8+ cytotoxic T cell responses and can activate components of the innate immune system, such as macrophages, eosinophils, and NK cells. DCs can also act on NK and NK T cells either directly through cell-cell interactions or through the production of cytokines. In particular IL-12 has been shown to be essential in DC-mediated activation of NK cells (2), but IL-15 and IL-18 have also been implicated (1). Furthermore, DCs play a role in the activation and differentiation of B cells either directly or by priming CD4 T cells of the Th2 phenotype, which then interact with B cells to induce antigen-specific antibody production.

Although tumors can be immunogenic, tumor antigens frequently are self antigens and induce tolerance. In this respect, detailed studies of tolerance in autoimmunity are particularly relevant to tumor biology (3–5). In addition, tumors are generated in a milieu that is not sufficiently inflammatory for effective DC activation and maturation.

Table 2. Signaling of Mushroom Compounds or Fractions Through TLRs and CR3

Mushroom	Fraction	Cell type	Response	Anti-TLR2 Ab
Ganoderma lucidum	EORP	Murine macrophages	Pro-IL-1 expression	
G. lucidum	EORP	Murine B cells	IgM secretion	Partial inhibition
			Blimp-1 mRNA expression	Partial inhibition[b]
G. lucidum	GLPS	Murine macrophages	IL-1b production	
G. lucidum	GLPS	Murine B cells	B cell proliferation	
Agaricus blazei	ABH	Human PBMC	IL-12 secretion	[a]
		Human peripheral CD14+ cells	Intracellular IL-12 production	
A. blazei	ABH	Bone marrow-derived DCs	Phenotypic maturation	
Phellinus linteus	PG, 150 kDa proteoglycan	Bone marrow-derived DCs	Phenotypic maturation	
			Intracellular IL-12 production	
P. linteus	PG, 150 kDa proteoglycan	Bone marrow-derived DCs	Phenotypic maturation	Inhibition of unspecified extent
			Intracellular IL-12 production	Almost complete inhibition

[a] Response unchanged; in the presence of an anti-TLR antibody means that the response is not mediated by this receptor; in the presence of anti-CD11b/antiCD18 antibody means that the response is not mediated by CR3; in C3H/HeJ mice indicates that the response is not mediated by TLR4.
[b] Simultaneous neutralization of TLR2 and TLR4 did not yield greater effects than neutralization of one or the other, suggesting that both receptors use a common signaling pathway.

Furthermore, cancer patients frequently exhibit suppressed immune responses, including defective antigen presentation by DCs (6). The development of DC-based cancer vaccines has been one of the approaches to overcoming this immunosuppression. It involves immunizing cancer patients with their own DCs after these have been loaded with tumor antigens ex vivo and stimulated under optimal conditions for the induction of vigorous and long-lasting T cell responses (7). Although the approach seems promising, clinical trials have failed to provide evidence that DC-based vaccines are superior to standard chemotherapy. One of the reasons may be that the protocols for ex vivo DC maturation are not yet fully optimized. They frequently include only cytokine cocktails, whereas recent data suggest that stimulation through toll-like receptors (TLRs) or other receptors recognizing pathogen-associated molecular patterns may be required for optimal DC activation.

In a systematic comparison of agonists specific for different TLRs, TLR2, 3, 4, 5 and 7 were found to differentially induce the mRNA expression of certain cytokines, including IL-12p35, in DCs (8). When combinations of agonists were used, it could be shown that signaling through TLR2 was able to inhibit TLR4-induced IL-12 p35 expression, and this was mediated by the regulatory cytokine IL-10, which was released in markedly higher amounts by DCs treated with a TLR2 agonist compared to those treated with TLR3 or TLR4 agonists. Note that different TLR2 agonists also vary in their ability to induce IL-10 and IL-12, and the picture becomes even more complex once CD40-CD40L interactions, such as would occur in vivo between DCs and naïve T cells, are taken into account (9). **Not surprisingly, the in vivo effects of combinations of TLR agonists may differ from those obtained in even the more complex in vitro systems (114).**

Because they are fungal molecules and as such are likely to be recognized by a variety of pattern recognition receptors, mushroom polysaccharides or peptidoglycans may offer the potential to improve existing protocols for the ex vivo induction of DC maturation for the purpose of DC-based cancer vaccines. To date, however, there is little information on the recognition of mushroom compounds by TLRs on DCs. Only a 150 kDa proteoglycan from *Phellinus linteus* (PG, see Table 1) has been shown to induce the expression of MHC II and costimulatory molecules and the production of IL-12 by murine bone marrow-derived DCs via both TLR2 and TLR4 (10, 11), although the complement receptor 3 (CR3) may also have contributed (12) (see also Table 2). Upregulation of CD40 expression of murine bone marrow-derived DCs incubated with fraction H of *Agaricus blazei* mycelia extract (ABH, see Table 1) *i* did not involve TLR4 (13). In contrast, induction of IL-12 production in human macrophages by this extract was shown to be mediated by TLR4 and CD14, but not by TLR2 (14). It remains to be established whether differences between species, cell-type specific receptor recognition of the same compound, or different ABH constituents are responsible for this divergent receptor usage. Recognition of certain mushroom compounds by TLR2 and/or TLR4 has also been demonstrated in other cell types and using other mushroom compounds, as summarized in Table 2.

Effect of Mushroom Compounds on DCs *In Vitro*. Consistent with the ability of tumors to suppress DC functions, incubation of differentiating human peripheral blood monocyte-derived DCs with tumor cell supernatant inhibited their phagocytosisand phenotypic maturation as

Table 2. (Extended)

Mushroom	Anti-TLR4 Ab	C3H/HeJ mice	Anti-CD11b/anti-CD18 Ab	Reference
Ganoderma lucidum	Complete inhibition	Complete inhibition	[a]	(99)
G. lucidum	Partial inhibition Partial inhibition[b]			(75)
G. lucidum		Complete inhibition		(72)
G. lucidum	Partial inhibition (33%)			(72)
Agaricus blazei	Complete inhibition at highest dose Complete inhibition			(14) (14)
A. blazei		[a]		(13)
Phellinus linteus			Partial inhibition	(12)
P. linteus	Inhibition of unspecified extent Almost complete inhibition			(10, 11)

assessed by HLA-DR, CD86 and CD83 expression (15). It also suppressed the production of IFNγ, IL-10, IL-12 and the pro-inflammatory cytokine tumor necrosis factor (TNF)α by mature DCs. The presence of the protein-bound polysaccharide K (PSK) isolated from *Coriolus versicolor* (Fr.) Quél was able to overcome the defective phagocytosis of differentiating DCs and to restore the expression of HLA-DR and CD86, though not of CD83. The production of IFNγ, TNFα, and IL-10 of tumor cell supernatant-treated mature DCs remained at similar levels in the presence and absence of PSK, whereas IL-12 synthesis was completely restored.

