Expert Opinion





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Vaccines & Antibodies

# Yeast whole glucan particle (WGP) β-glucan in conjunction with antitumour monoclonal antibodies to treat cancer

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Beta-glucans, biological response modifiers (BRMs) derived from the cell walls of yeast and other sources, have been demonstrated to prime leukocyte complement receptor 3 (CR3), thus enabling these cells to kill tumours opsonised with complement fragment iC3b. Many tumours activate complement via the classical pathway mediated by antitumour monoclonal antibodies (mAbs) or natural antibodies. Studies into the cellular and molecular mechanisms of action have demonstrated that orally administrated yeast β-glucans are ingested and processed by macrophages. These macrophages secrete the active moiety that primes neutrophil CR3 to kill iC3b-opsonised tumour cells. Extensive studies in preclinical animal tumour models have demonstrated the efficacy of combined oral particulate yeast β-glucan with antitumour mAb therapy in terms of tumour regression and long-term survival. It is proposed that the addition of β-glucan will further improve the clinical therapeutic efficacy of antitumour mAbs in cancer patients.

**Keywords:** β-glucan, antitumour mAbs, biological response modifier, chemotaxis, complement receptor 3 (CR3), immunotherapy

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## 1. Introduction

The field of tumour immunotherapy has had a chequered history where periods of great enthusiasm were dashed by failures in the clinic. Some of these problems can be ascribed to the complexity of the cellular and molecular mechanisms of immune function and regulation [1]. Other challenges to immunotherapy include the heterogeneity of the malignant process both among different patients and in the same patients at different disease stages [2]. There is an increasing awareness that the immune destruction of tumours requires a combination of effector mechanisms, and that a single vaccine, cytokine or biological response modifier (BRM) is unlikely to be successful in the majority of patients. For example, most tumour vaccines are aiming to elicit strong antigen-specific cytotoxic T lymphocyte (CTL) responses, but the fact that most tumour cells are lacking the expression of the major histocompatibility complex (MHC) class I molecule, or that the level of MHC class I is significantly downregulated, reduces the efficacy of these vaccines [3,4].

Humanised antitumour monoclonal antibodies (mAbs), such as Herceptin® (trastuzumab, Genentech, Inc., CA, USA) and Rituxan® (rituximab, Biogen Idec, MA, USA), are now accepted clinical practice in patients with Her-2/neu+ metastatic mammary carcinoma and B cell lymphoma, respectively [5-7]. In addition, several new humanised mAbs, such as Erbitux® (cetuximab, ImClone Systems, Inc., NY, USA), are approved with more limited indications [8]. The

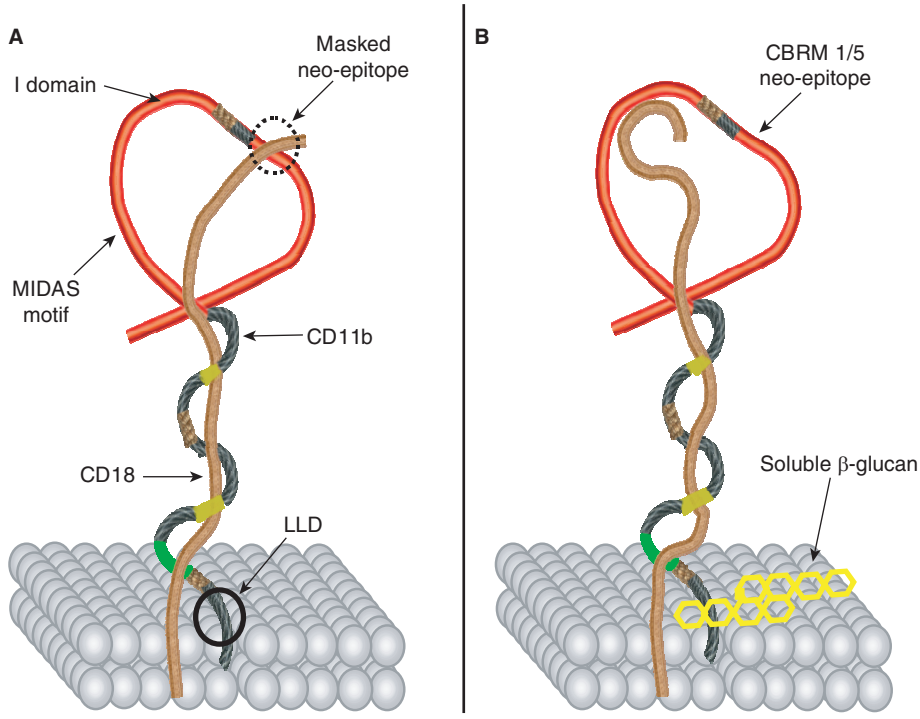


**Figure 1. Schematic illustration of CR3.** CR3 (Mac-1, $\alpha_m\beta_2$ integrin) is a heterodimer consisting of the $\alpha_m$ subunit (CD11b) and the $\beta_2$ subunit (CD18). CD11b contains two salient domains that are relevant to β-glucan and antitumour mAb immunotherapy. The I (inserted) domain is located near the N-terminus, mediates the adhesion functions of CR3 and binds with high affinity to iC3b and ICAM-1. The LLD is located near the C-terminus and binds to small molecular weight soluble β-glucans derived from the processing of β(1,3;1,6) yeast glucan or β(1,3;1,4) barley glucan. The binding of β-glucan to the LLD primes CR3 for cytotoxicity against iC3b-opsonised tumour cells. Panel **A** demonstrates CR3 in the non-activated state in which the CBRM 1/5 neo-epitope induced by β-glucan binding to the LLD is hidden. Panel **B** demonstrates activated CR3 as a consequence of the binding of β-glucan to the LLD, and the induction of the CBRM 1/5 neo-epitope. The induction of this neo-epitope is also dependent on the binding of divalent cations within the MIDAS motif. Following the neutralisation of background neutrophil activation signals, such as LPS, CBRM 1/5 induction may serve as a surrogate marker for β-glucan-dependent neutrophil priming.
ICAM: Intercellular adhesion molecule; LLD: Lectin-like domain; LPS: Lipopolysaccharide; mAb: Monoclonal antibody; MIDAS: Metal ion-dependent adhesion site.

