

β-GLUCAN ASSAY METHODS

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CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application Serial No. 61/912,275, filed December 5, 2013, and U.S. Provisional Patent Application Serial No. 62/005,335, filed May 30, 2014, each of which is incorporated herein by reference.

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SUMMARY

This disclosure provides methods for analyzing a sample from a subject for a biomarker that is indicative of the subject's immune response to β-glucan.

15 In some embodiments, the method generally includes analyzing a biological sample obtained from a subject for a biomarker anti-β-glucan antibody compared to a reference standard, computing an anti-β-glucan antibody concentration or Relative Antibody Unit (RAU) value for anti-β-glucan antibody in the sample, and identifying the subject as biomarker negative for β-glucan immunotherapy if the anti-β-glucan antibody concentration or RAU value is less than a predetermined anti-β-glucan antibody concentration or RAU value for the biomarker anti-β-glucan antibody, wherein the predetermined RAU value is selected based on a correlation of
20 specificity and sensitivity to at least one endpoint that stratifies biomarker-positive subjects and biomarker-negative subjects, and wherein anti-β-glucan IgG₂ is to be administered to the biomarker negative subject.

In other embodiments, the method generally includes analyzing a sample obtained from a
25 subject for a biomarker anti-β-glucan antibody compared to a reference standard, and identifying the subject as biomarker negative for β-glucan immunotherapy if the sample contains an amount of the anti-β-glucan antibody that is less than a predetermined cutoff value for the biomarker anti-β-glucan antibody, wherein the predetermined cutoff value is selected based on a correlation of specificity and sensitivity to at least one endpoint that stratifies biomarker-positive subjects
30 from biomarker-negative subjects, and wherein anti-β-glucan IgG₂ is to be administered to the biomarker-negative subject.

In some embodiments, the biomarker anti- β -glucan antibody can be IgG. In some of these embodiments, the predetermined RAU value can be 200.

In some embodiments, the biomarker anti- β -glucan antibody can be IgM. In some of these embodiments, the predetermined RAU value can be 300.

- 5 In some embodiments, a subject identified as biomarker positive may be administered with a composition that includes β -glucan.

In some embodiments, a subject identified as biomarker positive may be administered with a composition that includes anti- β -glucan IgG₂.

In some embodiments, the β -glucan is derived from yeast such as, for example, a β -1,3/1,6 glucan. In certain embodiments, the β -glucan can include $\beta(1,6)$ -[poly-(1,3)-D-glucopyranosyl]-poly- $\beta(1,3)$ -D-glucopyranose.

In some embodiments, analyzing the sample for a biomarker anti- β -glucan antibody can involve using an enzyme-linked immunosorbent assay (ELISA).

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. 1A. An arbitrary value of 160 was assigned to the reference serum (i.e., 160 Relative Antibody Units [RAU]/mL). Thus, the highest point on the calibration curve, the 1:400 dilution, results in a value of 400 mRAU/mL and at the lowest point on the calibration curve, the 1:51200 dilution, results in a value of 3.125 mRAU/mL). Mean RAU/mL = 365.

FIG. 1B. IgG anti- β -glucan antibody concentration working standard curve.

FIG. 1C. IgM anti- β -glucan antibody concentration working standard curve.

FIG. 2. Whole blood from eight healthy volunteers with low levels of IgG (RAU <200) and IgM (RAU <100) anti-beta glucan antibodies were spiked with increasing concentrations of IVIG (0, 2.5, 5 and 10 mg/ml). Plasma was removed and RAU values determined for each sample.

FIG. 3. A sample of blood from four healthy volunteers with low levels of IgG (RAU <200) and IgM (RAU <100) anti-beta glucan antibodies was spiked with increasing concentrations of IVIG (0, 2.5, 5 and 10 mg/ml). Whole blood cells were treated with IMPRIME PGG at 10 μ g/mL and incubated at 37 $^{\circ}$ C for 30min and analyzed for neutrophil binding.

FIG. 4. Whole blood cells from eight healthy volunteers with low levels of IgG (RAU <200) and IgM (RAU <100) anti-beta glucan antibodies were cultured with IMPRIME PGG (10 mg/ml) and IVIG (0, 2.5, 5 and 10 mg/ml) and evaluated for IMPRIME PGG binding and

mg/ml) and IVIG (0, 2.5, 5 and 10 mg/ml) and evaluated for IMPRIME PGG binding and function (30 min incubation for complement analysis; 2 hrs for binding and receptor modulation; overnight culture for cytokines).

FIG. 5. Whole blood cells from eight healthy volunteers with low levels of IgG (RAU <200) and IgM (RAU <100) anti-beta glucan antibodies were cultured with IMPRIME PGG (10 mg/ml) and IVIG (0, 2.5, 5 and 10 mg/ml) and evaluated for IMPRIME PGG induced SC5b-9 release.

FIG. 6. Whole blood cells from eight healthy volunteers with low levels of IgG (RAU <200) and IgM (RAU <100) anti-beta glucan antibodies were incubated with IMPRIME PGG (10 mg/ml) and IVIG (0, 2.5, 5, and 10 mg/ml) and evaluated for IMPRIME PGG induced CD62L shedding (flow-cytometry) on neutrophils and IL-8 production (ELISA).

FIG. 7. The RAU required for binding and function varies by individual. Hence, the objective of the biomarker cutoff is to identify an RAU level that will exclude individuals who will not respond to IMPRIME PGG and enrich for biomarker positive subjects that have a higher probability to respond. Binding and functional data indicate the most activity to occur in the $\geq$ IVIG-5 group (RAU 233-610) and therefore, further analysis for the IgG cutoff should be focused on the RAU range above 200.

FIG. 8. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level.

FIG. 9. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level. The circled volunteers were high neutrophil binders with low IgG RAU but had high IgM RAU, thus, can be removed from IgG RAU analysis.

FIG. 10. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level. An IgG RAU cutoff of 235 dissociated volunteers whose RAU was sufficient to facilitate binding (biomarker positive) versus volunteers whose RAU was not sufficient to facilitate binding (biomarker negative).

FIG. 11. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level. The circled individuals are IgG biomarker negative but high IgM RAU.

FIG. 12. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level. The non-blue volunteers circled in green were already biomarker positive based on high IgG RAU, thus, can be removed from IgM RAU analysis.

FIG. 13. Healthy volunteer neutrophil binding of IMPRIME PGG plotted by IgG RAU level. An IgM RAU cutoff of 330 dissociated volunteers whose RAU was sufficient to facilitate binding (biomarker positive) versus volunteers whose RAU was not sufficient to facilitate binding (biomarker negative).

FIG. 14. Correlation of Anti-beta Glucan Antibody Levels and C4a Production in Whole Blood from Healthy Donors. The amount of C4a in WB cultures stimulated 30 minutes with IMPRIME PGG (10 μ g/ml) from individual healthy donors are presented as fold change over citrate control in the Y axis. The mean fold change $\pm$ SEM are presented for each group in the X axis. 8/10 individuals (80%) with an IgG RAU of ≥ 235 and 0/7 individuals with an IgG RAU of < 235 demonstrated a ≥ 1.5 -fold C4a production with IMPRIME PGG, supporting a significant correlation between high RAU and greater complement activation (**p=0.0069).

FIG. 15. Correlation of Anti-beta Glucan Antibody Levels and C5a Production in Whole Blood from Healthy Donors. The amount of C5a in WB cultures stimulated 30 minutes with IMPRIME PGG (10 μ g/ml) from individual healthy donors are presented as fold change over citrate control in the Y axis. The mean fold change $\pm$ SEM are presented for each group in the X axis. 10/10 individuals with an IgG RAU of ≥ 235 and 1/7 individuals (14%) with an IgG RAU of < 235 demonstrated a ≥ 1.5 -fold C5a production with IMPRIME PGG, supporting a significant correlation between high RAU and greater complement activation(*p=0.0381).