Note that PSK is one of a variety of mushroom compounds or fractions that have been shown to induce the morphological, phenotypic and functional maturation of normal human monocyte-derived or murine bone marrow-derived DCs in vitro ((16) and see also Table 3). Compared to lipopolysaccharide (LPS), some are weaker inducers of DC maturation and particularly of DC cytokine production (12, 13, 17), whereas others are as effective or even more potent (17–19).

Th1/Th2 Polarization. The ability of DCs to polarize the immune response towards a Th1 or type 1 pattern (characterized by the synthesis of IFNγ and IL-2) crucially depends on their ability to produce IL-12 (1). As summarized in Table 3, almost all mushroom compounds investigated to date, whether from *Ganoderma lucidum*, *Coriolus versicolor*, *Phellinus linteus*, or *Agaricus blazei*, were able to induce IL-12 production of DCs. Whereas some also induced IL-10 synthesis, proteoglycans from *Phellinus linteus* and *A. blazei* did not. Some of the data presented in Table 3 also indicate that the production of IL-12 and IL-10 by DCs stimulated with mushroom compounds does not predict the cytokine pattern induced in allogeneic T cells. For example, ABH from *A. blazei* mycelia did not induce IL-12 or IL-10 in DCs, but ABH-treated DCs stimulated IFNγ production in allogeneic T cells (13). Most notably, DCs pre-incubated with ABH before stimulation with LPS induced significantly higher IFNγ production in allogeneic T cells than DCs stimulated with LPS only or pre-incubated with LPS before addition of ABH. This is the more surprising since ABH added to DCs before LPS stimulation markedly decreased the amount of IL-12p40 produced by these DCs.

Conversely, a polysaccharide fraction from *G. lucidum* mycelium enhanced DC production of IL-12 and IL-10, but these DCs had no effect on the IFNγ or IL-4 secretion of allogeneic T cells, even though they enhanced T cell proliferation. A protein-bound polysaccharide from *G. lucidum*, PS-G (see Table 1), stimulated DC production of IL-12 and IL-10 and these DCs induced significantly higher secretion of IFNγ than LPS-treated DCs (19). Interestingly, PS-G-treated DCs also stimulated allogeneic T cells to release considerable amounts of IL-10, whereas LPS did not. Intracellular cytokine staining confirmed that a significantly higher percentage of CD3+ T cells was positive for IL-10 after co-incubation with PS-G-treated compared to LPS-treated DCs.

To date, only one of the mushroom compounds that could induce DC maturation in vitro has also been investigated for its effects on DCs and the cytokine pattern associated with their activation in vivo, namely PG, a 150 kDa proteoglycan from *Phellinus linteus* (11). When administered intraperitoneally (i.p.) for 19 days following the implantation of MCA-102 tumor cells, PG inhibited tumor growth by ~40% and this was associated with the induction of phenotypic maturation of $CD11c^{+}$ DCs. In particular the frequency of activated$CD8\alpha^{+}$ DCs was increased in the spleen and lymph nodes of tumor-bearing mice. It has been reported that DCs expressing the CD8α molecule predominantly drive Th1 responses, whereas DCs lacking CD8α expression preferentially induce Th2 response (20, 21), but more recent data suggest that DC subpopulations may not be as specialized as previously thought (22). In vivo administration of PG also significantly raised the number of DCs positive for intracellular IL-12

Table 3. Effect of Mushroom Compounds on DC Maturation and Functions

Mushroom	Fraction or compound	Type of DC	Antigen uptake	MHC expression	CD expression	Cytokine production	Effect on allogeneic T cells	Reference
Ganoderma lucidum	PS-G	Human monocyte-derived	↓	↑ HLA-DR	↑ CD40, CD54, CD80, CD83, CD86	↑ IL-12p70 and p40 and IL-10	Increased IFNγ and IL-10 production (greater induction than in response to LPS)	(19)
G. lucidum	Hot water extract of mycelium	Human monocyte-derived	↓	↑ HLA-DR	↑ CD40, CD80, C86 (others not examined)	n.d.[a]	**No effect** on proliferation or production of IL-4 or IFNγ	(87)
G. lucidum	Polysaccharides purified from the hot water extract of mycelium	Human monocyte-derived	↓	↑ HLA-DR	↑ CD40, CD80, C86 (others not examined)	↑ IL-12p70 and IL-10 (with half as much IL-12, but similar levels of IL-10 compared to LPS)	Increased proliferation, but production of intracellular or secreted IFNγ or IL-4 was not significantly affected (although intracellular IFNγ was higher than in controls)	(18)
Coriolus versicolor	PSK (protein-bound polysaccharide K)	Human monocyte-derived	↓	↑ HLA II,	↑ CD40, CD80, CD86, CD83	↑ IL-12 (no other cytokines were assessed)	Increased proliferation	(16)
Phellinus linteus	PG, a 150 kDa proteoglycan	Murine bone marrow-derived	↓	↑ MHC II (both number of positive cells and expression intensity) ↑ Number of MHC I-positive cells	↑ CD80, CD86 (others not examined)	↑ IL-12p70, but IL-10 remained undetectable	Increased proliferation and secretion of IL-2	(10, 12)
Agaricus blazei	Water-soluble proteoglycan	Murine bone marrow-derived	↓	↑ MHC II	↑ CD80, CD86 (others not examined)	↑ IL-12p70, but IL-10 remained undetectable	Increased proliferation and secretion of IL-2	(17)
A. blazei	ABH	Murine bone marrow-derived	n.d.	↑ MHC II	↑ CD40, CD80 (though to a far lesser extent than LPS)	No effect on IL-12p40, IL-10, TNFα, or IL-1	Increased proliferation and IFNγ production, no effect on IL-4 production	(13)

[a] n.d., not determined.

and IFNγ (11). Furthermore, it augmented the percentage of CD4+ T cells in spleen and lymph nodes and, to a lesser extent, the percentage of CD8+ T cells and upregulated expression of the activation marker CD28 on both subsets. In addition, it enhanced intracellular levels of the Th1 cytokine IFNγ and decreased intracellular production of the Th2 cytokine IL-4 in lymph node and spleen T cells. The administration of DCs pulsed with tumor lysate one week before inoculation with MCA-102 tumor cells delayed tumor growth. Remarkably, no tumor growth at all was seen on day 28 in DC-immunized mice that had received PG together with the tumor cells, and 3 of 5 PG-treated mice remained free of tumors on day 60. This suggests that the maturation of DCs, particularly the induction of IFNγ and IL-12 production and the resulting polarization of T cells towards a type 1 cytokine pattern, play an important role in the tumor inhibiting effects of PG from *Phellinus linteus*. It also suggests that PG can contribute to the maintenance of vigorous protective T cell responses in mice that had received a DC-based cancer vaccine.