effector mechanisms mediated by these antitumour mAbs are diverse and include inhibition of growth factor activity, facilitation of antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC), and the creation of immunoconjugates with toxins or radioisotopes [9-12]. However, antibody therapy is not uniformly effective, even in patients whose tumours express a high level of tumour antigen. For example, clinical studies in patients with advanced metastatic breast cancer have indicated that single-agent Herceptin treatment alone yields a response rate of < 25% [13]. Developing novel strategies to maximise the efficacy of antitumour mAbs is necessary to overcome this leading cause of death.

β-glucans belong to the family of BRMs and have existed for centuries in traditional Asian medicine. They have been used for the treatment of malignancy clinically (with varying and unpredictable success) for decades, particularly in Japan [14-18]. *In vitro* studies demonstrated that soluble yeast β-glucan binds to a lectin domain within the COOH-terminal region of the CD11b subunit of complement receptor 3 (CR3, CD11b/CD18, $\alpha_m\beta_2$ integrin, Mac-1; **Figure 1**) [19,20]. Furthermore, studies have indicated that β-glucans prime neutrophils or natural killer (NK) cells for cytotoxicity against iC3b-opsonised tumours as a result of complement activation by antitumour mAbs or natural antibodies [21,22]. Dual ligation of neutrophil CR3 mediated by the I-domain ligand, iC3b, and the lectin-like domain (LLD) ligand, β-glucan, leads to degranulation and cytotoxic responses [23,24]. Thus, β-glucan-mediated tumour immunotherapy utilises a novel mechanism by which innate immune effector cells are primed to kill iC3b-opsonised tumour cells. This review will demonstrate various sources and the structure of β-glucans, discuss the mechanisms of action for combined antitumour mAbs and β-glucan tumour immunotherapy, and summarise therapeutic efficacy data in animal models. Finally, this review will discuss potential human clinical applications and possible challenges for human cancer therapy.

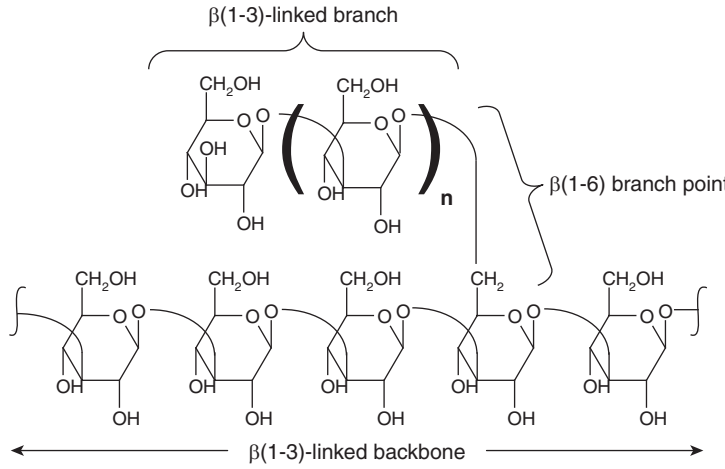


**Figure 2. Schematic illustration of the structure of yeast β-glucan demonstrating the β(1,3)-linked backbone with β(1,6) branches.**

## 2. Composition and structure of β-glucans

### 2.1 β-Glucan source and structure

β-Glucans are glucose polymers produced by a variety of plants and microorganisms, including oat, barley, mushroom, seaweed, some bacteria and blue-green algae, and yeast [25,26]. They have been used in traditional medicines for centuries, but it was not until the middle of the twentieth century that various β-glucans were isolated, structurally characterised and examined for the mechanism of their biological activities. β-Glucans derived from different sources have some variations in their structure. The basic structure, found in bacterial β-glucans, consists of linear glucose polymers with β(1,3) linkages. Some, such as oat and barley β-glucans, are primarily linear, with large regions of β(1,4) linkages separating shorter stretches of β(1,3) structures. Others (e.g., mushroom) have short β(1,6)-linked branches coming off of the β(1,3) backbone. Yeast β-glucans have β(1,6) branches that are further elaborated with additional β(1,3) regions. These seemingly minor structural difference can have large implications for the activity of the β-glucan. For example, differences in the length of the polysaccharide chain, the extent of branching and the length of those branches can result in the difference between material extractable by hot water (mushroom β-glucan) and an insoluble cell wall component (yeast β-glucan) [27].

### 2.2 Yeast β-glucan

For the rest of this review, β-glucan will refer to yeast β-glucan unless otherwise noted. Yeast β-glucans are long polymers of β(1,3) glucose, with 3 – 6% of the backbone glucose units possessing a β(1,6) branch (**Figure 2**) [28]. Their unique structure allows these polymers to form triple helices in solution, which have the ability to self-associate into large aggregates [26]. β-Glucans form a distinct layer in the yeast cell wall and will survive a variety of treatments necessary to remove the rest of the yeast cell and cell wall components [29]. This results in a yeast cell 'ghost', a hollow β-glucan sphere, roughly the same size (2 – 4 μm) as the original yeast cell (**Figure 3**).