FIG. 16. Correlation of anti-beta glucan RAU levels and SC5b-9 production in whole blood from healthy donors. The amount of SC5b-9 in healthy donor whole blood cultures incubated 30 minutes with IMPRIME PGG (10 μ g/ml). SC5b-9 levels (Y-axis) presented as fold change over citrate control stratified by IgG RAU status (X axis). 11/11 (i.e., 100%) individuals with an IgG RAU of ≥ 235 versus 0/9 (i.e., 0%) individuals with an IgG RAU of < 235 demonstrated a ≥ 2 -fold SC5b-9 production, supporting a significant correlation between high RAU and greater complement activation (**p=0.0082).

FIG. 17. Correlation of Anti-beta Glucan Antibody Levels and Increase in Surface Expression of CR3 in PMN of Whole Blood from Healthy Donors. Surface expression of CR3 on CD15+ neutrophils in WB cultures stimulated 30 minutes with IMPRIME PGG (10 μ g/ml) from individual healthy donors are presented as MFI percentage change over citrate control in the Y axis. The mean MFI percentage change $\pm$ SEM are presented for each group in the X axis. In comparison to the individuals with an IgG RAU of < 235 (N = 7), neutrophils from

individuals with an IgG RAU of ≥ 235 ($N = 11$) demonstrated significant increase in surface CR3 levels (* $p=0.0211$).

FIG. 18. Correlation of Anti-beta Glucan Antibody Levels and Reduction of CD88 Expression in PMN of Whole Blood from Healthy Donors. Surface expression of CD88 on CD15+ neutrophils in WB cultures stimulated 30 minutes with IMPRIME PGG (10 $\mu\text{g/ml}$) from individual healthy donors are presented as MFI percentage change over citrate control in the Y axis. The mean MFI percentage change $\pm$ SEM are presented for each group in the X axis. In comparison to the individuals with an IgG RAU of < 235 ($N = 8$), neutrophils from individuals with an IgG RAU of ≥ 235 ($N = 13$) demonstrated significant reduction in surface CD88 levels (* $p=0.0197$).

FIG. 19. Healthy donor whole blood was incubated with IMPRIME PGG (10 $\mu\text{g/ml}$) and surface expression of CD62L on CD15+ neutrophils assayed. The percent CD15⁺CD62L^{lo} cells (individual and mean $\pm$ SEM) is presented on the Y-axis stratified by RAU status on the X axis. Neutrophils from individuals with an IgG RAU of ≥ 235 demonstrated significant loss of CD62L expression (** $p=0.005$).

FIG. 20. Healthy donor whole blood was incubated overnight with IMPRIME PGG (10 $\mu\text{g/ml}$) then plasma IL-8 levels measured. IL-8 levels (Y-axis) presented as fold change over citrate control stratified by IgG RAU Status (X axis). 12/13 (i.e., 92%) individuals with an IgG RAU of ≥ 235 versus only 3/13 (i.e., 23%) individuals with an IgG RAU of < 235 demonstrated a ≥ 2 -fold IL-8 production with IMPRIME PGG, supporting a significant correlation between high RAU and greater IL-8 production (** $p=0.0028$).

FIG. 21. Correlation of Anti-beta Glucan Antibody Levels and SC5b-9 Production in Whole Blood from Healthy Donors. The amount of SC5b-9 in WB cultures stimulated 30 minutes with IMPRIME PGG (10 $\mu\text{g/ml}$) from individual healthy donors are presented as fold change over citrate control in the Y axis. The mean fold change $\pm$ SEM are presented for each group in the X axis. In comparison to the individuals with an IgM RAU of < 235 ($N = 9$), neutrophils from individuals with an IgM RAU of ≥ 330 ($N = 3$) demonstrated significant increase in SC5b-9 production (** $p=0.0003$).

FIG. 22. SDS-PAGE (4-20% under reducing conditions) of purified "gold reference" IgG and IgM anti- β -glucan antibody stained with Coomassie Blue. Protein standards, with molecular weight (MW) expressed in kilodaltons (kDa), are shown in Lane 3.

FIG. 23. (A) Correlation of IgG anti- β -glucan antibody concentrations with neutrophil binding of IMPRIME PGG in whole blood of healthy subjects; (B) Correlation of IgM anti- β -glucan antibody concentrations with neutrophil binding of IMPRIME PGG in whole blood of healthy subjects.

FIG. 24. (A) Correlation of IgG anti- β -glucan antibody concentrations with monocyte binding of IMPRIME PGG in whole blood of healthy subjects; (B) Correlation of IgM anti- β -glucan antibody concentrations with monocyte binding of IMPRIME PGG in whole blood of healthy subjects.

FIG. 25. (A) Correlation of IgG anti- β -glucan antibody concentrations with neutrophil binding of IMPRIME PGG based on high vs. low binder status; (B) Correlation of IgM anti- β -glucan antibody concentrations with neutrophil binding of IMPRIME PGG based on high vs. low binder status.

FIG. 26. (A) Correlation of IgG anti- β -glucan antibody concentrations with monocyte binding of IMPRIME PGG based on high vs. low binder status; (B) Correlation of IgM anti- β -glucan antibody concentrations with monocyte binding of IMPRIME PGG based on high vs. low binder status.

FIG. 27. (A) IgG ROC curve analysis based on neutrophil binding; (B) IgM ROC curve analysis based on neutrophil binding.

FIG. 28. (A) IgG ROC curve analysis based on monocyte binding; (B) IgM ROC curve analysis based on monocyte binding.

FIG. 29. IgG anti- β -glucan antibody subclass neutrophil binding based on high vs. low binder status.

FIG. 30. IgG anti- β -glucan antibody subclass neutrophil binding.

FIG. 31. Results of *in vivo* infusion of intravenous immunoglobulin (IVIG) to increase anti- β glucan antibody for IMPRIME PGG treatment of low binder patient. (A) IgG RAU; (B) PMN binding and monocyte binding; (C) C5a fold increase.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

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. Patent No. 7,981,447.

Biomarker research demonstrated differences among subjects in the ability of their neutrophils and monocytes to bind IMPRIME PGG. Binding of IMPRIME PGG to these cells correlated with the subjects' immunomodulatory response to IMPRIME PGG. Moreover, IMPRIME PGG binding to neutrophils and monocytes involves the presence of a specific level of natural anti- β -glucan antibodies.

This disclosure provides a simple ELISA method to quantitatively measure anti- β -glucan IgG and IgM antibodies in patient serum samples. The ELISA method may be used as a biomarker assay. Cutoff levels for the biomarker assay identify biomarker positive and biomarker negative subgroups of healthy volunteers and these cutoff points correlate with binding, function, and clinical outcomes.

Early studies evaluating binding of IMPRIME PGG to neutrophils from healthy volunteers revealed subjects with different binding capabilities. High neutrophil binding (e.g., IMPRIME PGG bound to >20% of neutrophils) of IMPRIME PGG is found in ~40% of healthy volunteers. Monocyte binding of IMPRIME PGG parallels the binding potential of neutrophils in healthy volunteers. *In vitro*, high neutrophil binders and high monocyte binders produce more IL-8 than low binders.