When tumor-bearing mice were treated with i.p. injections of the D-fraction of *Grifola frondosa* for 19 consecutive days, their DCs produced significantly higher amounts of IL-12p70 and IFNγ than those from saline-treated controls (23). Only IFNγ production was enhanced in lymph node DCs. Administration of the D-fraction was also associated with a significantly increased proportion of CD8α^+ DCs, but did not affect the CD8α^- fraction in the spleen and inguinal lymph nodes. Next, the authors isolated DCs from tumor-bearing mice that had been treated with D-fraction and injected the DCs into normal mice. When recipients of these DCs were subsequently inoculated with colon 26 carcinoma cells, tumor development was completely inhibited throughout the 28 day observation period. This protocol resembles a DC-based cancer vaccine, except that the DCs isolated from tumor-bearing animals are loaded with antigen in vivo rather than ex vivo and the vaccine is given before rather than after the development of tumors. The results suggest that the administration of the D-fraction to tumor-bearing DC donors was able to stimulate their DCs in such a way as to render them capable of inducing vigorous and long-lasting T cells responses when transferred into recipient mice. This is supported by the finding that recipients of DCs from D-fraction treated tumor-bearing animals showed enhanced expression of the activation marker CD28 on their CD4+ T cells and to an even greater extent on their CD8+ T cells. In addition, this protocol was associated with significantly increased production of IFNγ, IL-12 and TNFα by spleen and lymph node cells in response to Con A stimulation. Notably, it also increased IL-4 production, i.e., a clear Th1 pattern did not emerge. Note that the mouse strain used in this study, BALB/c mice, is genetically predisposed towards Th2 immune responses (24). A polarization from a carcinoma-induced Th2 towards a Th1 response, including suppressed splenic IL-4 production, was observed when the D-fraction was administered to tumor-bearing C3H/HeN mice, a strain with a Th1 default cytokine pattern (25).

More recently, the effectiveness of a combination of suboptimal doses of the chemotherapy agent mitomycin C (MMC) and the D-fraction was examined in tumor-bearing C3H/HeJ mice (26). The D-fraction significantly increased the spontaneous IL-12 production by whole spleen cells both in otherwise untreated and in MMC-treated mice. It also resulted in enhanced spleen cell IL-2 production compared to saline-treated controls and induced even higher levels of IL-2 synthesis from splenocytes of MMC-treated animals. In addition, IFNγ mRNA expression in whole spleen cells was decreased by MMC compared to saline treatment and was restored by the combination of MMC and D-fraction, but was highest in animals treated with the D-fraction only. Gene expression of IL-4 was increased by MMC, decreased to the levels seen in saline-treated controls in animals that received both MMC and D-fraction, and further decreased in those treated with D-fraction only. This resulted in the highest IFNγ:IL-4 mRNA ratio in fraction-D treated animals and the lowest in MMC-treated mice. These findings suggest that the D-fraction induced a predominant Th1 pattern in tumor bearing C3H/HeJ mice, including those treated with MMC.

The same researchers also investigated the cytokine pattern of Con A-stimulated splenocytes in normal C3H/HeJ mice that had been i.p. injected with the D-fraction of *Grifola frondosa* for 17 consecutive days (27). Compared to saline-treated controls, splenocytes from D-fraction-treated animals exhibited slightly, but significantly enhanced production of both IFNγ and IL-10 compared to those of saline-treated controls, whereas IL-4 and IL-12 synthesis were similar in the two groups. No clear cytokine pattern emerges from these data; however, D-fraction-treated animals had significantly elevated serum concentrations of IgE, which is associated with a Th2 pattern. In contrast to the data obtained in tumor-bearing animals, this suggests a stronger induction of Th2 than Th1 responses by the D-fraction in normal mice. Of particular note, this strain is genetically predisposed towards a type 1 pattern.

When immature DCs were treated in vitro with PS-G from *Ganoderma lucidum*, then co-cultured with autologous CD4+ T cells, the supernatants contained significantly increased levels of IFNγ, whereas production of the Th2 cytokine IL-5 was not affected (28). In the same study, mice immunized with ovalbumin (OVA) plus PS-G showed significantly higher levels of OVA-specific IgG2a, but similar levels of IgG1, compared to mice immunized with OVA alone. Isotype switching to IgG2a is induced by IFNγ, and production of this cytokine by OVA-stimulated splenocytes was significantly higher in mice treated with OVA plus PS-G compared to mice immunized with OVA alone, while IL-5 production was not markedly affected. These data suggest that PS-G can induce Th1 dominant immune responses not only in vitro but also in vivo in the mouse strain studied. Of note, BALB/c was the mouse strain

used and is characterized by a genetically determined preference for Th2 immune responses (24).

The effects of extracts of *Phellinus linteus* (PL) or *P. linteus* grown on brown rice (PB) on IgE synthesis have been studied in normal mice (29). The preparations consisted of the combined ethanol extract and the ethyl acetate extract of residue left after ethanol extraction and were administered orally for 4 weeks. Both extracts resulted in significantly decreased levels of IgE in serum and spontaneous as well as Con A-induced IgE production in mesenteric lymph node cells. They also enhanced the Con A-induced production of IFNγ in mesenteric lymph node cells, but IL-2 synthesis was not affected by PL and decreased by PB. Both extracts significantly reduced Con A-induced secretion of IL-4 and IL-10 in mesenteric lymph node lymphocytes. The same groups of researchers had previously reported that PL and PB reduced not only serum total IgE concentrations but also spontaneous and Con A-induced IgE production by spleen lymphocytes (30). They also increased Con A-induced IFNγ production in splenocytes, but neither significantly affected IL-2 synthesis in this system. In contrast to the results obtained with mesenteric lymph node cells, neither extract had a significant effect on Con A-induced IL-4 or IL-10 levels in whole spleen cells. Overall, these data suggest that PL can skew the immune response towards a type 1 pattern, particularly in mesenteric lymph nodes, which play an important role in food allergies but also in the intestinal immune responses to microbes.

