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(54) **METHODS FOR IDENTIFYING BETA-GLUCAN BINDING TO IMMUNE CELLS**

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G01N 33/50	(2006.01)
G01N 33/554	(2006.01)
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See application file for complete search history.

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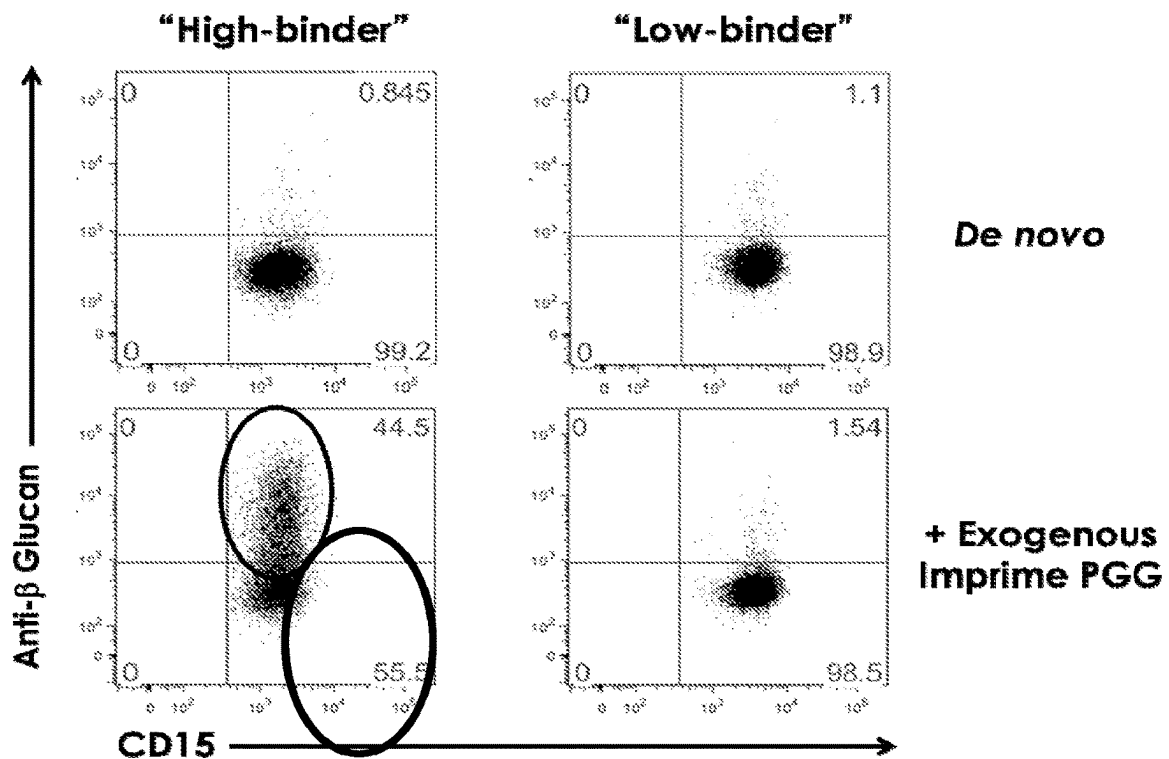
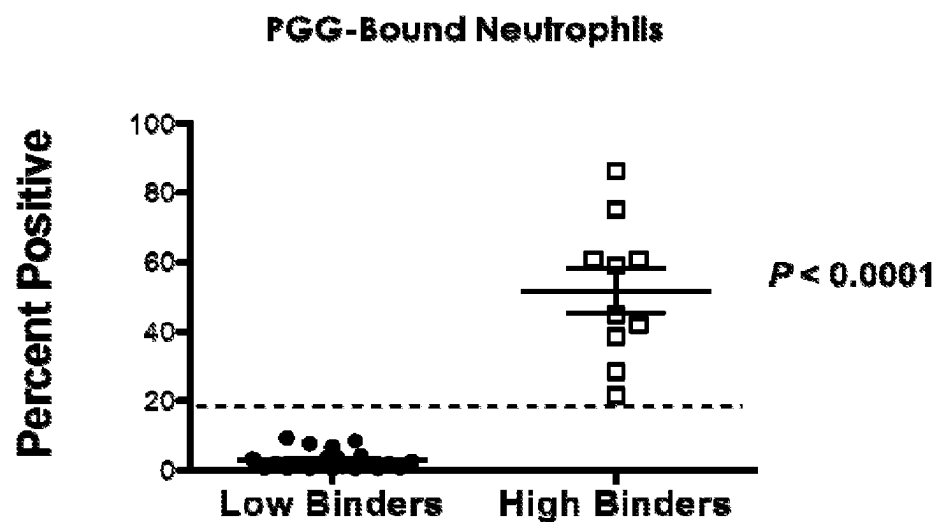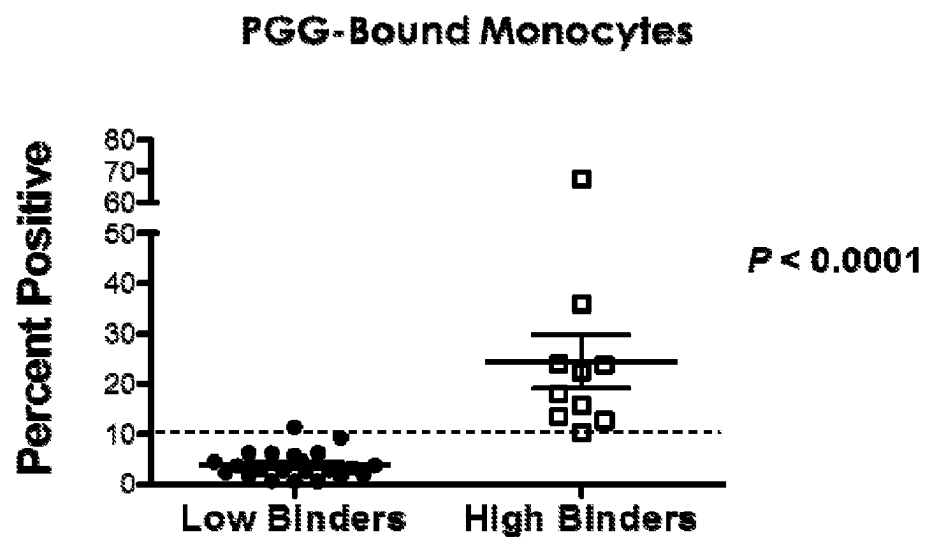* cited by examiner

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(57) **ABSTRACT**

This disclosure describes, in one aspect, a method for identifying β-glucan binding to immune cells of a subject. Generally, the method includes obtaining a blood sample from the subject, the blood sample comprising immune cells, adding soluble β-glucan to at least a portion of the blood sample and incubating the mixture under conditions allowing the soluble β-glucan to bind to the immune cells, and detecting soluble β-glucan hound to the immune cells. In another aspect, this disclosure describes a method that generally includes identifying the subject as a low binder of β-glucan, and co-administering to the subject a soluble β-glucan and an antibody preparation capable of converting the subject from a low binder to a high binder.