Early preparations used to investigate the biological activity of β-glucan were only partially purified, containing significant quantities of mannans and mannoproteins. Later preparations were more pure and were often ground to a smaller particle size (submicron) to facilitate injection of the material into animal models. Alpha-Beta Technology, Inc. developed a cost-effective process to generate purified yeast β-glucan ghosts, and referred to this material as whole glucan particles (WGPs; **Figure 3**). Whole yeast and particulate β-glucan showed enough biological activity in early experiments in a variety of models to encourage further efforts to develop soluble β-glucan [30]. Efforts to develop a water-soluble version of yeast β-glucan began with chemical modification of the chain, by sulfation or phosphorylation [27,31]. Later process development work resulted in soluble, unmodified yeast β-glucan preparations that have been used in more detailed *in vivo* studies of β-glucan's biological effects [32-37].

As a major cell wall component, β-glucan comprises ~ 12 – 14% of the dry cell weight of yeast. Glucan preparations are first fractionated based on alkali solubility. Alkaline extractions dissolve the yeast cytoplasmic material, cell-wall mannans and proteins, and a minor glucan component, and leave intact the WGP. The WGP is then hydrolysed, purified by ultrafiltration and gel permeation chromatography to yield homogenous poly(1-6)-β-D-glucopyranosyl-(13)-β-D-glucopyranose (PGG)-Glucan. PGG-Glucan is a molecule of ~ 150 kDa and is comprised of a β-D-(1-3)-linked glucopyranosyl backbone with β-D-(1-6)-linked β(1-3) side chains. Three preparations of yeast β-glucan will be discussed in detail here in the context of their biological activity:

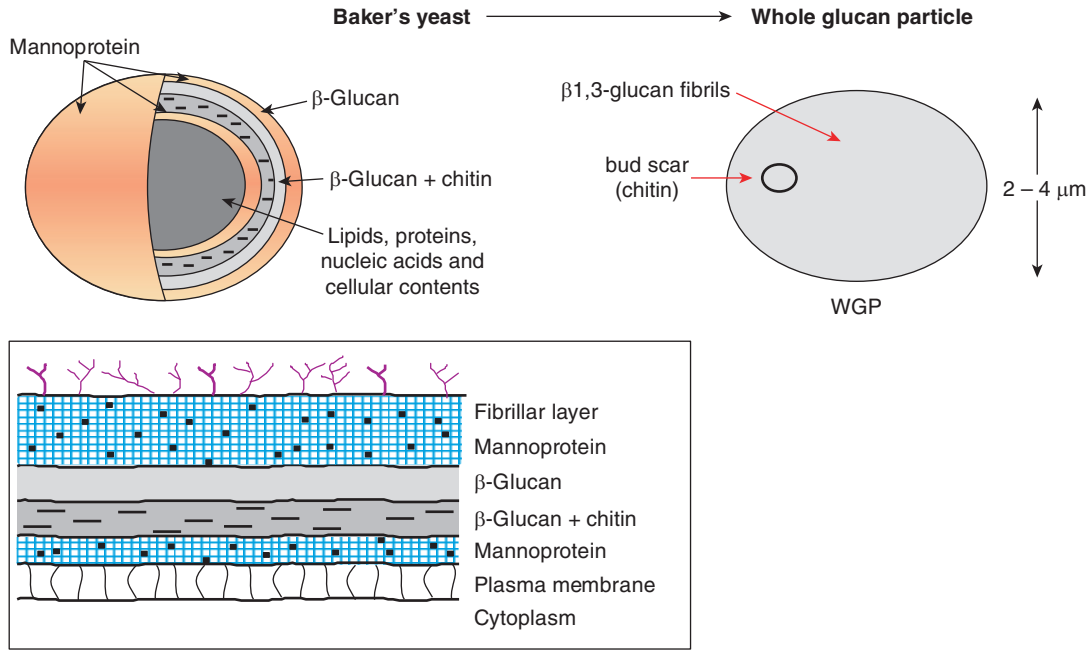


**Figure 3. Cross-sectional depiction of the baker's yeast cell wall.** Outside of the cell is a fibrillar mannoprotein layer that masks and presents a barrier to exposure of the β(1,3)-glucan to the external environment. Beneath the mannoprotein layer are layers of β-glucan and β-glucan chitin, which are outside the plasma membrane. It is this β-glucan layer that actually provides the shape and structural rigidity of the yeast cell. The baker's yeast can be processed by alkaline extraction to produce a WGP, which is a highly purified yeast cell wall ghost that is 2 – 4 mm in diameter and composed of β(1,3)-glucan fibrils.
WGP: Whole glucan particle.

### 2.3 WGP β-glucan

WGP β-glucan is an insoluble particle, purified from the cell walls of a common form of yeast, *Saccharomyces cerevisiae*, and is administered orally. It is a WGP, which is highly purified and maintains its *in vivo* spherical morphology. This hollow sphere of β(1,3;1,6)-glucan is generally 2 – 4 μm in diameter.

### 2.4 Betafectin PGG

Betafectin PGG is a soluble, intravenously-administered pharmaceutical-grade β-glucan compound derived from a proprietary strain of yeast. It is a triple helical molecule of PGG with an average molecular weight (MW) of 150 kDa. There is substantial preclinical data showing that Betafectin PGG stimulates a significant immune response and is efficacious in infectious disease, haematopoiesis and cancer immunotherapies.

### 2.5 Neutral soluble glucan

Betafectin PGG can be broken down into a single helical compound of small molecular weight (< 20 kDa) known as neutral soluble glucan (NSG). NSG directly binds to CR3 LLD, whereas more complex structures do not. This binding has been demonstrated to trigger a specific innate immune cell response.