Others investigated the effects of dietary supplementation with hot water extracts of *Ganoderma tsugae* mycelia and fruiting bodies harvested either 2–3 weeks (baby *G. tsugae*) or 4–8 weeks after fruiting (mature *G. tsugae*) in a murine model of allergic airway inflammation (31, 32), which is associated with a Th2 immune response. The experimental diets were fed for a total of 5 weeks before and during sensitization and challenge at levels of 0.2 or 0.4% for baby *G. tsugae* and 0.33 or 0.66% for mature *G. tsugae*. Allergic airway inflammation was induced by i.p. sensitization with ovalbumin (OVA) followed by challenge with aerosolized OVA. All of the extracts decreased the OVA-induced influx of leukocytes into the lungs compared to unsupplemented animals (32). They also reduced the levels of histamine and prostaglandin E2, but did not significantly affect the concentrations of the pro-inflammatory cytokines IL-1β and IL-6 in the bronchoalveolar lavage fluid (BALF). Notably, *G. tsugae* supplementation further increased the OVA challenge-induced concentrations of Th2 cytokines (IL-4, IL-5) in BALF and tended to decrease IL-2 concentrations, which were also increased by OVA challenge. Serum levels of OVA-specific IgE and IgG2a (Th2 and Th1-associated types of Ig, respectively) were not significantly altered. This enhancement of type 2 cytokine responses in a murine model of allergic inflammation does not recommend *G. tsugae* as an appropriate immunomodulator for decreasing allergic symptoms.

In a similar study, mice were immunized with the important allergen Dp2 from house dust mites (*Dermatophagoides pteronyssinus*) and were gavaged daily with a commercial preparation of *Ganoderma lucidum* (or a variety of other treatments) until intratracheal challenge with Dp2 28 or 35 days later (33). When mice were examined two days after the challenge, oral administration of *G. lucidum* was associated with increased numbers of IL-5 and IFNγ-producing peripheral CD4 T cells, although the ratio of IL-5:IFNγ-positive cells was decreased compared to buffer-treated animals. *G. lucidum* resulted in decreased serum levels of IgG1 and increased concentrations of IgG2a, which is consistent with the augmented serum IFNγ levels in *G. lucidum*-treated animals. The *G. lucidum* preparation also increased the IFNγ concentration in BALF slightly, but significantly. These findings suggest that this *G. lucidum* preparation was able to somewhat skew the immune responses towards a type 1 pattern, despite the increase in the Th2 cytokine IL-5. However, it did not significantly affect airway hypersensitivity to methacholine.

Several mushrooms such as *Ganoderma lucidum*, *Flammulina velutipes*, and *Volvariella volvacea* contain fungal immunomodulatory proteins (FIPs) with similarities in their amino acid sequence and their ability to modulate immune functions in vitro and in vivo, including after oral administration (34–37). One of these, FIP-fve from *Flammulina velutipes*, induced T cell proliferation in a manner that was dependent on antigen-presenting cells, suggesting that it might also have an effect on DCs (37). The production of IFNγ was dramatically increased when T cells were stimulated with FIP-fve in vitro, and mice gavaged with this protein exhibited significantly increased serum IFNγ concentrations in a time and dose-dependent manner. When given intragastrically to mice before and during sensitization and challenge with OVA, FIP-fve reduced OVA-specific IgE serum levels, but increased those of IgG2a. Compared to PBS-treated controls, splenocytes from FIP-fve-treated animals produced significantly less IL-5 and more IFNγ in response to OVA stimulation. Anaphylaxis symptoms induced by intragastric administration of OVA were significantly attenuated in FIP-fve-treated mice, as were plasma histamine levels. The effects of FIP-fve on OVA-specific IgE and IgG2a levels and plasma histamine concentrations were largely prevented by anti-IFNγ antibodies, suggesting that they were mediated by increased production of this Th1 cytokine.

In summary, numerous mushroom compounds are capable of inducing the phenotypic and functional maturation of DCs in vitro. Almost all of them also induce IL-12 production, but their effects on the Th1/Th2 polarization of the resulting T cell responses in vitro and in vivo are variable and appear to be highly compound-specific. In vivo studies suggest that PG from *Phellinus linteus* and the D-fraction from *Grifola frondosa* have potential as DC-stimulating agents in DC-based cancer vaccine protocols, but much further research is needed before their clinical use can be considered. In studies in a murine model of food

Table 4. Effects of Mushroom Extracts on NK Cells In Vitro

Mushroom	Extract/fraction	Effects	Reference
G. lucidum	EORP	Enhanced number of CD3-CD16+CD56+ NK cells in umbilical cord mononuclear cells; increased NK cytotoxicity in highly enriched CD56+ NK cells at an E:T[a] ratio of 20:1, but not at 5:1 or 80:1	(101)
G. frondosa	Fractions of the hot water extract of mycelia in submerged culture precipitated with 50% and 67% EtOH, respectively	Stimulated NK activity in human PBMC at an E:T ratio of 50:1	(108)
A. blazei	Aqueous extracts (40, 60, 80, or 100° C) of the fruit body	No effect	(44)
	Fraction of the aqueous extract soluble in 30% EtOH, but insoluble in 50% EtOH	Augmented NK activity in murine splenocytes at an E:T ratio of 100:1 (no other ratios were tested)	
A. blazei	Aqueous extract (37° C) of fruiting body or ABPC (see Table 1)	Stimulated NK activity in DC-rich splenocytes; induced IFNγ production by spleen cells	(43)
Phellinus linteus	73 kDa polysaccharide protein complex	Enhanced NK activity of purified NK cells at an E:T ratio of 50:1, but not 25:1 or 10:1)	(74)
Pleurotus ostreatus	Proteoglycan fraction of mycelia	Increased NK activity in non-adherent splenocytes at an E:T ratio of 10:1	(109)

[a] E:T, effector to target.

allergy, FIP-fve from *Flammulina velutipes* shows promise in dampening allergic responses. In contrast, experiments with extracts of *Ganoderma tsugae* and *Ganoderma lucidum* have yielded results suggesting that neither of these compounds is appropriate for the treatment of allergies.

NK Cells. DCs can activate NK cells, and their ability to produce IL-12 has been shown to be essential in this process (2). There are numerous reports that the anti-tumor effects of whole mushrooms or mushroom extracts or fractions are associated with significantly enhanced NK cell activity ex vivo (38–42). There is a growing list of mushroom compounds capable of stimulating NK cell activity or, in some cases, NK cell numbers in vitro (summarized in Table 4) and in vivo. Encouragingly, much of the current research in animal models uses the oral route of administration, and results from human supplementation studies are also becoming available. In addition, studies no longer exclusively focus on tumor-bearing animals, but include otherwise immunosuppressed mice as well as healthy animals. Most of the available in vivo studies focused on the cytotoxic activity of NK cells, only a few investigations examined the effects of mushroom compounds on NK cell numbers. Whenever available, results on NK cell counts are provided.