9 Claims, 6 Drawing Sheets

Figure 1

Figure 2

PGG-Bound Neutrophils

$P < 0.0001$

Figure 3

PGG-Bound Monocytes

$P < 0.0001$

Figure 4

$P = 0.005$
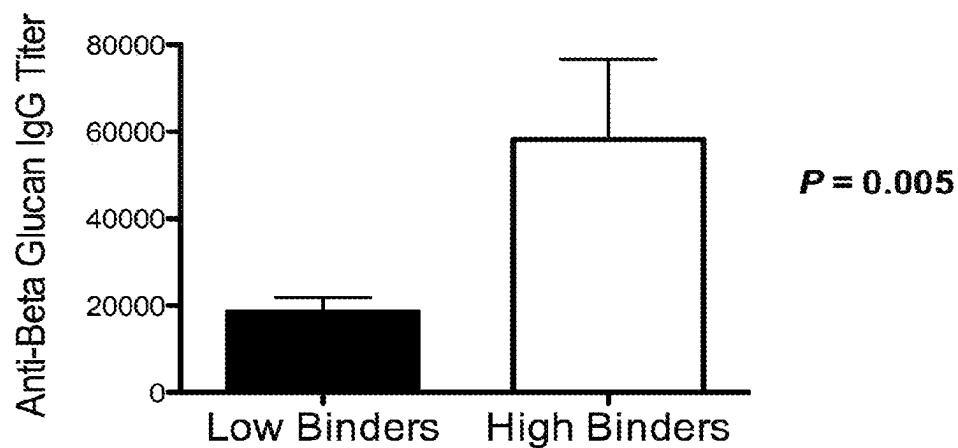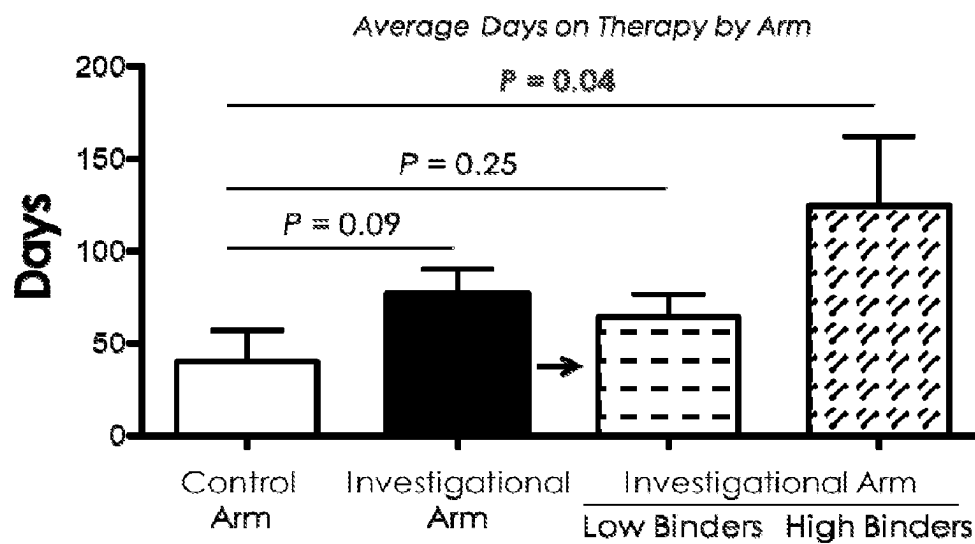
Figure 5

Figure 6

Figure 7

Figure 8

Imprime +
Low-binder
Serum

Imprime PGG® + Low-binder Serum
+ Increasing amounts of IVIG

Imprime +
IVIG + PBS
(No Serum)

Anti-β Glucan

CD15
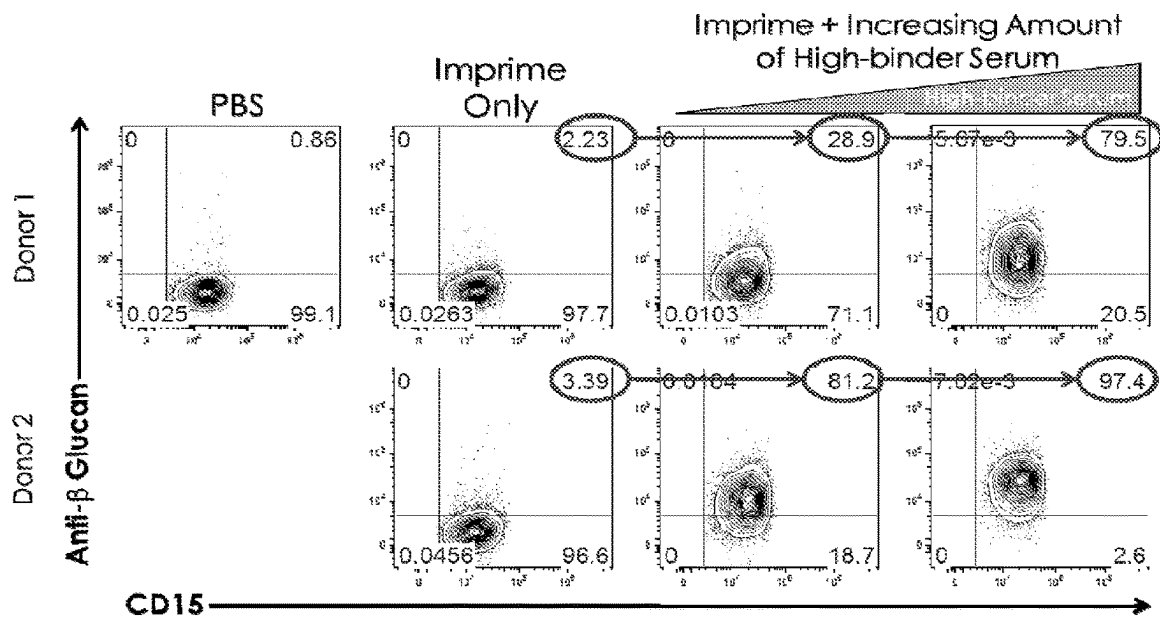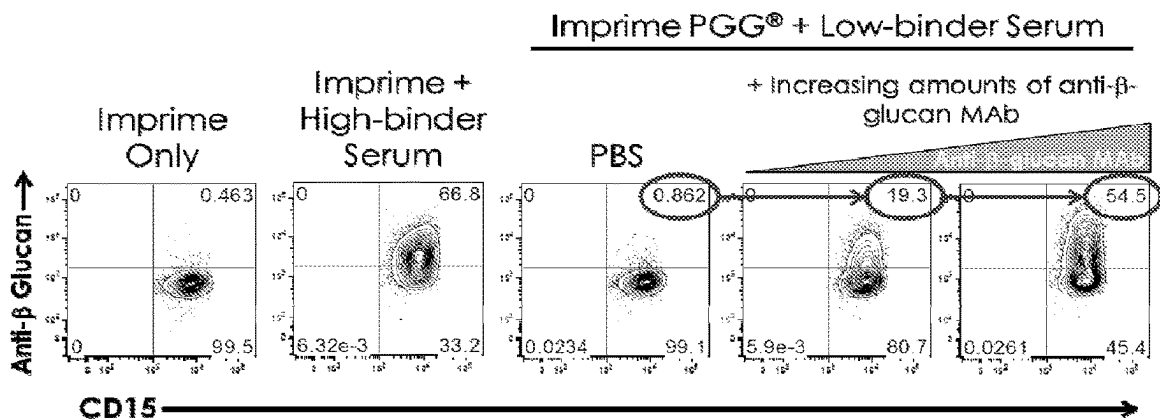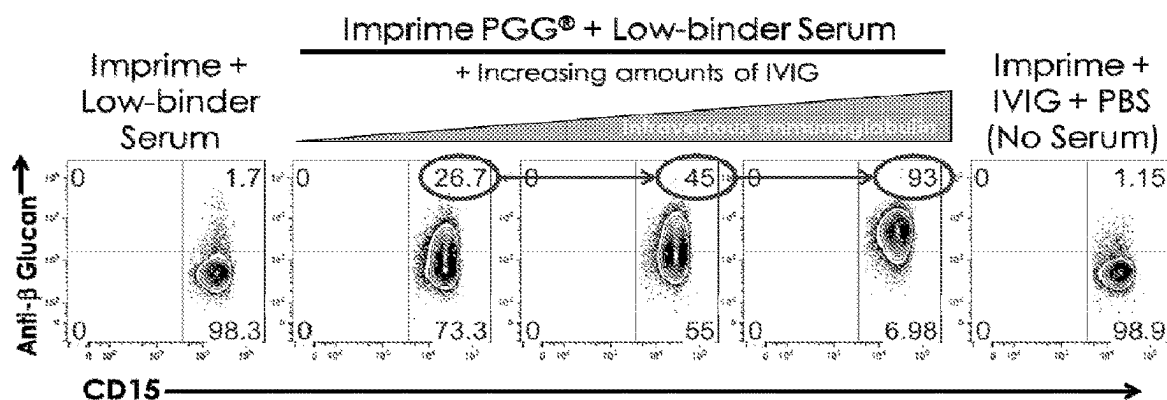
Figure 9