## 3. Mechanisms of action for the combined antitumour mAbs and β-glucan tumour immunotherapy

### 3.1 iC3b deposition on tumour cells mediated by antitumour mAbs

Most humanised antitumour mAbs use the IgG1 framework and are able to activate the classical pathway of complement following the binding of mAbs to tumour cells [8,9]. Some humanised mAbs mediate ADCC, such as anti-CD52 (Campath®; alemtuzumab, ILEX Pharmaceuticals, TX, USA) and anti-Her2/neu (Herceptin). The anti-CD20 mAb (rituximab) mediates its tumouricidal effect through both ADCC and CDC. Following the activation of complement mediated by these IgG1-based mAbs, iC3b is deposited on tumour cells that can be recognised by the leukocyte iC3b-receptor CR3. However, complement-mediated tumour destruction is not very efficient due to three main reasons. First, the level of antigen expression on tumour cells is variable. The low density of tumour antigen cannot enable the formation of IgG dimers, which are required for the attachment of C1q and subsequent complement activation [38]. Second, most tumour cells overexpress complement regulatory proteins, such as CD55 and CD59 [39,40,41]. CD55 (decay-accelerating factor) can compete with factor B for

binding to C3b on the cell surface, accelerate the decay of C3- and C5-convertases and prevent the activation of downstream complement proteins. CD59 inhibits the binding of C9 to the C5b678 complex and prevents membrane attack complex (MAC) formation. Lastly, CR3 priming for cytotoxic function requires dual ligation of the I-domain and lectin-like domain of CR3, as discussed later. In this case, CR3 binds to iC3b, but is not able to initiate tumour-killing activity due to the lack of LLD ligand, β-glucan.

### 3.2 CR3-dependent cell-mediated cytotoxicity (CR3-DCC)

CR3 is widely expressed on the surface of all phagocytes, including neutrophils, eosinophils and basophils, as well as on the surface of monocytes, macrophages and NK cells [42-44]. It has been shown that neutrophil CR3-dependent phagocytosis or degranulation in response to iC3b-opsonised yeast required ligation of two distinct binding sites in CR3, one for iC3b and a second site for β-glucan. Subsequent research mapped each of these binding sites to domains within the α-chain of CR3, CD11b (**Figure 1**). Furthermore, the lectin site was mapped to a region of CD11b located C-terminal to the I-domain [20,23]. Induction of CR3-dependent cell-mediated cytotoxicity (CR3-DCC) requires dual ligation of CR3 to both iC3b and yeast β-glucan [21,24]. C3-opsonised yeast present iC3b in combination with β-glucan, such that both of these domains of CR3 become attached to the yeast, stimulating phagocytosis and cytotoxic degranulation. In contrast to microorganisms, tumour cells lack β-glucan. The lack of similar CR3-binding β-glucan on human cells explains the inability of CR3 to mediate phagocytosis or cytotoxicity of tumour cells opsonised with iC3b. iC3b-opsonised tumour cells mediated by naturally occurring antitumour antibodies or antitumour mAbs engage only the I-domain of CD11b and not the lectin site. Soluble β(1,3)-glucan polysaccharides isolated from fungi can bind to the lectin site of CR3 with high affinity and prime the receptor for subsequent cytotoxic activation by iC3b-opsonised tumour cells that are otherwise inert in stimulating CR3-DCC. Mice administrated oral yeast or barley β-glucan in conjunction with antitumour mAbs displayed significant tumour regression and long-term survival [45,46]. Therapy failure in C3- or CR3-deficient mice ($C3^{-/-}$, $CR3^{-/-}$) indicates the requirement for both iC3b deposited on tumour cells mediated by complement-activating mAbs or naturally occurring antibodies and its receptor CR3 on phagocytes [45,46].

### 3.3 Oral WGP β-glucan trafficking

Soluble low molecular weight yeast glucans can prime CR3 directly to trigger neutrophil degranulation after receipt of the second signal from iC3b bound to the target cell surface. However, the small molecular weight β-glucans were shown to be rapidly excreted by the kidneys, thus limiting their bioactivity and clinical utility [47]. In contrast, an orally administrated particulate WGP β-glucan is expected to have a longer half-life *in vivo* and is highly desirable for its clinical utility. It was hypothesised that particulate β-glucans are processed and digested into small fragments that can prime CR3. Indeed, this hypothesis is supported by recent studies in mice [46]. The trafficking process of WGP yeast β-glucan can be divided into three steps (**Figure 4**). The first step is the phagocytosis phase. Orally fed WGP is ingested by gastrointestinal macrophages that transport them to lymphoid organs. Within 3 days of daily oral administration of WGPs, macrophages in the spleen and lymph nodes contain WGPs. After 4 days, WGP yeast β-glucans are observed in the bone marrow. The uptake of WGP by macrophages does not require CR3, as characterised by the similar percentage of WGP-containing macrophages in wild-type versus $CR3^{-/-}$ mice [46]. The CR3-independent uptake of orally administered WGPs may illustrate a potential role for other particulate β-glucan receptors, including Dectin-1, in the phagocytosis phase. Dectin-1 is a C-type lectin with an immunoreceptor tyrosine-based activation motif, and some Dectin-1-mediated functions have been observed to be MyD88-dependent [48]. Dectin-1 is expressed on most myeloid cells and mediates phagocytosis of non-opsonised particles containing limited bioavailable β-glucan (e.g., zymosan) and some live pathogenic yeast [49].

The second step is the processing and priming phase. WGP β-glucans are digested to release small fragments that are concentrated at the edges of the cytoplasm near the membrane. *In vitro* experiments conducted with cultures of the macrophage cell line J774 have demonstrated that the macrophages have begun breaking down the particles at day 3. Complete macrophage degradation of all visible cytoplasmic WGPs requires > 13 days. The soluble, biologically active components of WGP are released into the culture medium and can be measured using a β(1.3)-glucan-specific bioassay. Moreover, these small fragments of WGP yeast glucans are able to prime CR3 and kill iC3b-opsonised tumour cells. The processing of WGP by macrophages is presumably through an oxidative-dependent pathway, as macrophages do not have glucanase. WGP β-glucans can also stimulate macrophages to secrete cytokines such as TNF-α and IL-1, -12 and -6. The production of TNF-α and IL-12 is CR3-dependent, whereas the secretion of IL-6 is MyD88 pathway-dependent. IL-1β secretion is partially dependent on the CR3 pathway, but completely dependent on the MyD88 pathway (J Yan *et al.*, unpublished data). These pro-inflammatory cytokines can potentially enhance the activation of adaptive immunity, such as antigen presentation and T cell activation. Thus, the administration of WGP β-glucan links the activation of both innate and adaptive immunity.