Oral administration of powdered dried *A. blazei* fruiting bodies to mice increased the cytotoxic activity against NK-sensitive target cells of their liver and, to a lesser extent, spleen mononuclear cells (43). Augmented cytotoxicity was seen after 2 weeks, but not after 1 week, of supplementation and was INFγ-dependent as indicated by the fact that it was not observed in IFNγ-deficient mice. NK cells purified from liver of T cell-deficient RAG-2-/- mice also exhibited increased cytotoxic activity, further suggesting that NK cells rather than NK T cells were the cellular subset activated by *A. blazei*. It was reported that *A. blazei* administration increased the cytotoxicity of purified NK cells to a similar extent as seen in whole liver or spleen cells, arguing against a role of DCs in the stimulation of this activity. Nonetheless, an aqueous extract of *A. blazei* prepared at 37° C was shown to increase the production of IL-12 by isolated splenic DCs as well as splenic macrophages in vitro, and incubation of DC-rich splenocytes with this extract resulted in enhanced NK activity in an IFNγ-dependent manner. Of note, others found that hot water extracts of *A. blazei* obtained at temperatures between 40° and 100° C did not enhance NK cell activity in vitro, even though the same target cells were used ((44), see also Table 4). The 37° C aqueous extract of *A. blazei* when administered orally for 2 weeks significantly enhanced NK cytotoxicity in an IL-12 and IFNγ-dependent manner (43).

Oral administration of ABH from *A. blazei* mycelia for 21 days also increased splenic NK cell activity (14). In the same study, this extract was shown to induce IL-12p40 and p70 production in human PBMC in vitro, primarily in macrophages. Thus increased IL-12 secretion may have contributed to the augmented NK cell cytotoxicity seen in ABH-treated mice.

Intragastric administration of a commercially available hot-water extract of *A. blazei* increased splenic NK activity in normal mice (45). In tumor-bearing mice, NK cell activity was not examined, but their tumor-specific cytotoxic T lymphocyte activity was significantly enhanced and this was associated with marked inhibition of tumor growth (45). Their splenic production of IFNγ, an inducer of cytotoxic T

lymphocytes, was also significantly upregulated by *A. blazei* treatment.

When *A. blazei* was sequentially extracted with methanol, hexane, and dichloromethane and given orally to mice, only the hexane extract significantly inhibited Ehrlich tumor cell growth (46). Splenic NK activity was suppressed in tumor-bearing compared to normal mice, but was completely restored after 10 days of treatment with this *A. blazei* extract. After 30 days of treatment, however, NK activity was similar in all tumor-bearing animals regardless of *A. blazei* treatment and significantly lower than in normal control animals. This suggests that NK cells eventually become refractory to the stimulatory effects of this *A. blazei* extract during prolonged administration.

Another group isolated an anti-angiogenic substance from the methanol-soluble fraction of the chloroform-methanol extract of *A. blazei* and identified it as sodium pyroglutamate (47). When given orally to mice bearing Lewis lung carcinomas (LCCs), this compound not only inhibited tumor growth and metastasis but also increased the number of splenic NK cells at the lowest dose (30 mg/kg), but not at the two other doses (100 or 300 mg/kg). Note that NK cells were not decreased in untreated LCC-bearing mice, whereas the splenic lymphocyte counts, particularly CD4+ and CD8+ T cell counts were markedly reduced and this was reversed by sodium pyroglutamate at the dose of 30 mg/kg, whereas higher doses were mostly ineffective.

Intraperitoneal administration of the D-fraction of *Grifola frondosa* to tumor-bearing C3H/HeJ mice for 3 days resulted in significantly increased ex vivo NK cell activity compared to saline- or dextran-treated controls (48). This was associated with significantly elevated concentrations of plasma IFNγ and augmented spontaneous IL-12 production by cultured spleen cells. The increased expression of the activation marker CD86 on macrophages led the authors to speculate that this cell type constituted the most likely source of IL-12. Note, however, that other antigen-presenting cells, activated NK cells and T cells can also be major sources of IL-12. NK cells of D-fraction-treated mice also exhibited increased cell surface expression of the IL-12 receptor βI. Together these results suggest that the D-fraction increased the NK cytotoxicity via the induction of IL-12 and IFNγ. In a previous study, the same group of researchers had shown that i.p. administration of the D-fraction was able to enhance IFNγ and activation marker (CD69) expression by NK cells in normal mice (49).

Recently, the effects of i.p. administration of *Gl*-PS, a polysaccharide peptide from *Ganoderma lucidum* (see Table 1) were examined in mice that had been immunosuppressed by the administration of a single dose of the anti-tumor agent cyclophosphamide (Cy) (50). The cytotoxicity of NK cells and lymphokine-activated killer cells was significantly suppressed by Cy, but was restored to normal by the lower dose of *Gl*-PS (2.5 or 25 mg/kg) by day 7 after Cy treatment and was further enhanced beyond the normal levels on day 9 after Cy treatment. The high dose of *Gl*-PS (250 mg/kg) was not effective on day 7, but did not prevent the recovery seen in vehicle-treated animals on day 9. The numbers of splenic NK and NK T (NK1.1+) cells were significantly reduced in Cy-treated animals compared to normal controls, but remained normal in Cy-treated animals that received 2.5 or 25 mg/kg of *Gl*-PS, whereas the 250 mg/kg dose of *Gl*-PS in conjunction with Cy resulted in NK1.1+ cell counts even lower than seen in animals treated with Cy alone. NK T cell (CD3+NK1.1+) counts were depressed in Cy-treated animals compared to controls, and were increased only by the lowest dose of *Gl*-PS (2.5 mg/kg), though still not restored to normal levels.

Stress can have detrimental effects on a variety of immune functions, including NK activity, and a marked decrease in NK activity was observed in mice that had been submitted to restraint stress for 12 or 18 hours (51). Oral administration of CM6271, described as a freeze-dried "preparation" from the mycelia of *Tricholoma matsutake*, for 10 days before the restraint period promoted the recovery of NK cell activity such that CM6271-treated mice showed almost normal NK activity on day 7, whereas water-treated controls exhibited depressed NK activity even on day 10. Of note, treatment for 5 days before and 5 days after restraint was much less effective than administration for 10 days before restraint. The recovery of the splenic NK1.1+ cell counts was not significantly affected by the administration of CM6271. Oral administration of this preparation throughout the restraint stress period (20 days of 6 h of restraint daily) also significantly inhibited the stress-induced decrease in NK cell activity (52). Together, these results suggest that CM6271 requires repeated administration over prolonged periods of time before becoming effective, particularly when more severe depression of NK cell activity is induced by longer periods of stress. The biological activity was contained in the aqueous phase fraction of the alkaline extract of the mycelia preparation, and further fractionation resulted in the identification of a compound with a relative molecular mass of 360 kDa with a sugar:protein ratio of 16:1 (53). The sugar moiety consisted mostly of glucose with predominantly α-1,4 linkages. This fraction also accelerated the recovery of NK cell activity when administered for 10 days before an 18-hour restraint stress period. At a dose of 25 mg/kg/d, this fraction increased NK activity and serum IL-12 levels on day 7 after release from restraint to the same level as did 300 mg/kg/d of the parent compound. However, the fact that the efficacy of the parent compound, and presumably also of the purified fraction, is greatest when administered before stress induction makes its potential for enhancing the immunocompetence of stressed individuals somewhat doubtful.