Higher natural anti- β -glucan antibody levels correspond with neutrophil binding of IMPRIME PGG. High-binder serum/plasma can increase IMPRIME PGG binding to neutrophils from low binders. Anti- β -glucan antibodies in the high-binder serum can increase neutrophil IMPRIME PGG binding in low-binders in non-permissive binding conditions. For example, intravenous immunoglobulin (IVIG), which contains high natural anti- β -glucan antibody titers, can increase IMPRIME PGG binding to neutrophils from a low-binder. Natural anti- β -glucan IgG and/or IgM antibodies are involved in binding to neutrophils and monocytes. Without wishing to be bound by any particular theory, natural anti- β -glucan antibodies (e.g., IgG and IgM) bind to IMPRIME PGG. The IMPRIME PGG is opsonized *via* the classical pathway of complement activation. Opsonized IMPRIME PGG binds to the lectin-like domain of CR3 receptors on neutrophils and monocytes. The opsonization of IMPRIME PGG (i.e., iC3b deposition) occurs as a result of the classical pathway of complement activation after antibody binding. Several functional markers are modulated during the process of neutrophil and/or monocyte binding of IMPRIME PGG such as, for example, C4a, C5a and SC5b-9.

IMPRIME PGG binding to neutrophils and/or monocytes in whole blood samples can be reproducibly measured using assays that use, for example, flow cytometry. The use of whole blood for such assays, however, presents certain challenges. For example, it requires live, healthy cells so that blood samples need to be received and processed within 24 hours of collection. Thus, shipping conditions and environmental factors can damage the blood cells. Such assays also required control blood samples from known high binder and low binder subjects. Finally,

although a conceptually simple assay, flow cytometry technology is not common in many clinical labs. Thus, the flow cytometry-based assay is not a practical assay for clinical development.

This disclosure provides a method that involves simple quantitative ELISA to measure endogenous anti- β -glucan antibody levels in human serum samples. The method overcomes the challenges presented by the flow cytometry-based assay for the development of a practical clinical test. Serum-based ELISA assays are common clinical assays and can be performed by most clinical labs. Moreover, serum samples can be frozen resulting in easier storage, shipping and consistency in assay performance.

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, $\beta(1,6)$ -[poly-(1,3)-D-glucopyranosyl]-poly- $\beta(1,3)$ -D-glucopyranose.

In some embodiments, β -glucan bound 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. Patent No. 6,294,321.

Traditional approaches to measuring the amount of antibody in serum typically involve the use of an ELISA in the context of an assay in which a sample is serially diluted. The serial dilutions are subjected to the ELISA assay and the amount of antibody in the serum sample is estimated based on the greatest dilution of serum sample that generates a positive assay response. The resulting titer value is an estimate within dilutional ranges and not an exact measurement of the amount of antibodies. Serial dilution methods are frequently used in serology to determine the amount of antibody (titer level) in the serum. Determining titer levels is frequently used to evaluate antibody responses to vaccines. In serial dilution methods, however, one cannot measure directly the exact amount of antibody that is produced. Instead, one can only estimate the amount of antibody produced within a dilutional range. Titer levels are, therefore, not

amenable to standard statistical analyses in a straightforward manner without modifications of the data and/or the statistical analytical techniques.

In contrast, this disclosure provides an ELISA-based method that measures natural anti- β -glucan antibodies to IMPRIME PGG in human serum. The method involves quantitatively measuring the amount of natural anti- β -glucan antibodies as either Relative Antibody Units (RAU) or anti- β -glucan antibody concentration within statistical requirements. This is accomplished by generating a titer and correlating either RAU data or antibody concentration data (both determined from a calibration curve) in the same assay. Thus, the methods described herein involve generating internal standard curves from serum with an established level of natural anti- β -glucan antibody. Moreover, the methods provide a better method of quantitatively measuring relative or actual anti- β -glucan antibodies than measuring titers.

The methods can be used to measure anti- β -glucan IgG and/or anti- β -glucan IgM. The computation of RAU for IgG and/or IgM is shown in Table 7. The computation of antibody concentration for IgG and/or IgM is shown in Table 8 and Table 9, respectively. The methods provide intra-assay precision—i.e., reproducibility between plates. The methods also provide inter-assay precision—i.e., reproducibility between assays and/or assay days for control and test serum samples. The methods allow one to evaluate multiple samples analyzed on different test plates by a single operator. Thus, the methods provide reliable, reproducible results whether in the hands of a single operator analyzing multiple samples in a single day or in the hands of multiple operators over the course of multiple days.

The anti- β -glucan antibody level required for binding and function can vary by individual. FIG. 2 shows a dose-dependent increase in anti- β -Glucan IgG RAU levels with IVIG. The method involves using a biomarker cutoff to identify an RAU level that will exclude individuals who will not respond to IMPRIME PGG and enrich for biomarker positive subjects that have a higher probability to respond. Binding and functional data indicate the most activity to occur in the $\geq$ IVIG-5 group (RAU 233-610) and therefore, further analysis for the IgG cutoff should be focused on the RAU range above 200. (FIG. 7).

An initial study in 32 healthy volunteers was performed to establish the specific minimum level of natural anti- β -glucan antibodies (RAUs of IgG and/or IgM) necessary for binding and function of IMPRIME PGG in neutrophils or monocytes. The minimum levels quantified as anti- β -glucan antibody concentrations of IgG and/or IgM were further optimized

using a larger cohort of healthy subjects (n=143) and confirmed the significance of elevated anti- β -glucan antibody levels with respect to binding of IMPRIME PGG to neutrophils and monocytes, IMPRIME PGG-induced functional changes and clinical outcomes. Subjects with anti- β -glucan antibody levels conducive to IMPRIME PGG binding to neutrophils and monocytes are considered “biomarker positive.” The bioassay can allow one to identify subjects in a clinical setting with anti- β -glucan antibody levels that are too low for IMPRIME PGG binding to neutrophils and monocytes (“biomarker negative”) so that they can either receive alternative treatment or receive anti- β -glucan antibody treatments so that they better respond to therapy that involves IMPRIME PGG. Alternatively, a biomarker positive individual may be more immune competent than a biomarker negative individual and, thus, may respond better to immunotherapy drugs.

When using the RAU method, an individual can be biomarker positive by possessing an IgG RAU of at least a minimum IgG RAU predetermined value and/or possessing an IgM RAU of at least a minimum IgM RAU predetermined value. In some embodiments, the IgG RAU predetermined value can be at least 200 such as, for example, at least 205, at least 210, at least 215, at least 220, at least 225, at least 230, at least 235, at least 240, at least 245, at least 250, at least 255, at least 260, at least 265, at least 270, or at least 275. In some embodiments, the IgM RAU predetermined value can be at least 300 such as, for example, at least 305, at least 310, at least 315, at least 320, at least 325, at least 330, at least 335, at least 340, at least 345, at least 350, at least 355, at least 360, at least 365, at least 370, or at least 375. That is, an IgG RAU of, for example, at least 200 or an IgM RAU of, for example, at least 300 reasonably correlates with an individual that exhibits at least 5% of neutrophils binding β -glucan and the neutrophil and monocyte functional modulation associated with β -glucan “high binder” status.

For example, FIG. 8 shows a plot of the percent neutrophil binding for each of the 32 healthy volunteers as a function of the computed IgG RAU. The horizontal line at 5% neutrophil binding delineates “high binders” from “low binders.” To distinguish between those volunteers whose IgG RAU was too low to bind more than 5% neutrophils and those whose IgG RAU was sufficient to exhibit at least 5% of their neutrophils binding β -glucan, we established a cut off line at an IgG RAU of 235 (FIG. 10), the closest value under the lowest IgG RAU value showing higher than 5% of neutrophils binding β -glucan (239). We subsequently evaluated the level of the three individuals who had greater than 5% of neutrophils binding β -glucan, but were IgG

biomarker negative (FIG. 9, circled). These three individuals were IgG biomarker negative—i.e., IgG RAU of less than 235—but nevertheless exhibited at least 5% of their neutrophils binding β -glucan. When neutrophil binding was plotted as a function of IgM RAU, an IgM RAU cutoff value of 330 was established. (FIG. 13).