The final step is the effector phase. The β-glucan-primed neutrophils are chemoattracted by leukotriene $B_4$ ($LTB_4$) released from tumour endothelial cells, migrate into the tumour milieu and engage iC3b-opsonised tumour cells for cytotoxicity. *In vivo* experimental evidence indicated that C5a derived from complement activation initiates a cascade of chemoattractants and that the C5a-dependent chemotaxis of neutrophils is dependent on signal amplification by $LTB_4$.

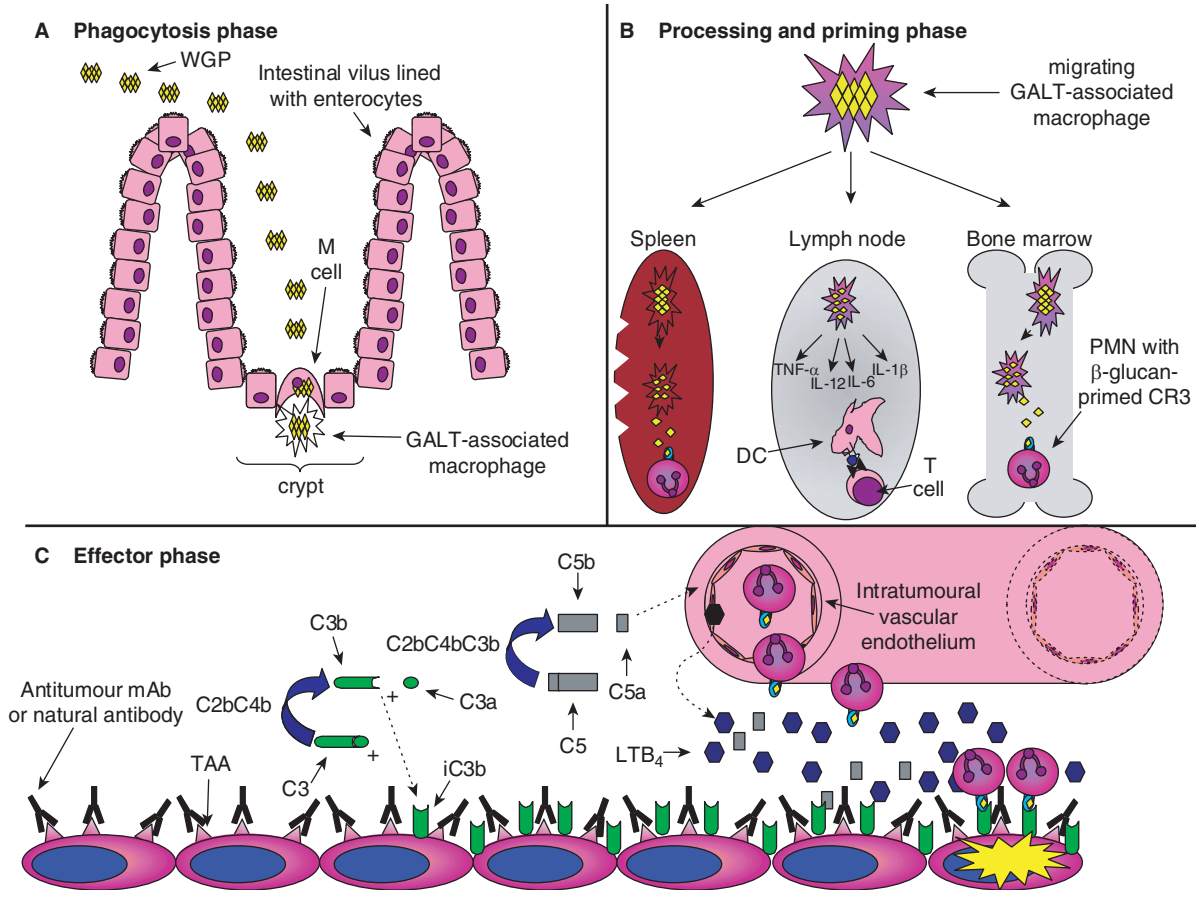


**Figure 4. A schematic illustration of the mechanism by which orally administered WGP is ingested, demonstrating the three phases characterised by: phagocytosis, processing and priming, and the effector phase.** Briefly, the phagocytosis phase is characterised by the CR3-independent uptake of WGP by gastrointestinal macrophages that are associated with M cells within the follicle-associated epithelium near the Peyer's patches. The processing and priming phase is characterised by the migration of macrophages from the GALT to lymphoid organs, including the lymph nodes, spleen and bone marrow. During this time, the particulate WGP is digested, most likely via oxidative pathways, and smaller soluble β-glucan is released, which primes the neutrophils in a CR3-dependent manner. In addition, the effector phase is characterised by the egress of β-glucan-primed neutrophils from the marginated pool in response to C5a produced in the tumour microenvironment as a result of complement activation by antitumour mAb. The homing of β-glucan-primed neutrophils to the tumour in response to C5a is amplified by the production of $LTB_4$ within the tumour milieu. Having reached the tumours, β-glucan-primed neutrophils can engage iC3b-opsonised tumour cells for cytotoxicity.
DC: Dendritic cell; GALT: Gut-associated lymphoid tissue; $LTB_4$: Leukotriene $B_4$; mAb: Monoclonal antibody; PMN: Polymorphonuclear leukocytes; TAA: Tumour-associated antigen; WGP: Whole glucan particle.