Supplementation of the diet of healthy 4-months old mice with 10% freeze-dried white button mushroom (*Agaricus bisporus*) resulted in augmented NK activity at all effector:target cell ratios tested compared to the control group that received a dietary mix providing the same energy, total carbohydrates, dietary fiber and protein as the

mushroom-supplemented mix (54). Although NK activity was also increased in the group receiving a 2% mushroom diet, the difference did not reach statistical significance. Note that *A. bisporus* feeding did not increase the percentage of NK cells in the spleen. The higher level of dietary supplementation was also associated with increased Con A-stimulated production of IFNγ and TNFα by splenocytes, whereas IL12-p70, IL-6 and IL-10 production was not significantly different between the groups.

NK Cells in Cancer Patients Treated with Mushroom Compounds. In human cancer patients (cervical, ovarian, endometrial), oral administration of ABM, an α-glucan from of *A. blazei* Murill fruiting body (see Table 1), was associated with increased peripheral NK activity, but lymphokine-activated killer cell activity and macrophage H_2O_2 production were not affected (55). Unfortunately, the authors provided no information on whether mycelia or fruiting bodies were used or on the extraction method.

An increase in NK activity has also been reported in 10 cancer patients who took the D-fraction of *Grifola frondosa* orally for 1 to 63 months without receiving concomitant radiotherapy or chemotherapy (56). However, since there were no controls in this study, it is difficult to determine whether the changes in NK cell activity were attributable to treatment with the D-fraction or were due to natural fluctuations in this parameter.

Supplementation with a *Ganoderma lucidum* polysaccharide fraction for 12 weeks was recently investigated in 41 patients with advanced colorectal cancer not currently undergoing chemotherapy or radiotherapy (57). After supplementation, none of the immune parameters evaluated, including NK activity, were significantly different from those measured at baseline. This contrasts somewhat with the findings of an earlier study, in which 34 patients with advanced-stage cancers in various tissue received Ganopoly (a polysaccharide fraction of *Ganoderma lucidum*, see Table 1) and showed significantly enhanced NK activity along with significant increases in plasma IL-2, IL-6, and IFNγ concentrations and decreases in plasma IL-1 and TNFα levels (58). However, when the same investigators tested this compound in a group of patients with advanced lung cancer, they saw no significant effect on NK cell activity or any other immune parameter measured, although some patients appeared to respond (59).

In summary, numerous mushroom compounds have been shown to increase NK activity in vitro and in experimental animals after oral administration or dietary supplementation. Fewer and more variable data are available on the effects of these compounds on NK cell numbers. Of particular note, several of these studies indicate that the effects of certain mushroom extracts on NK cell numbers and activity are greatest at low doses and decrease or even disappear at higher doses. This suggests that careful dosing studies are required before any of these compounds are used clinically. Failure to identify the optimal dosage may account for the lack of effectiveness in some of the clinical studies published to date.

Macrophages. DCs are not the only cell type producing cytokines that modulate the Th1/Th2 balance. Macrophages also are important producers particularly of IL-1, IL-6, TNFα, IL-10 and IL-12. It has long been known that many of the highly purified mushroom β-glucans with potent antitumor activity, such as lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune*, SSG from *Sclerotinia sclerotiorum*, or grifolan from *Grifola frondosa*, are potent activators of macrophages in vitro and in vivo (38). In addition, the antitumor activity of some of these compounds was shown to be mediated to a large extent by macrophages and their ability to induce certain cytokines, such as IL-1 and TNFα. Note, however, that TNFα can have both tumor-inhibiting and tumor-promoting effects and that other macrophage cytokines may play a more important role in tumor inhibition (60, 61). However, the elaboration TNFα and IL-1 is of great importance in providing protection in certain infectious diseases, as do other macrophage functions. Recent findings on the ability of various mushroom compounds to stimulate macrophage cytokine production and other activities in vitro are summarized in Tables 5 and 6.

Also in vitro, extract of reishi polysaccharides (EORP, see Table 1) from *Ganoderma lucidum* (also called reishi) induced the cell surface expression of TLR4 in the J774.1 murine macrophage cell line**,** whereas LPS down-regulated it (62). EORP also increased cell surface expression of CD14, whereas LPS had no effect. LPS recognition is mediated by a receptor complex including CD14 and TLR4, and EORP increased cell-surface binding and subsequent internalization of LPS. Thus, if EORP exhibited the same effect in vivo it would be expected to augment bacterial clearance. When administered i.p., EORP enhanced the increase in serum IL-1 concentration induced by a subsequent injection of LPS in mice. It did not, however, augment the toxicity of LPS; and this was shown to be due to the simultaneous induction of IL-1 receptor antagonist (IL-1Ra), a natural antagonist of IL-1 activity. Thus, whether EORP can provide protection from bacterial infections by increasing IL-1 secretion or whether the effect of IL-1 induction is neutralized by the simultaneous increase in IL-1Ra secretion remains to be established.

AbM "gold label," a commercial extract prepared from *A. blazei* (see Table 1), dose-dependently induced the in vitro production of TNFα, IL-1β, IL-6 and IL-8, but not IL-10 and IL-12, in human peripheral monocytes (63). Gene expression in a human monocyte cell line (THP-1) treated with the same extract showed potent upregulation of the *IL1B* and *IL8* genes along with several chemokine genes and various other immune-related genes (64). Of note, weak induction of *TLR2* gene expression was also observed with both the *A. blazei* extract and LPS, whereas expression of the gene encoding the important β-glucan receptor dectin-1 was unaffected. The *A. blazei* extract did not induce the *IL6*

Table 5. Effect of Mushroom Compounds on Macrophage Cytokine Production In Vitro

Mushroom	Fraction or compound	Type of macrophage	TNFα	IL-1	IL-6	IL-12	IL-10	Other effects	Refs
Ganoderma lucidum	EORP	Human monocytes murine J774A.1 macrophage line		↑↑[a]				↑↑ IL-1 converting enzyme activity	(99)
G. lucidum	Spray-dried mycelia	RAW264.7	↑						(110)
G. lucidum	*Gl*-PS			↑↑					(72)
Phellinus linteus	73 kDa proteoglycan	Murine peritoneal	↑↑	↑↑	↑↑				(74)
Coriolus versicolor	PSP, a polysaccharide peptide from mycelia	Murine peritoneal	↑	↑					(69)
Agaricus blazei	ABH	Human sorted CD14+ macrophages after stimulation of PBMC				↑↑ (intracellular)			(14)
A. blazei	Commercial semi-particulate aqueous extract (“gold label”)	Human monocytes	↑↑	↑↑	↑↑	No effect	No effect	↑↑ IL-8	(63)
A. blazei	AbM “gold label”	THP-1, a human monocytic cell line	↑↑	↑↑	↑	↑ (p40)	No effect	↑↑ IL-8	(64)
A. blazei	50% EtOH fraction of aqueous extract of the fruiting body[b]	Rat bone marrow macrophages	↑↑					↑↑ IL-8	(111)
	Unfractionated aqueous extract of the mycelia[c]		↑						(111)
Lentinus edodes	Lentinan, a purified polysaccharide	Murine bone marrow macrophages infected with *L. monocytogenes*	↑↑			↑↑ (IFNγ-induced)			(112)