When using the antibody concentration method, which is further optimized, an individual can be biomarker positive by possessing an IgG anti- β -glucan antibody concentration of at least a minimum IgG anti- β -glucan antibody concentration predetermined value and/or possessing an IgM anti- β -glucan antibody concentration of at least a minimum IgM anti- β -glucan antibody concentration predetermined value. IMPRIME PGG binding and anti- β -glucan antibody concentration measurements from serum derived from the same draw of whole blood were evaluated in healthy volunteers, N=143. The range of IgG and IgM anti- β -glucan antibody concentrations determined for the N=143 individuals was 1.13-209.8 μ g/ml (7.8-1447.8 RAU/ml) and 5.3-2032.7 μ g/ml (12.8-4878.4 RAU/ml), respectively. Both neutrophils and monocytes demonstrated greater than 5% binding more frequently at approximately 14 μ g/ml (100 RAU/ml) and 42 μ g/ml (100 RAU/ml) IgG and IgM anti- β -glucan antibody levels, respectively.

Therefore, in some embodiments, the IgG anti- β -glucan antibody concentration predetermined value can be at least 14 μ g/ml (100 RAU) such as, for example, at least 15, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, at least 50, at least 55, at least 60, at least 65, at least 70, at least 75 or at least 80 μ g/ml (100 RAU). In some embodiments, the IgM anti- β -glucan antibody concentration predetermined value can be at least 42 μ g/ml such as, for example, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, at least 270, at least 280, at least 290, at least 300, at least 310, at least 320, at least 330, at least 340, at least 350, at least 360, at least 370, at least 380, at least 390, at least 400, at least 410, at least 420, at least 430, at least 440, at least 450, at least 460, at least 470, at least 480, at least 490, at least 500, at least 510, at least 520, at least 530, at least 540 or at least 550 μ g/ml (100 RAU). That is, an IgG anti- β -glucan antibody concentration of, for example, at least 14 μ g/ml or an IgM anti- β -glucan antibody concentration of, for example, at least 42 μ g/ml reasonably correlates

with an individual that exhibits at least 5% of neutrophils or monocytes binding β -glucan and the neutrophil and monocyte functional modulation associated with β -glucan “high binder” status.

FIG. 23 and FIG. 24 show plots of the percent neutrophil and monocyte binding for each healthy volunteer as a function of the computed (A) IgG and (B) IgM anti- β -glucan antibody concentrations. As shown, IMPRIME PGG binding to neutrophils and monocytes increases as IgG and/or IgM anti- β -glucan antibody concentrations increase.

As shown in FIG. 25 and FIG. 26, there was strong correlation between IgG and IgM anti- β -glucan antibody concentrations and both neutrophil and monocyte binding at a 5% binding level, distinguishing high and low IMPRIME PGG binders. There was no correlation between anti- β -glucan antibody concentrations and age, gender, or total immunoglobulin (data not shown).

Next, exploratory cutoffs of anti- β -glucan antibody concentrations were investigated in the 143 healthy volunteers. Using a 5% level for both neutrophil and monocyte binding of IMPRIME PGG, the correlation between the sensitivity and specificity of anti- β -glucan concentrations was determined using ROC curve analysis (FIG. 27 and FIG. 28). At an initial exploratory specificity of 95% for binding, anti- β -glucan antibody concentration cutoffs were determined and used to calculate both the prevalence of the biomarker in the healthy population and its sensitivity for determining binding status. The results are shown in Table 1.

Table 1

Parameter	Based on Neutrophil Binding		Based on Monocyte Binding	
	IgG	IgM	IgG	IgM
Cutoff (μ g/ml)	51	118	40	126
Prevalence	36%	20%	41%	15%
(Biomarker Positive)	46% Overall		48% Overall	
Sensitivity	64%	33%	62%	22%
(Biomarker Positive)	75% Overall		66% Overall	

Positive IgG anti- β -glucan biomarker status is highly correlated with *in vitro* IMPRIME PGG biological activity using either set of cutoffs. However, as indicated by ROC curve

analysis, similar sensitivities at different cutoffs indicate a range of anti- β -glucan antibody concentrations that correlate with the biological activity of IMPRIME PGG.

Therefore, various anti- β -glucan antibody concentration cutoffs were investigated and functional endpoints were used to determine whether the cutoffs reasonably segregate individuals into high binder and low binder (biomarker positive and biomarker negative) status. IL-8 production was used as the functional endpoint to explore the range of IgG anti- β -glucan antibody concentration cutoffs. Results are shown in Table 2.

Table 2

Cutoffs (RAU/μg per ml)	Binding to Neutrophils	Binding to Monocytes	IL-8 Production	Approximate Specificity/Sensitivity
100/14	<0.0001	<0.0001	0.0132	44.90/92.55
125/18	<0.0001	<0.0001	0.0019	59.18/88.30
150/22	<0.0001	<0.0001	0.0002	69.39/79.79
175/25	<0.0001	<0.0001	<0.0001	77.55/74.47
200/29	<0.0001	<0.0001	<0.0001	81.63/72.34
276/40	<0.0001	<0.0001	<0.0001	97.96/60.64
351/51	<0.0001	<0.0001	<0.0001	100/54.26
400/60	<0.0001	<0.0001	<0.0001	100/48.94
425/62	<0.0001	<0.0001	<0.0001	100/47.87
450/65	<0.0001	<0.0001	<0.0001	100/45.74
475/69	<0.0001	<0.0001	<0.0001	100/42.55
500/72	<0.0001	<0.0001	<0.0001	100/40.43
525/76	<0.0001	<0.0001	<0.0001	100/39.36
550/80	<0.0001	<0.0001	0.0009	100/35.11

Likewise, C4a/SC5b9 production was used as the functional endpoint to explore the range of IgM anti- β -glucan antibody concentration cutoffs. Results are shown in Table 3.

Table 3

Cutoffs (RAU/ μ g per ml)	Binding to Neutrophils	Binding to Monocytes	C4a Production	Approximate Specificity/Sensitivity
100/42	0.0001	<0.0001	N/A	67.35/68.09
150/63	<0.0001	<0.0001	N/A	77.55/54.26
200/83	<0.0001	<0.0001	0.1331	85.71/42.55
250/104	<0.0001	<0.0001	0.0587	93.88/30.85
300/125	<0.0001	<0.0001	0.0363	95.92/21.28
350/146	<0.0001	<0.0001	0.0869	97.96/14.89
400/167	0.0022	0.0007	N/A	97.96/11.7
450/188	0.0034	0.0004	N/A	100/8.51
500/208	0.0034	0.0004	N/A	100/7.44
550/229	0.0016	0.0003	N/A	100/6.38

As is evident from the data, specific IgG anti- β -glucan antibody concentration or RAU cutoff can be selected depending on the combination of specificity and sensitivity deemed to be necessary for stratifying or selection of patients in a clinical trial using ROC curve analyses. For example, a cutoff of 276 RAU/ml or 40 μ g/ml would have a specificity of approximately 98% and sensitivity of 61% based on a ROC curve analysis of neutrophil and monocyte binding of IMPRIME PGG in healthy volunteers.