Binding of Imp Conjugates to PMN in LB's WB

| Citrate Ctrl | Imp Ref | 1711 Imp-8TH1704 | 1717 Imp-Erbitux |

CD15-PMN

BFDiv Binding

Figure 10

METHODS FOR IDENTIFYING BETA-GLUCAN BINDING TO IMMUNE CELLS

CROSS-REFERENCE TO RELATED APPLICATION

This application is a division of U.S. patent application Ser. No. 14/398,019 (U.S. Pat. No. 11,229,701), filed Oct. 30, 2014, which is a national stage entry of International Application No. PCT/US2013/031606, filed Mar. 14, 2013, which claims priority to U.S. Provisional Patent Application Ser. No. 61/640,834, filed May 1, 2012, U.S. Provisional Patent Application Ser. No. 61/640,842, filed May 1, 2012, and U.S. Provisional Patent Application Ser. No. 61/640, 397, filed Apr. 30, 2012, each of which is incorporated herein by reference.

SUMMARY

This disclosure describes, in one aspect, a method for identifying β-glucan binding to immune cells of a subject. Generally, the method includes obtaining a blood sample from the subject, the blood sample comprising immune cells, adding β-glucan to at least a portion of the blood sample and incubating the mixture under conditions allowing the β-glucan to bind to the immune cells, and detecting β-glucan bound to the immune cells.

In some embodiments, the β-glucan may be derived from yeast. In some embodiments, the β-glucan can include a β-1,3/1,6 glucan such as β(1,6)-[poly-(1,3)-D-glucopyrano-syl]-poly-β(1,3)-D-glucopyranose. In some embodiments, detecting β-glucan bound to the immune cells can include contacting the sample with a monoclonal antibody that specifically binds to the β-glucan. In some of these embodiments, the monoclonal antibody comprises BfD I, BfD II, BfD III, or BfD IV.

In some embodiments, the method can further include obtaining a second blood sample from the subject, the blood sample comprising immune cells, adding β-glucan to at least a portion of the second blood sample and incubating the mixture under conditions allowing the β-glucan to bind to the immune cells, and detecting β-glucan bound to the immune cells.

In another aspect, this disclosure describes a method of improving β-glucan immunotherapy for a subject. Generally, the method includes identifying the subject as a low binder, and co-administering to the subject a β-glucan and an antibody preparation capable of converti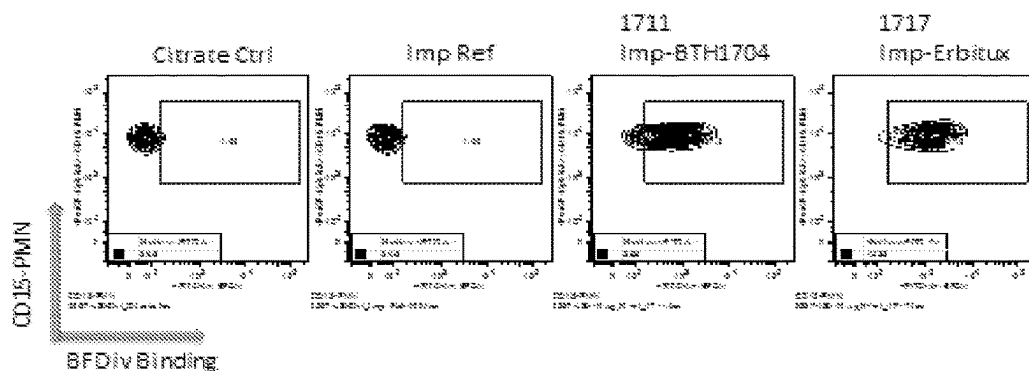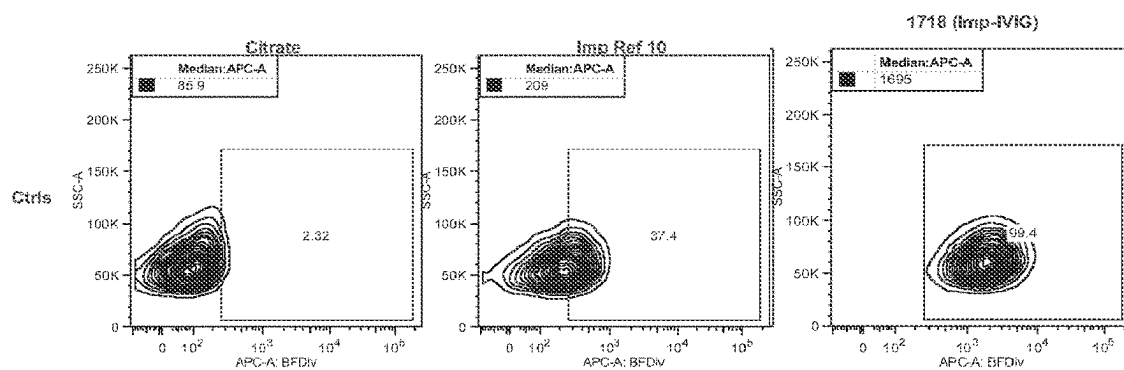ng the subject from a low binder to a high binder. In some embodiments, identifying the subject as a low binder can include obtaining a blood sample from the subject, the sample comprising an anti-β-glucan antibody titer, measuring the anti-β-glucan antibody titer of at least a portion of the blood sample, and identifying the subject as a low binder if the anti-β-glucan antibody titer is less than a predetermined level. In other embodiments, identifying the subject as a low binder can include obtaining a blood sample from the subject, the blood sample comprising immune cells, adding β-glucan to at least a portion of the blood sample and incubating the mixture under conditions allowing the β-glucan to bind to the immune cells, detecting β-glucan bound to the immune cells, and identifying the subject as a low binder if β-glucan is bound to no more than 10% of the immune cells.

In some embodiments, the antibody preparation can include serum from a high binder. In some embodiments, the antibody preparation can include a monoclonal antibody that

specifically binds the β-glucan such as BfD I, BfD II, BfD III, or BfD IV. In some embodiments, the antibody preparation can include intravenous immunoglobulin. In some embodiments, the antibody preparation can include a β-glucan moiety conjugated to an antibody or antibody fragment such as the Fc portion.

In some embodiments, the β-glucan and the antibody preparation are co-administered simultaneously. In other embodiments, the β-glucan and the antibody preparation are co-administered at different times. In some embodiments, the β-glucan and the antibody preparation are co-administered at different sites.