[a] ↑↑, strongly induced; ↑, modestly induced.
[b] The fractions of the fruiting body extract obtained with lower EtOH concentrations had little or no effect on any of the tested parameters.
[c] The EtOH fractions of the mycelia extract had little or no effect on any of the tested parameters.

Table 6. Effects of Mushroom Compounds on Other Macrophage Functions

Mushroom	Fraction or compound	Type of macrophage	Phagocytosis	NO[a] production	iNOS expression	Lysosomal enzyme activity	Cytotoxicity	Refs
Ganoderma lucidum	EtOH-precipitated fractions of a hot water extract	Human monocytes	↑			↑		(108)
Agaricus blazei	50% EtOH fraction of aqueous extract of the fruiting body			↑				(111)
Phellinus linteus	153kDa proteoglycan	Murine peritoneal		↑				(77)
P. linteus	73 kDa proteoglycan	Murine peritoneal		↑	↑		↑ Against B16 melanoma cells	(74)
Coriolus versicolor	Various polysaccharide fractions	Murine peritoneal		No effect by themselves; ↑ IFNγ-induced NO production				(113)
Lentinus edodes	Lentinan, a purified polysaccharide	Murine bone marrow macrophages infected with a deletion mutant of *L. monocytogenes*		↑			↑ Killing of a deletion mutant of *L. monocytogenes* that is unable to escape the phagolysosome	(112)

[a] NO nitric oxide; iNOS inducible nitric oxide synthase.

and *TNF* genes, as determined by microarray analysis, whereas LPS did. Nonetheless, the *A. blazei* extract markedly enhanced IL-6 and TNFα along with IL-12p40 protein production, as determined by ELISA. Upregulation of IL-8 and IL-1β protein secretion was also confirmed. When this extract was administered orally to patients with chronic hepatitis C infection, microarray analysis of peripheral blood indicated that none of the genes that were prominently upregulated in vitro showed increased expression in vivo (65). However, a total of 14 genes whose products are involved in cell cycling and transcriptional regulation were induced by oral intake of this *A. blazei* extract.

Nonetheless, when this extract was given orally to mice before they were infected with *Streptococcus pneumoniae*, it decreased the bacterial load for the first four days of the 10-day observation period and enhanced the survival compared to PBS treatment (66). The observation that uninfected mice orally treated with the *A. blazei* extract exhibited increased serum levels of TNFα and MIP-2, the murine analog of IL-8, suggests that the activation of innate immunity participated in the protective effect in pneumococcal infection. The same extract, upon oral administration before the induction of peritonitis by i.p. injection of fecal matter, resulted in decreased septicemia and increased survival (67).

Dietary supplementation with 2 or 10% white button mushroom (*Agaricus bisporus*) did not have a significant effect on peritoneal macrophage production of IL-6, TNFα, prostaglandin E2, nitric oxide or H_2O_2 (54). Note that in another study, the phagocytic and cytolytic activity of peritoneal macrophages was enhanced in mice receiving supplementation with *A. bisporus* at a dietary level of 20%, but not at 10% (68). This suggests that the 10% supplementation level was insufficient to induce significant macrophage activation.

Other studies show enhanced macrophage activation and cytokine production after i.p. administration of the D-fraction of *Grifola frondosa* (27) and of a polysaccharide peptide isolated from mycelia of *Coriolus versicolor* in normal mice (69). In addition, i.p. administration of a new polysaccharide L-II purified from the fruiting body of *Lentinus edodes* restored the macrophage phagocytic activity of tumor-bearing mice (70).

To summarize, a variety of purified mushroom polysaccharides or polysaccharide fractions are able to enhance various macrophage functions in vivo, including the production of cytokines and other inflammatory mediators and phagocytic and cytolytic activity. Whether this translates into improved ability to overcome bacterial infections has only rarely been investigated, except for promising results with orally administered extracts of *A. blazei*.

B Cells. While many mushroom compounds activate predominantly T cells and macrophages, others selectively stimulate B cells in vitro. These include a proteoglycan (GLIS) and a polysaccharide (GLPS) from *Ganoderma lucidum* (71, 72), and 150 kDa and 73 kDa proteoglycans

from *Phellinus linteus* fruiting body (73, 74). Other mushroom compounds can activate both T cells and B cells in vitro. Among these, EORP of *G. lucidum* was found to stimulate the proliferation and activation marker (CD86) expression of murine splenic B cells and to induce their differentiation into IgM-secreting plasma cells (75). Blimp-1, a main regulator of B cell differentiation, was markedly induced by EORP. In partial contrast, this polysaccharide fraction did not activate human peripheral B cells or induce their proliferation or differentiation into plasma cells; however, it upregulated Blimp-1 mRNA expression and markedly stimulated IgM and IgG production. The difference between murine and human B cells is most likely attributable to differential regulation of B cell activation by Blimp-1. A polysaccharide from *Phellinus linteus* mycelia, which has been shown to induce T cell proliferation and macrophage and NK cell activities (76), could also stimulate T-cell dependent antibody production and increase the number of antibody-forming cells in vitro (76, 77).

There is relatively little data on the in vivo effects of mushroom compounds on B cells, although some data are beginning to accumulate. Oral administration of an aqueous (80–85° C) extract of *A. blazei* fruiting body to normal Balb/cByJ mice markedly enhanced serum IgG levels, although it did not affect the proportion of B cells in the spleen (78). In OVA-immunized mice, *A. blazei* administration significantly raised the serum levels of OVA-specific IgG, but not IgM. It also augmented OVA-specific proliferative responses and TNFα secretion of splenocytes and increased the OVA-induced delayed-type hypersensitivity response, suggesting that oral intake of *A. blazei* stimulated both humoral and cellular immune responses.