Moreover, therapeutic failure was observed in tumour-bearing $BLT^{-/-}$ mice ($LTB_4$ receptor deficient) treated with WGP and antitumour mAbs (D Allendorf, J Yan *et al.*: C5a-mediated leukotriene $B_4$-amplified neutrophil chemotaxis is essential in tumour immunotherapy facilitated by antitumour mAb and β-glucan, submitted for publication, 2005).

### 3.4 Primary effector cells for combined antitumour mAbs and WGP β-glucan mediated immunotherapy

Previous *in vitro* studies have demonstrated that human and mouse monocytes/macrophages, granulocytes and NK cells express CR3 and can each carry out β-glucan-mediated CR3-DCC against iC3b-opsonised tumour cells [21,50]. Indeed, it has been shown that the *in vivo* recruitment of granulocytes is relatively equivalent in therapy versus PBS control group tumours [22]. Granulocytes are recruited by tumours independently of mAb and β-glucan therapy, perhaps because of natural antibody activation of complement within tumours that releases the potent chemotactic factor C5a. Complement activation releases C3a and C5a, which function to recruit eosinophils, mast cells (C3a), neutrophils and macrophages (C5a). To demonstrate that granulocytes, more so than other cell types, are responsible for β-glucan-mediated antitumour cytotoxicity *in vivo*, a tumour therapy protocol was undertaken in animals depleted of granulocytes by administration of rat antimouse Gr-1 mAb. Tumour regression mediated by antitumour mAb plus β-glucan was completely abrogated by neutrophil depletion, indicating that neutrophils are primarily

responsible for β-glucan-mediated tumour regression *in vivo* [45]. The lack of C3aR and C5aR on NK cells probably explains the absent requirement for NK cell function in mAb and β-glucan-mediated tumour immunotherapy [51,52].

### 3.5 Chemotaxis of β-glucan primed neutrophils to the tumour milieu

Having demonstrated that granulocytes were essential for WGP-mediated tumour regression, their homing to the tumour milieu from the bone marrow, where their surface CR3 is primed for cytotoxicity by soluble β-glucan, was considered. Complement activation in the tumour microenvironment resulting from the opsonisation of tumours by antitumour mAb (or naturally occurring antibodies) not only results in the deposition of iC3b on the tumour cells, but also the production of C3a and C5a – small, soluble, highly chemotactic peptides known as anaphylatoxins. The cognate receptors for the anaphylatoxins, C3aR and C5aR, respectively, are expressed on the surface of granulocytes, although in differing relative amounts depending on cell type. Neutrophils express much more C5aR than C3aR and are therefore more sensitive to gradients of C5a, whereas eosinophils and basophils express more C3aR than C5aR and are, thus, more sensitive to C3a gradients [53-56]. To test the hypothesis that C5a-mediated neutrophil chemotaxis is essential in β-glucan-mediated tumour therapy, wild-type Balb/c mice implanted with Ptas64 mammary adenocarcinoma were treated with a cyclic peptide antagonist of the C5aR, or a sham peptide, in addition to oral WGP and 11C1 (mouse IgG2a antimurine mammary tumour virus) mAb. Animals receiving the C5aR antagonist in addition to combined immunotherapy were observed to have significantly larger tumours compared with those animals receiving the sham peptide in addition to immunotherapy (D Allendorf, J Yan *et al.*: C5a-mediated leukotriene $B_4$-amplified neutrophil chemotaxis is essential in tumour immunotherapy facilitated by antitumour mAb and β-glucan, submitted for publication, 2005).

These data have indicated the importance of neutrophil chemotaxis, particularly in response to C5a, in combined WGP and antitumour mAb immunotherapy. However, the half-life of the anaphylatoxins is very brief due to inactivation by serum carboxypeptidase N and internalisation by $C5aR^+$ cells [57]. Thus, it is possible that the chemotactic signal induced by C5a can be potentiated by other chemoattractant molecules. The receptor for $LTB_4$, BLT-1, had been shown to be necessary for the influx of neutrophils into the peritoneal cavity in response to intraperitoneal (i.p.) administration of zymosan, a yeast extract that activates the alternative pathway of complement [58]. To that end, BLT-$1^{-/-}$ mice or their wild-type littermates were implanted with the lymphoma RMA-S and treated with 14G2a, directed against the highly conserved $G_{D2}$ ganglioside tumour antigen, with or without WGP. Wild-type animals receiving daily oral WGP in addition to 14G2a were observed to have significantly smaller tumours compared with other wild-type animals receiving 14G2a only and to BLT-$1^{-/-}$ animals receiving WGP in addition to 14G2a (D Allendorf, J Yan *et al.*: C5a-mediated leukotriene $B_4$-amplified neutrophil chemotaxis is essential in tumour immunotherapy facilitated by antitumour mAb and β-glucan, submitted for publication, 2005). In addition, tumours removed at the end of the therapy period demonstrated a paucity of infiltrating neutrophils in treated BLT-$1^{-/-}$ compared with treated wild-type animals. These experimental results confirmed another observation, that BLT-$1^{-/-}$ animals had 70% fewer infiltrating neutrophils with respect to wild-type animals in response to i.p. administration of recombinant C5a (rC5a) (D Allendorf, J Yan *et al.*: C5a-mediated leukotriene $B_4$-amplified neutrophil chemotaxis is essential in tumour immunotherapy facilitated by antitumour mAb and β-glucan, submitted for publication, 2005).

## 4. Preclinical animal models

Preclinical animal models have demonstrated the efficacy of combined antitumour mAbs and orally administered WGP β-glucan, as well as contributed to our understanding of the cellular and molecular mechanisms of action.