In some embodiments, the β-glucan may be derived from yeast. In some embodiments, the β-glucan can include β-1,3/1,6 glucan such as β(1,6)-[poly-(1,3)-D-glucopyrano-syl]-poly-β(1,3)-D-glucopyranose.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**. Flow cytometry data showing differential β-glucan (PGG) binding to polymorphonuclear leukocytes in healthy human whole blood.

FIG. **2**. Data showing differential β-glucan binding to neutrophils in healthy human whole blood.

FIG. **3**. Data showing differential β-glucan binding to monocytes in healthy human whole blood.

FIG. **4**. Data comparing anti-β-glucan antibody titers of low binders and high binders.

FIG. **5**. Comparison of the average number of days on therapy for patients in control and investigational arms of two-armed, open-label, randomized, multi-center study.

FIG. **6**. Data showing that high binder serum can increase β-glucan binding to PMNs obtained from a low binder.

FIG. **7**. Data showing the anti-β-glucan antibodies can increase β-glucan binding to PMNs from a low binder.

FIG. **8**. Data showing intravenous immunoglobulin can increase β-glucan binding to PMNs from a low binder.

FIG. **9**. Data showing binding of PGG-antibody conjugates to PMNs.

FIG. **10**. Data showing binding of PGG-IVIG conjugates to PMNs.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes methods related to the use of soluble β-glucan as a component of immunotherapy. The methods described herein exploit the observation of differential binding of β-glucan by immune cells in different populations of healthy humans. Surprisingly, "high binders" of β-glucan exhibit higher titers of anti-β-glucan antibodies than "low binders." Thus, this disclosure describes methods of screening individuals to identify "high binders" and "low binders." This disclosure also describes methods that generally include converting a "low binder" to a "high binder" and, thus, increase the population for whom β-glucan-based immunotherapy can be effective. This disclosure also describes treatment regimens that include periodically moni-

toring a subject for their current "high binder" or "low binder" status, and adjusting the therapy provided to the subject, if necessary, to promote achieving or maintaining "high binder" status.

β-glucans are polymers of glucose derived from a variety of microbiological and plant sources including, for example, yeast, bacteria, algae, seaweed, mushroom, oats, and barley. Of these, yeast β-glucans have been extensively evaluated for their immunomodulatory properties. Yeast β-glucans can be present as various forms such as, for example, intact yeast, zymosan, purified whole glucan particles, solubilized zymosan polysaccharide, or highly-purified soluble β-glucans of different molecular weights. Structurally, yeast β-glucans are composed of glucose monomers organized as a β-(1,3)-linked glucopyranose backbone with periodic β-(1,3) glucopyranose branches linked to the backbone via β-(1,6) glycosidic linkages. The different forms of yeast β-glucans can function differently from one another. The mechanism through which yeast β-glucans exert their immunomodulatory effects can be influenced by the structural differences between different forms of the β-glucans such as, for example, its particulate or soluble nature, tertiary conformation, length of the main chain, length of the side chain, and frequency of the side chains. The immune stimulating functions of yeast β-glucans are also dependent upon the receptors engaged in different cell types in different species, which again, can be dependent on the structural properties of the β-glucans.

In general, β-glucan immunotherapies can include administering to a subject any suitable form of β-glucan or any combination of two or more forms of β-glucan. Suitable β-glucans and the preparation of suitable β-glucans from their natural sources are described in, for example, U.S. Patent Application Publication No. US2008/0103112 A1. In some cases, the β-glucan may be derived from a yeast such as, for example, Saccharomyces cerevisiae. In certain cases, the β-glucan may be or be derived from β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose, also referred to herein as PGG (IMPRIME PGG®, Biothera, Eagan, MN), a highly purified and well characterized form of soluble yeast-derived β-glucan. Moreover, β-glucan-based immunotherapies can involve the use of, for example, a modified and/or derivatized β-glucan such as those described in International Patent Application No. PCT/US12/36795. In other cases, β-glucan immunotherapy can involve administering, for example, a particulate-soluble β-glucan or a particulate-soluble β-glucan preparation, each of which is described in, for example, U.S. Pat. No. 7,981,447.

As noted above, yeast β-glucans have been extensively evaluated for their immunomodulatory properties. In particular PGG β-glucan has demonstrated preclinical activity against a variety of cancer types when administered in combination with tumor-related monoclonal antibodies (mAbs). Exemplary types of cancer and their associated tumor-related mAbs include, for example, T-cell lymphoma (anti-MUC1, anti-GD2), lung carcinoma (anti-MUC1), breast adenocarcinoma (anti-MMTV), ovarian carcinoma (bevacizumab), non-small-cell lung carcinoma (bevacizumab, cetuximab), colorectal cancer (cetuximab), chronic lymphocytic leukemia and non-Hodgkin's lymphoma (rituximab), and pancreatic carcinoma (cetuximab, anti-MUC1). β-glucans can prime neutrophils, which are then recruited to tumors marked with tumor-related antibody. The recruited neutrophils are activated by dual ligation of the

complement receptor 3 (CR3) by iC3b/C3—at the surface of mAb-marked tumor cells—and infused β-glucan, to kill tumor cells.

We discovered, however, that distinct populations of individuals exist: one population exhibits relatively high capacity of β-glucan binding to innate immune cells in whole blood; another population exhibits relatively low capacity of β-glucan binding to innate immune cells in whole blood. This observation was wholly unexpected based on data from mouse models of immunity and studies involving isolated human immune cells. Many individuals exhibit some level of β-glucan binding to immune cells from native, low level exposure to β-glucans. (e.g., FIG. 1, "De novo"). When exogenous β-glucan is administered, "low binders" exhibit a modest increase in the percentage of immune cells that bind β-glucan, while "high binders" exhibit a marked increase in the percentage of innate immune cells that bind β-glucan. (FIG. 1., "+Exogenous PGG"). FIG. 1 and FIG. 2 show data reflecting β-glucan binding to polymorphonuclear leukocytes (PMNs), and FIG. 3 (monocytes) shows that the differential binding applies to other innate immune cell populations as well. In addition, "high binders" also tend to produce more chemoattractant cytokines and chemokines such as, for example, IL-8, MCP, MIP-1, etc.

As used herein, status as a "high binder" refers to an individual who exhibits a predetermined percentage of a particular immune cell population that binds exogenously provided β-glucan. The immune cell population used to determine whether an individual is a "high binder" or a "low binder" can be, for example, polymorphonuclear lymphocytes (PMNs) or monocytes. An individual can be considered a "high binder" if at least 10% of the PMNs or monocytes in a blood sample from the individual bind exogenously provided β-glucan. Thus, an individual may be a "high binder" if at least 10%, at least 12%, at least 15%, at least 20%, at least 15%, or at least 40% of PMNs or monocytes in a blood sample from the individual bind exogenously provided β-glucan. (See, e.g., FIG. 2 and FIG. 3). In some cases, the exogenously provided β-glucan can include PGG provided to a final concentration of 10 µg/mL to 100 µg/mL. Status as a "low binder" refers to an individual who fails to exhibit "high binder" status.