Similarly, i.p. administration of ABM from *A. blazei* fruiting body together with antigen was found to significantly increase the number of antibody-producing cells without increasing the proportion of B cells in the spleen (79). This was associated with significantly increased mRNA expression of IL-1β and IL-6 in spleen cells. Both cytokines are involved in the differentiation of B cells into antibody-producing cells. The same *A. blazei* extract administered i.p. at the same time as an intramuscular immunization with a DNA vaccine for the hepatitis B virus core protein (HBcAg) significantly increased the antigen-specific antibody production after the second DNA vaccine injection (80). It also enhanced spleen T cell HBcAg-specific proliferative responses. Mice given a single i.p. injection of a *Phellinus linteus* mycelia polysaccharide together with i.p. administration of the antigen also exhibited increased antigen-specific antibody production (76).

Dietary supplementation of broiler chicks with poorly characterized extracts of water-soluble polysaccharides from *Lentinus edodes* or *Tremella fuciformis* for 7 days before infection with *Eimeria tenella* was found to enhance the specific IgA, IgM and particularly IgG responses to this intestinal protozoan parasite (81). The cellular immune response as assessed by antigen-specific proliferation of splenocytes was also significantly augmented.

In addition to mushroom compounds that enhance B cell activation, there are also some that suppress B cell functions. For example, a polysaccharide isolated from the spores of *Ganoderma lucidum* significantly decreased the number of antibody-producing cells in mouse spleen when given i.p. for four days following the injection of the antigen (82). In addition, it suppressed mitogen-induced proliferation of both B and T cells. It has also been reported that i.p. administration of the D-fraction of *Grifola frondosa* to tumor-bearing mice was associated with decreased expression of the activation marker CD69 on B cells, while increasing it on T cells, isolated from inguinal lymph nodes (25). CD69 expression on spleen lymphocytes was not affected.

In addition to these polysaccharides, a FIP protein isolated from *Ganoderma lucidum*, LZ-8, was able to significantly suppress antigen-specific antibody production when injected i.p. into mice (36). As noted above, FIP-fve from *Flammulina velutipes* significantly decreased OVA-specific IgE when administered orally (37). In addition, extracts of *Phellinus linteus* when given orally for 4 weeks to normal mice decreased total serum IgE levels and IgE production in mesenteric lymph nodes (29).

In summary, data concerning the modulation of B cell responses by mushroom compounds are still limited. It is of particular interest, that ABM extracted from *A. blazei* and various poorly characterized preparations from other mushrooms stimulated B cell antibody production, whereas several other compounds have been shown to suppress B cell activation in vivo. Since enhanced B cell function may provide improved protection against pathogens, but suppression of B cell functions would be highly desirable in certain autoimmune diseases and in allergies, further intense research seems warranted.

Potential for Study. After 50 years of mushroom research, numerous fungal compounds have been shown to possess immunomodulatory activity, including polysaccharides (mostly β- and some α-glucans), proteoglycans, proteins, and various constituents of small molecular mass. In vivo data are available for far fewer of them, and clinical studies are rare, often include small numbers of subjects, frequently are not randomized, mostly are not placebo-controlled, and hardly ever are double blind. Most disappointingly, the research is not systematic. New bioactive fractions or compounds are continually isolated, but—with a few notable exceptions—research on these substances remains confined to the occasional in vitro study.

There are very few comparative studies of different mushroom species or similar compounds isolated from either the same or different species in the same model in order to determine which substance is best suited to a particular purpose. There are also few series of studies that systematically investigate all facets of a particular compound or fraction, e.g., which cell types it activates, what

the consequences of this activation are, what the mechanisms of action are and, most importantly, whether the effects seen in vitro can be recapitulated in vivo. Optimal dosage and route and timing of administration have not been thoroughly established even for the better-known compounds, some of which are used in adjuvant therapy for cancer patients.

Mushrooms—either lyophilized or extracted by various methods—are being sold as dietary supplements, but information on their safety and efficacy is largely lacking. There can be pronounced differences in the bioactivity of mushrooms of the same species depending on strain (83, 84), growing conditions (e.g., wild versus cultured or different growing regions) (85, 86), developmental stage (mycelia versus fruiting body, immature versus mature basidiocarp), the part of the mushroom that is used (pileus or stipe) (85), and whether spores are included or not (18, 84, 87). Factors such as the temperature used for aqueous extraction (44, 84, 88) and the ethanol concentration (44) have a decisive role in determining the composition and, therefore, the bioactivity of the resulting extract and may even make the difference between beneficial and toxic effects (88). This underscores the urgent need to systematically determine what constitutes the best strain for a particular purpose and the best method for extracting the constituents responsible for this activity.

There are other areas in which additional research is called for. To date, there is only a single investigation of the absorption of orally administered soluble glucans, including the fungal scleroglucan from *Sclerotium glucanum*, in animals (89). After intragastric administration of fluorescently labeled glucans, fluorescence was quickly detected in plasma peaking after only 15 minutes in the case of scleroglucan, but after 3 or 4 hours for the other glucans tested. Bioavailability of scleroglucan was estimated to be 4.9%. Of note, the authors used glucans labeled at the reducing terminus and they did not investigate whether the fluorescence they detected in plasma was associated with glucans of the original molecular mass or with smaller remnants such as had been detected in earlier studies after the administration of other polysaccharides (90–92). Research on the large number of other bioactive β-glucans is urgently needed. Similar efforts should be undertaken to clarify the mechanisms of absorption and extent of bioavailability of other mushroom compounds, such as α-glucans and certain immunomodulatory proteins.

Although several β-glucan receptors have been identified and dectin-1 has emerged as the most important one on macrophages and possibly dendritic cells, these data have been obtained with compounds and substances that may have little in common with mushroom β-glucans (39). There is only one study specifically addressing the binding of a mushroom β-glucan to dectin-1 (93). It showed that schizophyllan, a gel-forming 1,6-branched 1,3-β-glucan from *Schizophyllum commune*, specifically bound to this receptor. Grifolan and SSG, two other 1,6-branched 1,3-β-glucans from *Grifola frondosa* and *Sclerotinia sclerotiorum*, respectively, were able to inhibit SPG binding, suggesting that they were also recognized by dectin-1.

TNFα and IL-12 production by macrophages in response to zymosan requires cooperation between dectin-1 and TLR2 (94, 95) and possibly other TLRs. However, TLR2 does not directly recognize certain β-glucans (95). Of note, mouse strains differ in their expression of the two major isoforms of dectin-1 and the stalkless dectin-1B isoform was always associated with higher production of TNFα in response to zymosan binding (96). If this also applies to mushroom glucans, it should be taken into account in the design and interpretation of studies investigating the β-glucan induced TNFα response. More research on the interaction of mushroom compounds with dectin-1 and the collaboration between this β-glucan receptor TLRs as well as other pattern recognition receptors will be necessary before a more detailed understanding of the different bioactivities of the various mushroom polysaccharides can be reached.

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