A reasonable range of IgM anti- β -glucan antibody concentrations or RAU cutoffs based on functional analyses, such as C4a production used here, may be 200-350 RAU/ml or 83-146 μ g/ml. As above, a specific IgM anti- β -glucan antibody concentration or RAU cutoff can be selected depending on the combination of specificity and sensitivity deemed to be necessary for stratifying or selection of patients in a particular clinical trial. For example, a cutoff of 250 RAU/ml or 104 μ g/ml would have a specificity of approximately 94% and sensitivity of 31% based on a ROC curve analysis of neutrophil and monocyte binding of IMPRIME PGG in healthy volunteers.

As additional support, using the cutoffs shown in Table 1, biomarker status correlates with functional changes induced by IMPRIME PGG, including activation of complement

components C3a, C5a, SC5b9, modulation of neutrophil CR1, CR3, CD88, and CD62L surface marker expression, and IL-8 induction. The results are shown in Table 4.

Table 4

Function	Comparison by Overall Biomarker Status ¹	
	Neutrophil-Derived Cutoff ²	Monocyte-Derived Cutoff ³
C4a Fold Increase (n=32)	0.0002	0.0002
C5a Fold Increase (n=32)	0.0037	0.0037
SC5b9 Fold Increase (n=32)	<0.0001	<0.0001
IL-8 Fold Increase (n=129)	0.0006	0.0008
Neutrophil Binding (n=143)	<0.0001	<0.0001
Neutrophil CR1 Fold Increase (n=32)	0.2585	0.2085
Neutrophil CR3 Fold Increase (n=40)	0.0249	0.0249
Neutrophil CD88 Fold Decrease (n=32)	0.0074	0.0074
Neutrophil CD62L Fold Decrease (n=32)	0.0058	0.0058
Monocyte Binding (n=143)	<0.0001	<0.0001
Monocyte CR1 Fold Increase (n=36)	0.0023	0.0028
Monocyte CR3 Fold Increase (n=37)	0.0007	0.0007
Monocyte CD88 Fold Decrease (n=37)	0.0801	0.0801

¹ p values

² Neutrophil IgG anti- β -glucan antibody concentration cutoff of 51 μ g/ml and IgM cutoff of 118 μ g/ml

³ Monocyte IgG anti- β -glucan antibody concentration cutoff of 40 μ g/ml and IgM cutoff of 126 μ g/ml

Biomarker cutoffs were then applied to patients in two clinical trials studying treatment of lung cancer that included IMPRIME PGG. In the first randomized, phase 2 study, 59 stage IV NSCLC patients received cetuximab, carboplatin, and paclitaxel without (Control) or with IMPRIME PGG 4 mg/kg on Days 1, 8 and 15 of each 3-week treatment cycle for the first 4 to 6 cycles. Maintenance treatment with cetuximab alone or in combination with IMPRIME PGG was continued until disease progression. Cutoffs at various points within the ranges established by healthy volunteer data and functional data above were investigated. The results are shown in Table 5.

Table 5

IgG/IgM cutoffs	No. of Biomarker+	Median Overall Survival (months)		Survival Difference
		Biomarker+	Biomarker-	
IgG: 51 µg/ml IgM: 118 µg/ml	25	429	261	168
IgG: 30 µg/ml IgM: 60 µg/ml	41	289	162	127
IgG: 40 µg/ml IgM: 80 µg/ml	32	373	217.5	155.5
IgG: 60 µg/ml IgM: 130 µg/ml	23	378	244	134

Median Overall Survival (OS) was determined by investigators at the clinical trial sites. For some patients, biomarker status changes during the course of treatment. Therefore, biomarker positive status was given to patients that measured above the cutoff at either the first or second cycles.

As an example, using a cutoff of 51 µg/ml IgG anti-β-glucan antibody concentration, 118 µg/ml IgM anti-β-glucan antibody concentration 25 patients are biomarker positive. Biomarker-positive patients had a median overall survival of 429 days compared to 261 days for biomarker-

negative patients. Thus, the survival difference between the two groups is 168 days, which is a significant difference. Of the examples given here, that cutoff produces the best separation in this clinical trial. The remaining example cutoffs produce smaller median overall survival differences between the two groups. However, in some cases, it may be ideal to have more patients treated by lowering the cutoff. And as discussed above, using ROC curve analysis, the specificity and sensitivity can be changed as needed for customizing patient separation.

In the second randomized, phase 2 study, 58 stage IV NSCLC patients received bevacizumab, carboplatin, and paclitaxel without (Control) or with IMPRIME PGG 4 mg/kg on a similar treatment cycle as the above study. Maintenance treatment with bevacizumab alone or in combination with IMPRIME PGG was continued until disease progression. Cutoffs at various points within the ranges established by healthy volunteer data and functional data above were investigated. The results are shown in Table 6.

Table 6

IgG/IgM cutoffs	No. of Biomarker+	Median Overall Survival (months)		Survival Difference
		Biomarker+	Biomarker-	
IgG: 60 µg/ml IgM: 100 µg/ml	18	553	357	196
IgG: 38 µg/ml IgM: 60 µg/ml	26	474	437.5	36.5
IgG: 80 µg/ml IgM: 120 µg/ml	16	419	483	-64
IgG: 50 µg/ml IgM: 80 µg/ml	25	474	437.5	36.6

For this clinical trial, the optimal cutoff occurs at 60 µg/ml IgG anti-β-glucan antibody concentration and 100 µg/ml IgM anti-β-glucan antibody concentration. As shown by the remaining examples, the cutoffs on either side of that cutoff quickly break down and do not provide practical differences.

Again, depending on the specificity and sensitivity desired (calculated by ROC curve analysis), one can select an appropriate cutoff to achieve stratification of patients that respond to IMPRIME PGG treatment without supplementation with anti- β -glucan antibodies.

Separation of patients based on biomarker status (high binder vs. low binder) can be accomplished using cutoffs within the ranges set forth above. Thus, biomarker status can be used as predictor of successful β -glucan immunotherapy.

As noted above, the biomarker status of some patients can change over the course of therapy. We therefore evaluated cutoff values for patients who exhibited biomarker-positive status after one cycle of therapy versus patients who exhibited biomarker-positive status after any cycle—after any one of C1/C2/C3—of therapy.

Table 7 Overall Survival of biomarker-positive patients exhibiting biomarker-positive status after one cycle of chemotherapy

IgG/IgM Cutoffs (μ g/ml)	Median OS (bevacizumab)		Median OS (cetuximab)	
	BM +	BM -	BM+	BM-
34/146 (235/330 RAU)	401	471.5	283	275
35/100 (242/240 RAU)	478	350	308	263
40/100 (276/240 RAU)	482	343	373	247.5
45/100 (311/240 RAU)	514	338.5	375.5	251
50/100 (345/240 RAU)	514	338.5	375.5	251
55/100 (380/240 RAU)	546	343	373	254.5
60/100 (414/240 RAU)	553	338.5	308	266.5
35/110 (242/264 RAU)	478	350	308	263
40/110 (276/264 RAU)	482	343	378	247.5
45/110 (311/264 RAU)	514	338.5	387.5	251
50/110 (345/264 RAU)	482	343	387.5	251
55/110 (380/264 RAU)	514	350	378	254.5
60/110 (414/264 RAU)	546	343	308	266.5
35/120 (242/288 RAU)	456	374.5	308	263
40/120 (276/288 RAU)	474	357	378	247.5
45/120 (311/288 RAU)	478	350	387.5	251
50/120 (345/288 RAU)	474	357	387.5	251
55/120 (380/288 RAU)	474	364	378	254.5
60/120 (414/288 RAU)	478	360.5	308	266.5

As an example, using a cutoff of 60 µg/ml IgG anti-β-glucan antibody concentration, 100 µg/ml IgM anti-β-glucan antibody concentration to separate biomarker-positive from biomarker-negative patients, biomarker-positive patients receiving bevacizumab therapy had a median overall survival of more than 200 days more than biomarker-negative patients using the same cutoff values. Similarly, using a cutoff of 45 µg/ml IgG anti-β-glucan antibody concentration, 110 µg/ml IgM anti-β-glucan antibody concentration, biomarker-positive patients receiving cetuximab therapy had a median overall survival of more than 130 days more than biomarker-negative patients using the same cutoff values. As described above with respect to the data presented in Table 5 and Table 6, one can select an appropriate cutoff value for a desired combination of specificity and sensitivity, as needed for customizing patient separation, using ROC curve analysis.