An early study demonstrated that natural antibodies present in mice from a variety of genetic backgrounds directed against syngeneic subcutaneous implanted tumours were required for tumour regression in response to intravenous (i.v.) administration of a highly purified low molecular weight soluble zymosan-derived polysaccharaide (SZP) [22]. In mice with low titres of natural antitumour antibody, tumour regression was not observed. Similarly, antibody-deficient severe combined immunodeficiency (SCID) mice were also refractory to treatment with SZP β-glucan. However, passive immunisation with either purified natural antibody in SCID mice or purified antitumour mAbs in mice with low natural antitumour antibody titres restored SZP β-glucan-mediated tumour regression. Moreover, SZP β-glucan-dependent antitumour responses were not observed in either $C3^{-/-}$ or $CR3^{-/-}$ animals [22].

In a similar manner, daily i.v. administration of NSG in addition to antitumour mAbs was shown to enhance both tumour regression and long-term survival with respect to treatment with antitumour mAb alone [45]. However, the small molecular weight β-glucans, including SZP β-glucan and NSG, have been shown to be rapidly excreted in the urine, thus limiting their bioactivity and clinical utility [47]. An orally administered β-glucan is highly desirable for its clinical practicality, and recent studies have demonstrated the efficacy of both orally administered mushroom-derived β-glucan [59,60] and barley β-glucan [46,61,62]. Preclinical animal studies demonstrated that orally administered barley β-glucan of very high purity enhanced the cytotoxicity of tumours opsonised with antitumour mAb and iC3b, and produced significant regression and survival benefit in mice bearing either syngeneic tumours or in SCID mice bearing human tumour xenografts [46,62]. Thus, particulate WGP β-glucan was utilised in preclinical animal models in

combination with exogenous administration of antitumour mAb to test the efficacy for tumour therapy.

In a subcutaneous C57Bl/6 model of lymphoma, RMA-S-MUC1, the combination of daily oral WGP and weekly administration antitumour mAb 14G2a resulted in significant tumour regression of ≥ 80% compared with treatment with mAb alone [46]. It is notable that treatment with the 14G2a mAb alone yields no tumour regression, thus ruling out the contribution of ADCC, an antitumour mechanism observed with some antitumour mAbs. In the same experiment, groups of animals received daily oral barley β-glucan in addition to 14G2a, and the tumour regression induced by the orally administered WGP was comparable to that of the animals receiving barley β-glucan. However, when observed for long-term survival, 100% of mice receiving oral WGP in addition to 14G2a lived 8 weeks beyond therapy cessation compared with 50% of mice receiving oral barley β-glucan in addition to 14G2a. Groups of $CR3^{-/-}$ ($CD11b^{-/-}$) mice were also included and treatment with neither oral WGP nor oral barley β-glucan resulted in tumour regression or survival benefit [46]. This important observation confirms similar observations made with i.v. administered SZP and NSG, and reiterates the necessity of the lectin domain to bind soluble β-glucan in order to prime CR3 for cytotoxicity.

Another experiment employed the implantation of Lewis lung carcinoma cells engineered to express human MUC1 (LL/2-MUC1) into groups of wild-type and complement $C3^{-/-}$ mice on the C57Bl/6 genetic background. In this experiment, animals were treated, or not, with BCP8 (mouse IgG2a anti-human MUC1) and either WGP or barley β-glucan. The addition of either WGP or barley β-glucan to BCP8 was observed to significantly enhance tumour regression relative to treatment with BCP8 alone [46]. WGP yielded a marginal, but nonsignificant, increase in regression compared with barley β-glucan. In this particular model, animals receiving either WGP or oral barley β-glucan alone were observed to have smaller tumours, although statistically nonsignificant, than mice receiving no treatment or BCP8 alone. This observation is attributed to the persistence of natural antibody directed against either the parent Lewis lung carcinoma cells or to the human MUC1 protein that was expressed on the cells. $C3^{-/-}$ mice receiving BCP8 and either oral WGP or oral barley β-glucan exhibited no tumour regression. In contrast to the observation in wild-type animals, $C3^{-/-}$ mice receiving only WGP or barley β-glucan exhibited no tumour regression. Thus, despite the presence of natural antibody, the absence of C3 resulted in therapeutic failure. Analysis of long-term survival indicated that the addition of either oral WGP or oral barley β-glucan to BCP8 enhanced survival to 5 weeks beyond the cessation of therapy in ~ 40% of wild-type animals. Unlike the RMA-S-MUC1 model, there was no relative survival advantage derived from the addition of WGP compared with barley β-glucan [46]. This is most likely due to the inherent aggressiveness of the Lewis lung cancer. The addition of oral WGP or barley β-glucan to BCP8 failed to produce a survival advantage in $C3^{-/-}$ animals, and 100% of these animals had died within 2 weeks of the cessation of therapy. Thus, daily oral WGP was shown to have equivalent efficacy as NSG in terms of both tumour regression and long-term survival, but yielded the convenience of oral dosing.

In summary, data from preclinical animal models suggest that the combination of WGP β-glucan and antitumour mAbs exhibit therapeutic efficacy by linking innate $CR3^{+}$ granulocytes with the activation of adaptive immunity. However, careful selection and optimisation of tumour therapy protocols is necessary. For example, it is important to utilise implantable tumours with very stable expression of tumour antigens.

## 5. Relevance to clinical medicine

### 5.1 Antitumour mAbs in clinical studies

Antitumour mAbs represent one of the earliest targeted therapies in clinical cancer care [8]. At present, eight antitumour mAbs with anticancer indications have been approved by the FDA for usage in the US [8]. Many more are in various stages of clinical and preclinical development. The clinical success in solid tumours of antitumour mAbs as immunotherapy *per se* has been limited mostly by the realisation that the effector mechanisms of these drugs have less to do with engaging the cytotoxic capacity of the immune system, but rather with inhibiting growth factor receptor function, inducing apoptosis and sensitising malignant cells to conventional chemotherapy [8,63]. Thus, to achieve more clinically relevant responses, most antitumour mAbs have been administered with pre-existing protocols utilising conventional chemotherapy. Although antitumour mAb monotherapy has the advantage of tumour-specific cytotoxicity with decreased systemic toxicity, the addition of conventional chemotherapy, while increasing efficacy, also has increased treatment-related toxicity.