Moreover, "high binders" can exhibit higher titers of anti-β-glucan antibodies than "low binders." (FIG. 4). A typical anti-β-glucan antibody titer for a "high binder" can be a titer of at least 25,000 such as, for example, at least 30,000, at least 35,000, at least 40,000, at least 45,000, at least 50,000, at least 55,000, or at least 60,000. (See, e.g., FIG. 4). Anti-β-glucan antibody titer typically refers to IgG. In some cases, however, the presence of IgM can compensate for a lower IgG titer to help establish "high binder" status. As is described in more detail below, "high binder" status can influence an individual's response to β-glucan therapy. One can artificially induce a natural "low binder" to exhibit a "high binder"-like response to β-glucan therapy by providing the individual anti-β-glucan antibody as part of the β-glucan therapy.

Thus, in one aspect, this disclosure describes screening an individual to identify whether the individual, at that point in time, exhibits "high binder" or "low binder" status. The method generally includes obtaining a blood sample from the individual, adding β-glucan to at least a portion of the sample, incubating the mixture under conditions allowing the β-glucan to bind to the immune cells, and detecting β-glucan bound to the immune cells.

The blood sample includes a sufficient portion of the blood to include immune cells. Thus, in some embodiments,

the blood sample can be whole blood. In other embodiments, the blood sample may be at least partially processed to remove one or more components of whole blood that are not necessary for the screening assay such as, for example, erythrocytes. Thus, in some embodiments, the blood sample can include a blood product such as, for example, any blood fraction that includes at least a portion of the buffy coat.

In some embodiments, the β-glucan may be derived from yeast such as, for example, Saccharomyces cerevisiae. In some embodiments, the β-glucan can include a β-1,3/1,6 glucan such as, for example, β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

In some embodiments, the β-glucan hound to the immune cells may be detected by contacting the sample with a monoclonal antibody that specifically binds to the β-glucan. The monoclonal antibody may be any monoclonal antibody that specifically binds to the β-glucan. As used herein, "specific" and variations thereof refer to having a differential or a non-general (i.e., non-specific) affinity, to any degree, for a particular target. Exemplary monoclonal antibodies that specifically bind β-glucan include, for example, monoclonal antibodies identified as BfD I, BfD II, BfD III, and/or BfD IV (Biothera, Eagan, MN), each of which is described in U.S. Pat. No. 6,294,321.

To assist in detecting the anti-β-glucan antibody that binds to the immune cell-bound β-glucan, the anti-β-glucan antibody can include a detectable label. Alternatively, bound anti-β-glucan antibody may be detected using a labeled secondary antibody. In either case, suitable labels include, for example, a fluorescent label, an enzymatic label, a colorimetric label, or a radiolabel.

The method can further include immobilizing the β-glucan-bound immune cells on a substrate. In some cases, the immune cells may be immobilized prior to being contacted with the β-glucan—e.g., by contacting at least a portion of the blood sample with the substrate prior to contacting the blood sample with the β-glucan. In other embodiments, the immune cells may be immobilized after being contacted with the β-glucan. In either case, the immune cells may be immobilized using any suitable material such as, for example, an immobilized antibody that specifically binds to the desired population of immune cells.

In some embodiments, the method can include a wash step to remove unbound assay components. For example, some embodiments can include a step of washing unbound β-glucan and/or unbound blood sample components from the β-glucan-bound immune cells. As another example, some embodiments can include a step washing unbound immune cells from the substrate, if present. As yet another example, some embodiments can include washing unbound β-glucan-specific antibodies from the β-glucan bound to the immune cells.

In some embodiments, the method can include repeated screening of a subject longitudinally to monitor the subject's status as a "high binder" or "low binder" over time. Some cancer patients receiving β-glucan immunotherapy exhibit a decrease in anti-β-glucan antibody titer over time with repeated β-glucan dosing. These patients also can exhibit a longitudinal decrease in β-glucan-bound immune cells (e.g., peripheral PMNs). This observation may be due, at least in part, to recruitment of β-glucan-bound immune cells out of circulation and into the tumor microenvironment. Alternatively, it may be due, at least in part, to changes in plasma factors that influence β-glucan binding to circulating immune cells (e.g., decreased anti-β-glucan antibodies over time).

In another aspect, this disclosure describes a method of converting a "low binder" to a "high binder." In a two-armed, open-label, randomized, multi-center study, 795 subjects with recurrent/progressive colorectal cancer after at least two previous chemotherapeutic treatments were divided into a control arm and an investigational arm. Subjects in the control arm received treatment with cetuximab. Subjects in the investigational arm received treatment with cetuximab +4 mg/kg PUG β-glucan. FIG. 5 shows that while subjects receiving β-glucan as part of their immunotherapy remained on therapy for a longer average period than subjects receiving only cetuximab, the effect was greatest in those subjects that were "high binders." In this context, length of therapy is an indication of therapy success so that a longer therapy time indicates a positive therapeutic outcome while a shorter length of therapy indicates poor outcomes. Thus, there is a clinical consequence to "high binder" status versus "low binder" status.

This clinical consequence of exhibiting "high binder" status versus exhibiting "low binder" status means that being able to convert an individual from a "low binder" to a "high binder" can have clinical consequences for that individual. FIG. 6 shows that "high binder" serum can increase β-glucan binding to immune cells (e.g., PMNs) of a "low binder." Increasing amounts of anti-β-glucan monoclonal antibody also can increase β-glucan binding to immune cells (e.g., PMNs) in serum from a "low binder." (FIG. 7). Also, intravenous immunoglobulin (IVIG), a blood product that contains pooled polyvalent IgG from many donors (typically many hundreds, even thousands, of donors and, thus, naturally containing anti-β-glucan antibodies), also can increase β-glucan binding to immune cells (e.g., PMNs) in serum from a "low binder." (FIG. 8). Consequently, one can convert an individual from exhibiting "low binder" status to exhibiting "high binder" status by providing the individual, as a component of immunotherapy, with a combination of β-glucan and a preparation that includes anti-β-glucan antibodies. Significantly, the conversion of an individual from "low binder" status to "high binder" status can improve clinical outcome of immunotherapy.

Thus, the method includes co-administering a β-glucan with an antibody preparation capable of converting the subject from a low binder to a high binder. As used herein, "co-administered" refers to two or more components of a combination administered so that the therapeutic or prophylactic effects of the combination can be greater than the therapeutic or prophylactic effects of either component administered alone. Two components may be co-administered simultaneously or sequentially. Simultaneously co-administered components may be provided in one or more pharmaceutical compositions. Sequential co-administration of two or more components includes cases in which the components are administered so that both components are simultaneously bioavailable after both are administered. Regardless of whether the components are co-administered simultaneously or sequentially, the components may be co-administered at a single site or at different sites.

A similar conversion of status from "low binder" to "high binder" can occur by administering to the subject a composition that includes a β-glucan moiety conjugated to any antibody or a portion of an antibody. FIG. 9 shows data illustrating relatively low PGG binding by PMNs in whole blood (Imp Ref, second panel) changing to high binding status by conjugating the PGG to either BTH1704 (anti-MUC1, U.S. Pat. No. 6,204,366, Biothera, Inc, Eagan, MN, third panel) or ERBITUX® (cetuximab) (Eli Lilly and Co., Indianapolis, IN, fourth panel) anti-tumor antibodies. FIG.

10 also illustrates relatively low PGG binding by PMNs in whole blood (Imp Ref, second panel) changing to high binding status by conjugating the PGG to intravenous immunoglobulin (IVIG, Biolegend, San Diego, CA).