Table 8 Overall Survival of biomarker-positive patients exhibiting biomarker-positive status after any cycle of chemotherapy

IgG/IgM Cutoffs (µg/ml)	Median OS (bevacizumab)		Median OS (cetuximab)	
	BM +	BM -	BM+	BM-
34/146 (235/330 RAU)	438	551	308	244
35/100 (242/240 RAU)	478	499	298.5	244
40/100 (276/240 RAU)	482	483	328	217.5
45/100 (311/240 RAU)	514	437.5	378	244
50/100 (345/240 RAU)	514	437.5	378	244
55/100 (380/240 RAU)	514	437.5	375.5	244
60/100 (414/240 RAU)	546	392	373	247.5
35/110 (242/264 RAU)	478	499	308	244
40/110 (276/264 RAU)	482	483	328	217.5
45/110 (311/264 RAU)	514	437.5	397	244
50/110 (345/264 RAU)	482	483	397	244
55/110 (380/264 RAU)	482	483	387.5	244
60/110 (414/264 RAU)	514	437.5	378	247.5
35/120 (242/288 RAU)	456	533	308	244
40/120 (276/288 RAU)	474	515	328	217.5
45/120 (311/288 RAU)	478	499	397	244
50/120 (345/288 RAU)	474	515	397	244
55/120 (380/288 RAU)	456	525.5	387.5	244
60/120 (414/288 RAU)	474	515	378	247.5

Here again, one can select an appropriate IgG and IgM cutoff value to maximize survival of biomarker-positive patients. For example, using a cutoff of 60 $\mu\text{g/ml}$ IgG anti- β -glucan antibody concentration, 100 $\mu\text{g/ml}$ IgM anti- β -glucan antibody concentration, biomarker-positive patients receiving bevacizumab therapy had a median overall survival of more than 150 days more than biomarker-negative patients using the same cutoff values. Using a cutoff either 45 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$ IgG anti- β -glucan antibody concentration, and either 110 $\mu\text{g/ml}$ or 120 $\mu\text{g/ml}$ IgM anti- β -glucan antibody concentration, biomarker-positive patients receiving bevacizumab therapy had a median overall survival of more than 150 days more than biomarker-negative patients using the same cutoff values.

In some clinical situations, however, overall survival may not necessarily be the most relevant clinical endpoint. In some cases, overall response rate (ORR) may be more relevant. As used herein, "overall response rate" refers to the percentage of patients that exhibit a measurable reduction in the size and/or proliferation of cancer after treatment. The data in Table 9 demonstrate that IgG and IgM cutoff values may be determined using ORR as the clinical endpoint. Table 9 reports combined data from the bevacizumab and cetuximab studies described above, reflecting mean overall survival and overall response rate as endpoints.

Table 9

IgG/IgM Cutoffs ($\mu\text{g/ml}$)	Median OS			%ORR		
	Bio+	Bio-	Diff.	Bio+	Bio-	Diff.
35/100	368.5	294	74.5	57.14%	40.38%	16.76%
40/100	387.5	286	101.5	59.57%	38.89%	20.69%
45/100	438	283.5	154.5	63.41%	38.33%	25.08%
50/100	438	283.5	154.5	63.41%	38.33%	25.08%
55/100	418	286	132	65.00%	37.70%	27.30%
60/100	446	286	160	63.16%	39.68%	23.48%
65/100	425.5	292	133.5	61.11%	41.54%	19.57%
35/110	373	293	80	57.14%	40.38%	16.76%
40/110	397.5	286	111.5	58.70%	40.00%	18.70%
45/110	454	283.5	170.5	62.50%	39.34%	23.16%
50/110	446	286	160	61.54%	40.32%	21.22%
55/110	438	289	149	63.16%	39.68%	23.48%
60/110	454	289	165	61.11%	41.54%	19.57%
65/110	454	293	161	58.82%	43.28%	15.54%

35/120	364	305	59	57.45%	40.74%	16.71%
40/120	387.5	292	95.5	59.09%	40.35%	18.74%
45/120	438	289	149	63.16%	39.68%	23.48%
50/120	418	292	126	62.16%	40.63%	21.54%
55/120	397.5	294	103.5	65.71%	39.39%	26.32%
60/120	417.5	294	123.5	63.64%	41.18%	22.46%
65/120	387.5	316	71.5	61.29%	42.86%	18.43%

Table 9 shows that the IgG/IgM cutoff values most effective for separating biomarker-positive from biomarker-negative patients may differ somewhat depending upon whether one uses overall survival or overall response rate as the clinical endpoint for making the separation. Thus, regardless of the clinical endpoint that is most relevant for the treatment of a given biomarker-positive patient, the methods described herein can provide IgG/IgM cutoff values for identifying biomarker-positive patients.

Once we established that biomarker status could be used as a predictor of successful β -glucan immunotherapy, we then looked at whether IgG subclasses played a role. IMPRIME PGG is a carbohydrate, and human IgG responses to carbohydrate antigens are primarily restricted to the IgG₂ subclass. IgG₂ is a poor complement activator and activates the classical pathway of complement activation only at antigen-antibody equivalence or when antibody is in excess. IMPRIME PGG has been shown to activate complement (C4a, C5a, SC5b9), but it does not cause cell lysis due to MAC formation on the cell surface. In addition, IMPRIME PGG, in the majority of the donors exhibits bell-shaped concentration – response curves. Binding, complement activation, and IL-8 production are optimal at 10 or 25 μ g/ml but lower at 100 μ g/ml. In some cases, this may be due to antigen-antibody being at equivalence or antibody being in excess at 10 μ g/ml or 25 μ g/ml, while the antigen is in excess at 100 μ g/ml.

To understand IgG subclasses as they relate to IgG anti- β -glucan antibodies, neutrophil binding was performed using secondary antibodies specific to each subclass of IgG anti- β -glucan antibodies. First, IgG subclasses from high binder and low binder serums were tested for binding to neutrophils. The results are shown in FIG. 29. As is evident from the results, IgG₂ subclass showed the strongest correlation to biomarker status. This finding was verified in the plots generated in FIG. 30. Neutrophil binding of IgG₂ anti- β -glucan antibodies from high binder serum produced a much stronger correlation than with IgG₁.

Therefore, in another embodiment, IgG₂ anti- β -glucan antibodies may be used as the predictive biomarker for IMPRIME PGG immunotherapy. In addition, for patients that are low binders, administration of IgG₂ anti- β -glucan may be used to improve their response to IMPRIME PGG immunotherapy.