### 5.2 Potential contributions of β-glucan to mAb and vaccine immunotherapies

The addition of β-glucan allows the complement system to participate in tumour cytotoxicity, thus re-engaging the immune system as an effector mechanism for antitumour mAbs, and adding an additional independent modality to combination cancer therapy. Rather than relying on CDC as mediated by the terminal component of complement activation, the MAC, β-glucan employs CR3-DCC, in which β-glucan primes neutrophil surface CR3 for cytotoxicity against iC3b opsonised tumour cells [9]. This mechanism is akin to the highly conserved host response against pathogenic yeast and fungi in which the phagocytosis of these pathogens is facilitated by the binding of iC3b to neutrophil, monocyte and macrophage CR3 [64]. This novel strategy has robust potential for enhancing the antitumour activity of mAbs. In addition, β-glucan could complement existing antitumour mAb protocols as its mechanism of action would not interfere with the effector mechanisms of other drugs. Indeed, β-glucan

in combination with antitumour mAb significantly enhanced the killing of renal cell carcinoma micrometastases [65]. At present, there is one ongoing protocol combining an orally administered barley β-glucan with a mAb directed against a conserved neuroblastoma tumour-associated antigen (TAA) [61].

Immunotherapy with β-glucan is not limited to protocols utilising passive immunisation with antitumour mAbs. Active immunisation generating a cognate antibody response has the advantage of producing high titres of polyclonal antibodies directed against multiple conserved TAAs expressed by malignant cells. Thus, antitumour immunity would be expected to be more complete as the immune response is directed against a variety of TAAs and not the single TAA targeted by antitumour mAbs. To date, there are examples of failed clinical trials in which active immunisation generated a non-protective antibody response [66-68]. Many of these active immunisation protocols sought to generate an immune response against the TAAs carcinoembryonic antigen (CEA) and MUC1. MUC1 is often expressed on lung and breast adenocarcinomas, whereas CEA is commonly expressed on colon adenocarcinomas. The prevalence of these diagnoses, as well as their high mortality rates, indicate that there are many thousands of patients who could potentially benefit from combined immunotherapy with β-glucan. It is likely that the addition of β-glucan to these otherwise failed active immunisation protocols may result in the conversion of a non-protective antibody response to a robust and durable protective antibody response.

## 6. Expert opinion and discussion

The data presented in this review suggest that the complement system can be manipulated in such a way that it can substantially contribute to maximise the therapeutic efficacy of antitumour mAbs. β-Glucan primed CR3-dependent cytotoxicity represents a novel mechanism by linking innate immune $CR3^+$ cells with adaptive immunity to eliminate iC3b-opsonised tumour cells. The experimental animal observations and a few clinical studies have demonstrated a significant therapeutic efficacy in murine breast, liver metastasis, lung, lymphoma tumour models and paediatric neuroblastoma patients. The demonstration of cellular and molecular mechanisms of action for the combined WGP β-glucan with antitumour mAbs not only improves the prospect of immunotherapeutic treatment, but also increases the number of mAbs that can be useful in the clinic. All of the antitumour mAbs that are able to activate complement can be used in combination with β-glucan for tumour therapy. Another exciting prospect is the combining of β-glucan with existing tumour vaccine strategies, as most tumour vaccines elicit humoral responses in addition to CTL responses, although antibodies have an unappreciable effect in vaccine-alone models.

There are some challenges to the success of combined immunotherapy utilising β-glucan and antitumour mAbs; namely, requisite for the success of this strategy is the stable expression of TAA. As observed with other immunotherapy approaches, a hallmark of cancer is genomic instability, and tumours are heterogeneous populations of cells with potentially mixed populations of TAA expression [63]. Thus, any single therapeutic strategy is a selection pressure for a tumour; sensitive cells will be effectively killed, but resistant variants will be selected. Therefore, the most successful anticancer treatments rely on a combination of many strategies. To that end, β-glucan can be used with antitumour cocktail mAbs that bind to multiple targets of TAA [69] or with cytokines, such as IFN-γ, that can increase the expression of TAA [70]. Another challenge for β-glucan-mediated immunotherapy is that most cancer patients have received or will receive chemo- or radiation therapy. Many chemotherapy drugs and radiotherapies are myelotoxic and produce pancytopenias. Preclinical studies have indicated that neutrophils are *sine qua non* for the success of combined β-glucan and antitumour mAb immunotherapy [45]; thus, the presence of neutropenia, in the absence of growth factor support, could limit the efficacy of therapies employing β-glucan. Thus, the timing that cancer patients receive β-glucan in conjunction with antitumour mAbs during the course of chemo- and radiation therapy is crucial. Lastly, preclinical animal studies have demonstrated that strategies to improve the chemotaxis of neutrophils into the tumour microenvironment in the setting of combined WGP and antitumour mAb immunotherapy should improve therapeutic efficacy for this therapy. Trafficking of immune cells, including macrophages, dendritic cells and CTLs, into the tumour microenvironment has proven to be difficult [71]. However, mechanistic studies demonstrate that C5a is a potent chemoattractant for neutrophil recruitment [72]. It is feasible to combine β-glucan-mediated tumour therapy with recombinant C5a (rC5a), synthesised C5a agonist or cytokines to increase neutrophil recruitment, such as granulocyte colony-stimulating factor. Nevertheless, the combined β-glucan with antitumour mAbs immunotherapy has demonstrated promising results in the therapeutic setting and might be curative for the cancer patients treated with this approach.

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