Thus, in another aspect, the method includes administering to a subject a composition that includes a β-glucan moiety conjugated to an antibody, a therapeutic antibody, an anti-tumor antibody, or an antibody fragment such as the Fc portion of an antibody. Modified and/or derivatized PGG, including PGG conjugates of a PGG moiety and an antibody are described in International Patent Application No. PCT/US12/36795, which may also be applied to conjugates of antibody fragments. The PGG moiety may be, or be derived from a β-1,3/1,6 glucan. In this context, "derived from" acknowledges that a conjugate may necessarily be prepared by creating a covalent linkage that replaces one or more atoms of the PGG β-glucan. As used herein, "derived from a β-1,3/1,6 glucan" refers to a portion of the PGG β-glucan that remains as part of a conjugate after replacing one or more atoms of the PGG to form the covalent linkage of the conjugate.

As used herein, "capable of converting a low binder to a high binder" refers to the property of modifying the binder status of an individual from "low binder" (e.g., below a predetermined threshold) to "high binder" (e.g., above a predetermined threshold). The status of a subject as a "low binder" or a "high binder" may be determined with reference to the extent to which β-glucan binds to immune cells in at least a portion of a blood sample obtained from the subject. Alternatively, the binder status of a subject may be determined with respect to the anti-β-glucan antibody titer measured in at least a portion of a blood sample obtained from the subject.

In some embodiments, the antibody preparation can include serum from a high binder.
In other embodiments, the antibody preparation can include intravenous immunoglobulin. In still other embodiments, the antibody preparation can include a monoclonal antibody that specifically binds the β-glucan. Exemplary monoclonal antibodies that specifically bind to β-glucan include, for example, BfD I, BfD II, BfD III, or BfD IV.

The β-glucan, the antibody preparation, and/or the combination of both components may be formulated in a composition along with a "carrier." As used herein, "carrier" includes any solvent, dispersion medium, vehicle, coating, diluent, antibacterial agent and/or antifungal agent, isotonic agent, absorption delaying agent, buffer, carrier solution, suspension, colloid, and the like. The use of such media and/or agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the β-glucan or the antibody, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the β-glucan and/or the antibody without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The β-glucan, the antibody preparation, and/or the combination of both components may be formulated into a pharmaceutical composition. In some embodiments, β-glucan and the antibody preparation may be provided in a single formulation. In other embodiments, the β-glucan and the antibody preparation may be provided in separate formulations. A pharmaceutical composition may be formulated in a variety of and/or a plurality forms adapted to one or more preferred routes of administration. Thus, a pharmaceutical composition can be administered via one or more known routes including, for example, oral, parenteral (e.g., intradermal, transcutaneous, subcutaneous, intramuscular, intravenous, intraperitoneal, etc.), or topical (e.g., intranasal, intrapulmonary, intramammary, intravaginal, intrauterine, intradermal, transcutaneous, rectally, etc.). A pharmaceutical composition, or a portion thereof, can be administered to a mucosal surface, such as by administration to, for example, the nasal or respiratory mucosa (e.g., by spray or aerosol). A pharmaceutical composition, or a portion thereof, also can be administered via a sustained or delayed release.

A formulation may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. Methods of preparing a composition with a pharmaceutically acceptable carrier include the step of bringing the β-glucan and/or the antibody preparation into association with a carrier that constitutes one or more accessory ingredients. In general, a formulation may be prepared by uniformly and/or intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

The β-glucan, the antibody preparation, and/or the combination of both components may be provided in any suitable form including but not limited to a solution, a suspension, an emulsion, a spray, an aerosol, or any form of mixture. The composition may be delivered in formulation with any pharmaceutically acceptable excipient, carrier, or vehicle. For example, the formulation may be delivered in a conventional topical dosage form such as, for example, a cream, an ointment, an aerosol formulation, a non-aerosol spray, a gel, a lotion, and the like. The formulation may further include one or more additives including such as, for example, an adjuvant, a skin penetration enhancer, a colorant, a fragrance, a flavoring, a moisturizer, a thickener, and the like.

In some embodiments, the β-glucan may be derived from yeast such as, for example, Saccharomyces cerevisiae. In some embodiments, the β-glucan can include a β-1,3/1,6 glucan such as, for example, β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

In some embodiments, the method can include administering sufficient β-glucan to provide a dose of, for example, from about 100 ng/kg to about 50 mg/kg to the subject, although in some embodiments the methods may be performed by administering the β-glucan in a dose outside this range. In some embodiments, the method includes administering sufficient β-glucan to provide a dose of from about 10 µg/kg to about 10 mg/kg to the subject such as, for example, a dose of about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, or about 10 mg/kg. In one particular embodiment, the method includes administering sufficient β-glucan to provide a dose of about 4 mg/kg.

Alternatively, the dose may be calculated using actual body weight obtained just prior to the beginning of a treatment course. For the dosages calculated in this way, body surface area (m^2) is calculated prior to the beginning of the treatment course using the Dubois method: $m^2 = (wt\ kg^{0.425} \times height\ cm^{0.725}) \times 0.007184$. In some embodiments, therefore, the method can include administering sufficient β-glucan to provide a dose of, for example, from about 0.01 mg/m^2 to about 10 mg/m^2.

In some embodiments, the method can include administering sufficient antibody that specifically binds the β-glucan to provide a dose of, for example, from about 100 ng/kg to about 50 mg/kg to the subject, although in some embodiments the methods may be performed by administering the antibody in a dose outside this range. In some embodiments, the method includes administering sufficient antibody to provide a dose of from about 10 μg/kg to about 5 mg/kg to the subject, for example, a dose of from about 100 μg/kg to about 1 mg/kg. In some embodiments, antibody that specifically binds the β-glucan can be administered in the form of intravenous immunoglobulin (IVIG), a blood product that contains pooled polyvalent IgG from many donors (typically many hundreds, even thousands, of donors and, thus, naturally containing anti-β-glucan antibodies). In such embodiments, WIG may be administered in a dose of from about 0.1 g/kg to about 2.0 g/kg such as, for example, 0.1 g/kg, 0.2 g/kg, 0.3 g/kg, 0.4 g/kg, 0.5 g/kg, 0.6 g/kg, 0.7 g/kg, 0.8 g/kg, 0.9 g/kg, 1.0 g/kg, 1.1 g/kg, 1.2 g/kg, 1.3 g/kg, 1.4 g/kg, 1.5 g/kg, 1.6 g/kg, 1.7 g/kg, 1.8 g/kg, 1.9 g/kg, or 2.0 g/kg. In certain embodiments, IVIG may be administered to provide a dose of about 0.4 g/kg to about 1.0 g/kg.

Alternatively, the dose may be calculated using actual body weight obtained just prior to the beginning of a treatment course. For the dosages calculated in this way, body surface area (m^2) is calculated prior to the beginning of the treatment course using the Dubois method: $m^2=(wt\ kg^{0.425} \times height\ cm^{0.325}) \times 0.007184$. In some embodiments, therefore, the method can include administering sufficient antibody to provide a dose of, for example, from about 0.01 mg/m^2 to about 10 mg/m^2.

In some embodiments, the β-glucan and antibody preparation may be co-administered, for example, from a single dose to multiple doses per week, although in some embodiments the method may be performed by co-administering the β-glucan and antibody at a frequency outside this range. In certain embodiments, the β-glucan and antibody may be administered from about once per year to once per week.

In some embodiments, the method further includes providing immunotherapy to the subject. The immunotherapy can include administering one or more tumor-related antibodies (e.g., cetuximab, bevacizumab, anti-MUC1). In this context, the co-administration of β-glucan and the antibody preparation can increase the efficacy of the immunotherapy.