Lastly, it was shown that *in vivo* infusion of intravenous immunoglobulin (IVIG) increases anti- β -glucan antibody levels and improves response to IMPRIME PGG treatment. A 54-year old patient undergoing combination treatment of weekly infusions of cetuximab and IMPRIME PGG for colorectal adenocarcinoma had low anti- β -glucan antibody levels measured in pre-treatment serum samples. In order to increase the patient's anti- β -glucan antibody levels, IVIG (1g/kg) was infused on the first day of a 28-day treatment cycle starting with cycle 7 and continuing until cycle 12. As shown in FIG. 31A, post treatment serum samples were analyzed for IgG anti- β -glucan antibody levels in RAU/ml by ELISA. Addition of IVIG increased the anti- β -glucan antibody concentration in the Day 1 sample of each cycle and then declined to baseline levels during the remaining weeks in each cycle. In FIG. 31B, *in vivo* binding of IMPRIME PGG binding to PMNs and monocytes was analyzed by FACS and calculated by the increase of IMPRIME PGG+ PMNs or monocytes in a post-IMPRIME PGG dose whole blood sample in comparison to a pre-dose sample. Binding varied with the level of anti- β -glucan antibody concentration measured with the highest binding for both PMNs and monocytes on days where the patient received an infusion of IVIG. In FIG. 31C, complement activation was measured by the fold increase of C5a as measured by ELISA in a post-IMPRIME PGG dose serum in comparison to a pre-dose sample. A two-fold increase in C5a level was only observed in patient samples corresponding with IVIG infusion.

The method described herein can identify individuals who are most likely to benefit from a therapeutic regimen that includes administration of β -glucan such as, for example, IMPRIME PGG. Thus, the assay can allow medical professionals to better tailor therapeutic treatments to an individual based, at least in part, on the subject's likelihood of responding to therapy that includes β -glucan. The assay may be used to screen individuals for inclusion in clinical studies to better define various subject populations.

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

Costar universal binding plates were coated with 50 μ L per well with IMPRIME PGG (Biothera, Eagan, MN) at 1 μ g/mL in purified water and incubated at 37°C for 30 minutes. The coated plate was then exposed to high intensity ultraviolet light at $>1500 \mu\text{W}/\text{cm}^2$ for 5 minutes at room temperature and placed in a 50°C forced air oven until dry before a second exposure to ultraviolet light for five minutes at room temperature. The plate was then blocked with 0.5% solution of bovine serum albumin (BSA) for greater than 30 minutes before washing with wash buffer (phosphate buffered saline [PBS] with 0.05% Tween-20). Each assay run included two assay plates. Each plate included a calibration curve made by serially diluting the reference human serum. The plates also included four test serum samples diluted identically as the reference serum. The dilution series for the calibration curve and the serum samples started with a 1:400 dilution and continued with serial 1:2 dilutions to the lowest dilution of 1:51,200. The dilutions were made in wash buffer. A dilution of 1:50 of the reference serum was used as the

highest anchor point on the curve. Each dilution of the reference and serum samples was evaluated in replicate wells on each of the two plates.

The samples were incubated on the assay plate at room temperature for 90 minutes to permit human IgG to bind to the plate-bound IMPRIME PGG. 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 antibody) was incubated in the wells to bind with human IgG bound to IMPRIME PGG antigen. The secondary antibody was allowed to incubate for 90 minutes before washing with wash buffer. After the wash buffer was removed from the wells, a peroxidase substrate was incubated in the wells and color development was quenched with ~1M phosphoric acid at 5 minutes color development. The optical density at 450 nm was measured using a microtiter plate reader and means from replicate wells calculated.

The results were computed in two ways:

1) Titer: The titer was determined as the greatest dilution factor of sample with an optical density reading greater than or equal to 0.1 OD.

2) RAU - An arbitrary value of 160 was assigned to the reference serum (160 Relative Antibody Units (RAU/mL). Thus a 1:400 dilution in the assay method results in a value of 400 mRAU/mL as the highest point on the calibration curve. The dilution values and the corresponding RAU values are stated in Table 10.

Table 10

<u>Dilution</u>	<u>Mean OD</u>	<u>Calculated Conc.</u> <u>(mRAU/ml)</u>	<u>Calc. Conc. ×</u> <u>Dil</u>	<u>Divide by 1000 =</u> <u>RAU/ml</u>
400	1.577	404.6		
800	1.493	322.2	257749	258
1600	1.369	239.3	382912	383
3200	1.031	119.9	383683	384
6400	0.715	64.3	411398	411
12800	0.393	29.5	378022	378
25600	0.204	14.7	377574	378
51200	0.105	8.0		

RAU values for samples and controls were calculated with a 4-parameter fit using the reference human serum dilution series as the calibration curve. The concentration in mRAU for each sample dilution that falls within the linear portion of the calibration curve was computed by interpolation from the calibration curve, followed by correction for dilution. Subsequently, an average concentration of each sample was arrived at from those back calculated multiple dilutions.

Results are shown in FIG. 1A.

Example 2

IgG and IgM anti- β -glucan antibody “gold reference standards” were purified from commercially available, 95% pure total IgG and IgM fractions derived from pooled normal human plasma (Athens Research and Technology, Athens, GA). The IgG and IgM fractions were passed over IMPRIME PGG affinity columns, concentrated, and characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purity. See FIG. 22. Serial dilutions of the “gold reference standards” were used to generate calibration curves by the method described below for generating standard curves.

Assay working reference standards were prepared from pooled normal human serum with anti- β -glucan concentrations determined by calibration against the “gold reference standards.” Assay controls were prepared from normal human serum from selected individual subjects with anti- β -glucan concentrations near defined locations on the working standard curves.

MAXISORP flat bottom 96-well plates (Thermo Fisher Scientific, Waltham, MA) are coated with 100 μ L per well of IMPRIME PGG at 3 μ g/mL in D-PBS (Corning Inc., Tewksbury, MA). The plates are covered, placed in a tightly sealed zip closure plastic bag and incubated at 4°C for a minimum of 15 hours and a maximum of 24 hours. The coated plates are then removed from refrigeration, aspirated and washed three times with wash buffer (0.05% Polysorbate 20 (Alfa Aesar, Ward Hill, MA) in PBS). Following the final aspiration, each coated plate is completely wrapped in a paper towel and tapped hard three times on a cushion of paper towels to remove the remaining liquid. The plate is then blocked with 250 μ L per well of StabilCoat Immunoassay Stabilizer (SurModics, Inc., Eden Prairie, MN) for 1 to 3 hours on the bench top. After blocking, the plates are aspirated to remove the contents and wrapped in a paper towel. The

tapping procedure to remove the remaining liquid, as described above, is repeated. The plates are uncovered and allowed to dry for a minimum of 45 minutes and up to three hours on the bench top.

Each assay included a working standard curve made by serially diluting a working reference sample. The dilution series of the working reference samples are shown in Tables 11 and 12. The assay also included control and test samples diluted 1:20, 1:400 and 1:1600. The dilutions were made in wash buffer. Each dilution of the reference and serum samples was evaluated in triplicate.

The samples were incubated on the assay plate at room temperature for 45 minutes on an orbital shaker with an orbital diameter of 2 mm set to 310 rpm to permit human IgG and/or IgM to bind to the plate-bound IMPRIME PGG. Following incubation, the wells were washed with wash buffer and an enzyme labeled secondary antibody (horseradish peroxidase conjugated affinity purified goat anti-human IgG or IgM, Fc gamma specific antibody) was incubated in the wells to bind with human IgG or IgM bound to IMPRIME PGG antigen. The secondary antibody was allowed to incubate for 45 minutes before washing with wash buffer. After the wash buffer was removed from the wells, a peroxidase substrate was incubated in the wells and color development was quenched with ~1M phosphoric acid at five minutes color development. The optical density at 450 nm was measured using a microtiter plate reader and means from replicate wells calculated.

IgG and IgM anti- β -glucan antibody concentrations ($\mu\text{g/ml}$) were determined by correlating the sample absorbance against the working standard curve generated from the working reference sample dilutions. The dilution values and the corresponding concentrations for a calibration curve are stated in Tables 11 and 12.