As used herein, the term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements; the terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims; unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Unless otherwise expressly specified that the features of a particular embodiment are incompatible with the features of another embodiment, certain embodiments can include a combination of compatible features described herein in connection with one or more embodiments.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The present invention is illustrated by the following examples. It is to be understood that the particular examples,

materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Materials

Imprime PGG® (β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose) (Biothera, Eagan, MN) was provided in a preservative-free, soluble β-glucan formulation prepared at a concentration of 1 mg/mL in 0.8% sodium chloride and 0.2% sodium citrate monobasic, at a pH of 6.4. The compound was stored at 4-8° C. until use.

Preparation of Samples

Whole Blood. Fresh whole blood was obtained from healthy volunteers that had provided informed consent prior to donation (New England Institutional Review Board, May 2007). The blood was collected in a Vacutainer® containing 158 USP Units Freeze-Dried Sodium Heparin (BD Biosciences; San Jose, CA).

Serum and Plasma. Whole blood was processed into serum or plasma by Vacutainer® tubes (BD Biosciences; San Jose, CA) collection with either serum separator (red top) or sodium heparin (green top) tubes. Tubes were mixed well, incubated at room temperature for 30 minutes, and then centrifuged at 2000 rpm (~1150×g) for 10 minutes. The supernatant (either serum or plasma) was then transferred to a fresh polycarbonate storage conical tube.

Anti-BG ELISA Method

A preliminary ELISA method modified from the monkey anti-β-glucan method (Noss et al., 2012 *Int. Arch. Allergy Immunol.*, 157:98-108) was used to test the human sera samples. Costar universal binding plates were coated with 50 μL of β-glucan at 1 μg/mL purified β-glucan diluted in purified water and incubated at 37° C. for 30 minutes. The coated plate was then exposed to high intensity ultraviolet light at >1500 μW/cm^2 for 5 minutes at room temperature and placed in a 50° C. forced air oven until dry before a second exposure to ultraviolet light at >1500 μW/cm^2 for five minutes at room temperature. The plate was then blocked with a 0.5% solution of Bovine Serum Albumin for >30 minutes before washing with wash buffer (phosphate buffered saline [PBS] with 0.05% Tween-20). Human serum samples were diluted into wash buffer added to the plate and subsequently serially diluted in wash buffer on the plate. Test samples diluted 1:400 were pipetted onto the test plate with seven additional serial 1:2 dilutions (serum dilutions between 1:400 and 1:12,800). Samples were incubated at room temperature for 30 minutes to permit human IgG to bind to the plate-bound β-glucan antigen. Following incubation the wells were washed with wash buffer and an enzyme labeled secondary antibody (horseradish peroxidase conjugated affinity purified goat anti human IgG, Fc gamma specific) was incubated in the wells to bind with the human IgG bound to the β-glucan antigen. The secondary antibody was allowed to incubate for 30 minutes before washing with wash buffer. After the entire wash buffer was removed from the wells a peroxidase substrate was incubated in the wells and color development was quenched with 1 M phosphoric acid at five minutes color development. The optical density (OD) at 450 nm was measured using a microtiter plate reader.

Determining Anti-β-Glucan Ab Titer

Resulting OD from replicate wells were averaged and the mean assay background subtracted. The greatest dilution

giving a background adjusted OD greater than or equal to 0.100 was considered the samples titer and was expressed as the inverse of that dilution. For definition of assay performance a value was assigned the standard reference serum and a reference curve was constructed on each assay plate. For example a test sample giving a background adjusted OD of 0.100 at a dilution of 1:12,800 was considered to have a titer of 12,800. Where samples were tested multiple times and the average of their titers fell between the serial 1:2 titer levels from 1:400 the next lowest titer level was reported as its titer. For example, one donor's serum from four donations was tested in five separate assays resulting in a mean titer of 28,160; its titer was reported to be 25,600.

Assay Standard Curve. A value of 160 Arbitrary Units per mL (AU/mL) was assigned to the standard human anti β-glucan. Thus a 1:400 dilution in the assay method results in a value of 400 mAU/mL as the highest point of a standard dilution curve additional serial 1:2 dilutions were prepared on the assay plate. Assay controls were diluted 1:100 in ELISA wash buffer for testing. Furthermore two dilutions of each control level were independently prepared for testing on each plate in parallel.

Statistical Analysis. Plotting standard concentration in mAU/mL versus mean background corrected optical density resulted in a standard reference curve. Using the ELISA software a 4-parameter fit was computed from the standard dose response curve to determine unknown values for samples, controls and test serum. Assay response values falling between the upper and lower inflection points of the standard curve (linear portion) were used to determine a samples test value. To compute the coefficient of variation (% CV); the standard deviation of a set of values was divided by the mean of the same set of values and the result multiplied by 100.

Binding of PGG to Cells of Whole Blood (WB)

One hundred microliters of WB from healthy donors was aliquoted into 5 mL polystyrene fluorescence activated cell sorter (FACS) tubes. These WB samples were stimulated with either Imprime PGG® (β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose) (10 μg/mL or 100 μg/mL) or citrate buffer, the vehicle control. The FACS tubes containing the samples were loosely covered with the corresponding caps and incubated for 30 minutes or two hours, at 37° C. in a humidified incubator (5% CO_2).

TABLE 1

Antibody Cocktail Used To Stain Whole Blood Samples

Antibody	Company; Clone #	Dilution or Final Concentration	For identifica-tion of:
Anti-CD15	Biolegend; W6D3	0.2 μg/mL	neutrophils
Anti-CD19	Biolegend; HIB19	0.63 μg/mL	B cells
Anti-CD14	Biolegend; HCD14	5 μg/mL	monocytes
Anti-CD14	Invitrogen; TüK4	1:50	monocytes
Anti-CD3	Biolegend; HIT3a	0.25 μg/mL	T cells
Anti-CD45	Biolegend; HI30	0.25 μg/mL	hematopoietic cells excluding erythrocytes and platelets
Goat F(ab')2 anti- mouse IgM	Jackson Immunolab	5 μg/mL	mouse anti-β glucan antibody

Proceeding incubation with the anti-β-glucan antibody BfD IV, the cells were incubated with the antibody cocktail which contains a secondary antibody for the recognition of BfD IV as well as antibodies for the recognition of various cell surface markers

After incubation, all samples were washed by adding 2 mL of 1×Dulbecco's phosphate buffered saline (DPBS) and

centrifuged at 1500-1700 rpm at 4° C. for five minutes. After two rounds of washes and aspirations, 5 μL of the anti-β-glucan antibody BfD IV (~100 μg/mL), was mixed into each tube and incubated at room temperature for 30 minutes. This primary antibody was washed off twice with 1×DPBS as described above and a cocktail of antibodies containing the secondary antibody as well as the specific cell surface markers (Table 1) was added and incubated for 30 minutes at room temperature in the dark. To lyse the red blood cells, 2 mL of 1×BD lysing solution (BD Biosciences; San Jose, CA) was added to each sample and gently vortexed. After an incubation period of one hour at room temperature, the samples were centrifuged at 1500-1700 rpm at 4° C. for five minutes. The BD lysing solution was aspirated and the cells were washed once with 1×DPBS and aspirated as described above. For fixation, 300-400 μL of 1% parafounaldehyde was added to each sample. The samples were acquired on the LSR II (BD Biosciences; San Jose, CA) within 20 hours of fixation. Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Example 2

Materials

Imprime PGG® (β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-(1,3)-D-glucopyranose (Biothera, Eagan, MN) was provided in a preservative-free, soluble β-glucan formulation prepared at a concentration of 1 mg/mL in 0.8% sodium chloride and 0.2% sodium citrate monobasic, at a pH of 6.4. The compound was stored at 4-8° C. until use.

Whole Blood (WB) Binding Assay

Fresh WB was obtained from healthy volunteers that had provided informed consent prior to donation (New England Institutional Review Board. Blood Donation Protocol No. 07-124). The blood was collected in a Vacutainer® containing 158 USP Units Freeze-Dried Sodium Heparin (BD Biosciences; San Jose, CA). Serum was collected in a Vacutainer® containing a thrombin-based clot activator (BD Biosciences; San Jose, CA). Approximately 20 minutes after collection, the vial was centrifuged at 2000 rpm for 10 minutes at room temperature. Serum was harvested from this vial and stored at 4° C. for use within 8 hours or at −80° C. for use after 8 hours.

The whole blood binding assay was performed by incubating whole blood samples with Imprime PGG® (β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose) for 30 minutes or two hours at 37° C. in a humidified incubator. After washing with 1× Dulbecco's phosphate buffered saline (DPBS), BfDIV, a mouse anti-β-glucan antibody, was added and incubated with the WB for 30 minutes at room temperature. After more rounds of washing, an antibody cocktail including a goat anti-mouse detection antibody and antibodies to surface molecules were added and incubated at room temperature in the dark for 30 minutes. Erythrocytes were lysed with BD Lyse and samples were resuspended in 1% paraformaldehyde. Samples were acquired on a flow cytometer and analyzed using FlowJo software (Ashland, OR).

WB and Serum Crossover Studies

For serum crossover studies, whole blood was spun down at 1200 rpm for 10 minutes and plasma removed. Blood cells were washed 1-2 times with 1× DPBS to remove remaining plasma. 50 μL of serum was added and mixed before addition of Imprime PGG® (β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose).

For incubation with anti-β-glucan IgG (BioSupplies, Australia), the lyophilized antibody was resuspended to 1

mg/mL with 1×DPBS and stored at −80° C. or 4° C. as a stock solution. Before being added to blood samples, the stock was diluted 1:10 to 100 μg/mL and 10 μL of this solution was added to 100 μL of blood. For incubation with IVIG, 10% IVIG (100 mg/mL) (PRIVIGEN, CSL Behrling, King of Prussia, PA) was added to the whole blood sample at the indicated final concentrations.

EXEMPLARY EMBODIMENTS

Embodiment 1. A method for identifying soluble β-glucan binding to immune cells of a subject, the method comprising:

    obtaining a blood sample from the subject, the blood
        sample comprising immune cells;
    adding soluble β-glucan to at least a portion of the blood
        sample and incubating the mixture under conditions
        allowing the soluble β-glucan to bind to the immune
        cells; and
    detecting β-glucan bound to the immune cells.

Embodiment 2. The method of Embodiment 1 wherein the soluble β-glucan is derived from yeast.

Embodiment 3. The method of Embodiment 1 or Embodiment 2 wherein the soluble β-glucan comprises a β-1,3/1,6 glucan.

Embodiment 4. The method of any preceding Embodiment wherein the soluble β-glucan comprises β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

Embodiment 5. The method of any preceding Embodiment wherein detecting soluble β-glucan bound to the immune cells comprises contacting the sample with a monoclonal antibody that specifically binds to the β-glucan.

Embodiment 6. The method of Embodiment 5 wherein the monoclonal antibody comprises BfD I, BfD II, BfD III, or BfD IV.

Embodiment 7. The method of any preceding Embodiment further comprising:

    obtaining a second blood sample from the subject, the
        blood sample comprising immune cells;
    adding soluble β-glucan to at least a portion of the second
        blood sample and incubating the mixture under condi-
        tions allowing the soluble β-glucan to bind to the
        immune cells; and
    detecting soluble β-glucan bound to the immune cells.

Embodiment 8. A method of improving β-glucan immunotherapy for a subject, the method comprising:

    identifying the subject as a low binder; and
    co-administering to the subject a soluble β-glucan and an
        antibody preparation capable of converting the subject
        from a low binder to a high binder.

Embodiment 9. The method of Embodiment 8 wherein identifying the subject as a low binder comprises:

    obtaining a blood sample from the subject, the sample
        comprising an anti-β-glucan antibody titer;
    measuring the anti-β-glucan antibody titer of at least a
        portion of the blood sample; and
    identifying the subject as a low binder if the anti-β-glucan
        IgG antibody titer is less than 20,000.

Embodiment 10. The method of Embodiment 8 wherein identifying the subject as a low binder comprises:

    obtaining a blood sample from the subject, the blood
        sample comprising immune cells;
    adding soluble β-glucan to at least a portion of the blood
        sample and incubating the mixture under conditions
        allowing the soluble β-glucan to bind to the immune
        cells;

    detecting soluble β-glucan bound to the immune cells;
        and
    identifying the subject as a low binder if β-glucan is
        bound to no more than 10% of the immune cells.

Embodiment 11. The method of any one of Embodiments 8-10 wherein the antibody preparation comprises serum from a high binder.

Embodiment 12. The method of any one of Embodiments 8-10 wherein the antibody preparation comprises a monoclonal antibody that specifically binds the soluble β-glucan.

Embodiment 13. The method of Embodiment 12 wherein the monoclonal antibody comprises BfD I, BfD II, BfD III, or BfD IV.

Embodiment 14. The method of any one of Embodiments 8-13 wherein the antibody preparation comprises intravenous immunoglobulin.

Embodiment 15. The method of any one of Embodiments 8-14 wherein the soluble β-glucan and the antibody preparation are co-administered simultaneously.

Embodiment 16. The method of any one of Embodiments 8-14 wherein the soluble β-glucan and the antibody preparation are co-administered at different times.

Embodiment 17. The method of any one of Embodiments 8-14 wherein the soluble β-glucan and the antibody preparation are co-administered at different sites.

Embodiment 18. The method of any one of Embodiments 8-16 wherein the soluble β-glucan is derived from yeast.

Embodiment 19. The method of any one of Embodiments 8-18 wherein the soluble β-glucan comprises a β-1,3/1,6 glucan.

Embodiment 20. The method of any one of Embodiments 9-19 wherein the soluble β-glucan comprises β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical

values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A method of improving β-glucan immunotherapy for a subject who is a low-binder of β-glucan, the method comprising:

identifying a subject who is a low binder of β-glucan; and

administering to said subject an antibody preparation comprising an anti-β-glucan antibody and a β-glucan immunotherapy, wherein the β-glucan immunotherapy comprises soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose and one or more tumor-related monoclonal antibodies;

wherein a subject is identified as a low-binder of β-glucan if β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose is bound to no more than 10% of the subject's immune cells, or wherein a subject is identified as a low binder of β-glucan if the anti-β-glucan IgG antibody titer of the subject is less than 20,000.

2. The method of claim 1, wherein the antibody preparation comprises serum from a high binder of β-glucan.

3. The method of claim 1, wherein the antibody preparation comprises a monoclonal antibody that specifically binds soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

4. The method of claim 1, wherein the antibody preparation comprises intravenous immunoglobulin.

5. The method of claim 1, wherein the soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose and the antibody preparation are administered simultaneously.

6. The method of claim 1, wherein the soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose and the antibody preparation are administered at different times.

7. The method of claim 1, wherein the soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose and the antibody preparation are administered at different sites.

8. The method of claim 1, wherein the soluble β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose is derived from yeast.

9. The method of claim 1, wherein the antibody preparation comprises intravenous immunoglobulin conjugated to β(1,6)-[poly-(1,3)-D-glucopyranosyl]-poly-β(1,3)-D-glucopyranose.

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