Table 11. IgG Dilutions for Standard Curve

<u>Dilution</u>	<u>Mean OD</u>	Mean Calculated Conc. (<u>ng/ml</u>)
1:10	2.859	42073.3
1:100	2.626	191.0
1:400	2.214	58.2
1:800	1.773	29.1

1:1600	1.161	13.7
1:3200	0.674	7.1
1:12800	0.202	2.0

Table 12. IgM Dilutions for Standard Curve

<u>Dilution</u>	<u>Mean OD</u>	<u>Mean Calculated Conc. (ng/ml)</u>
1:6.26	2.642	11176.3
1:50.08	2.133	1354.7
1:100.17	1.706	664.4
1:200.33	1.227	338.5
1:801.38	0.454	83.7
1:3204.76	0.158	20.8
1:12819	0.079	5.2

Standard curves derived from the data of Tables 11 and 12 are shown in FIGS. 1B and 1C, respectively. Once the antibody concentration of the test samples is determined by the standard curve, the concentrations can be converted to $\mu\text{g/ml}$ by multiplying the mean calculated concentration by the dilution factor and then divided by 1000.

Example 3

Whole blood (WB) was collected from healthy volunteers in heparin containing tubes (BD VACUTAINER sodium heparin tubes, Becton Dickinson, Franklin Lake, NJ). The IVIG used in the study was Privigen, a 10% solution of human polyvalent human immunoglobulin at 100 mg/mL (CSL Behring, King of Prussia, PA). Samples were spiked with dilutions of IVIG in PBS to final concentrations of 2.5 mg/mL, 5 mg/mL, and 10 mg/mL along with a PBS only control. Aliquots of the IVIG spiked blood were then made for IgG RAU assays, and IMPRIME PGG induction of complement pathway activation, neutrophil binding (described below in Example 4), activation marker expression, and IL-8 production assays.

Aliquots of the IVIG spiked WB were incubated with IMPRIME PGG at 10 $\mu\text{g/mL}$ or equivalent volume of citrate buffer (11 mM NaCitrate, 140 mM NaCl, pH 6.3) as a control for

30 minutes at 37°C. After incubation, cells were washed twice with PBS before adding 5 µL of BfD IV mouse anti-beta glucan IgM antibody per 100 µL of WB and incubating at room temperature for 15 minutes. The samples were then washed twice again with PBS and stained with an antibody cocktail containing antibodies for surface markers as well as an anti-mouse IgM for detection of BfD IV binding. Cells incubated 30 minutes at room temperature before adding 2 mL of FACS/Lyse (eBiosciences, San Diego, CA) and incubating at room temperature for 15 minutes. The cells were then washed two times with PBS before fixing with 1% paraformaldehyde and analyzing on LSRII flow cytometer. FACS data analyzed by FlowJo software.

Results are shown in FIGS. 2-7.

Example 4

Binding of IMPRIME PGG in WB and the detection of glucan binding on cell surface were performed essentially as described above in Example 2.

Results are shown in FIGS. 8-13, 23-30, 31A and 31B.

Example 5

Whole blood (WB) was collected from healthy volunteers in heparin containing tubes as described in Example 3. The tube was mixed well and stored on ice until ready to use. Aliquots of WB were incubated with IMPRIME PGG at a final concentration of 10 µg/mL or 100 µg/mL, or with equivalent volume of PBS or Citrate Buffer. Whole glucan particle (WGP) at 10 µg/mL was used as a positive control. Treated WB were incubated at 37°C in a humidified 5% CO₂ incubator for 30 minutes or 120 minutes. Immediately at the end of each time point, WB was centrifuged at 2000 *x rpm* (or 1150 *x g*) for 10 minutes at 4°C. The supernatant (plasma) was collected, and transferred to a 1.5 mL eppendorf tube and kept on ice. The plasma was used the same day in complement modulation study as described in the following section or was frozen at -70°C until ready to use.

Complement C4a modulation study. The MicroVue C4a EIA kit (Quidel Corp., San Diego, CA) was used for the quantitation of C4a in plasma according to the vendor's instruction. In brief, Standards, Controls, and 1:20 diluted test specimens (untreated or various treated plasma preparations) were added to microassay wells pre-coated with a specific anti-C4a

monoclonal antibody. After incubation at room temperature for 60 minutes, the plate was washed and a wash cycle. Horseradish peroxidase (HRP)-conjugated murine anti-human C4a monoclonal antibody was added to each test well and incubated for another 60 minutes at room temperature. Following incubation, the plate was washed and a wash cycle, before the addition of a chromogenic enzyme substrate TMB to initiate the enzymatic reaction. The plate was incubated at room temperature for 60 minutes and the enzyme reaction was subsequently quenched with the provided stop solution. The color intensity was measured spectrophotometrically at 450 nm. The concentration of C4a present in the test specimens were calculated from the standard curve generated with the provided standards, and analyzed using a 4-parameter regression analysis.

Results are shown in FIG. 14.

Complement C5a modulation study. The MicroVue C5a EIA kit (Quidel Corp., San Diego, CA) was used for the quantitation of C5a in plasma according to the vendor's instruction. In brief, Standards, Controls, and 1:60 diluted test specimens (untreated or various treated plasma preparations) were added to microassay wells pre-coated with a specific anti-C5a monoclonal antibody. After incubation at room temperature for 60 minutes, the plate was washed and a wash cycle. Horseradish peroxidase (HRP)-conjugated murine anti-human C5a monoclonal antibody was added to each test well and incubated for another 60 minutes at room temperature. Following incubation, the plate was washed and a wash cycle, before the addition of a chromogenic enzyme substrate TMB to initiate the enzymatic reaction. The plate was incubated at room temperature for 60 minutes and the enzyme reaction was subsequently quenched with the provided stop solution. The color intensity was measured spectrophotometrically at 450 nm. The concentration of C5a present in the test specimens were calculated from the standard curve generated with the provided standards, and analyzed using a 4-parameter regression analysis.

Results are shown in FIG. 15 and FIG. 31C.

Sc5b-9 modulation study. The MicroVue SC5b-9 Plus EIA kit (Quidel Corp., San Diego, CA) was used to measure the amount of the SC5b-9 complex present in plasma specimens according to the vendor's instruction. Briefly, Standards, Controls, and 1:20 diluted test specimens (untreated or various treated plasma preparations) were added to microassay wells pre-coated with a specific anti-SC5b-9 monoclonal antibody. The plate was incubated at room

temperature for 60 minutes followed by five washes. The plate was then incubated at room temperature for 30 minutes with the provided SC5b-9 Plus Conjugate that contained a horseradish peroxidase-conjugated murine anti-human Ab specific for SC5b-9. The plate was then washed five times, incubated with a chromogenic enzyme substrate TMB for 60 minutes at room temperature to initiate the enzymatic reaction and subsequently quenched with the stop solution. OD₄₅₀ was measured. Results were calculated from the generated standard curve using linear regression analysis.

Results are shown in FIG. 16.

Modulation of cell surface complement receptors. Binding of IMPRIME PGG to WB was studied at 10 µg/mL and 100 µg/mL, and at both 30 minutes and 120 minutes. After binding, cells were washed twice with PBS and then incubated with CD88-APC, CD35-PE, and CD11b-PB (BioLegend) for 30 minutes at room temperature. RBC were lysed by incubating with 2 mL of FACS/Lyse (eBiosciences, San Diego, CA) at room temperature for 15 minutes. Cells were washed twice with PBS, fixed with 1% paraformaldehyde and analyzed on LSRII flow cytometer. FACS data analyzed by FlowJo software.

Results are shown in FIG. 17 and FIG. 18.

Example 6

Binding of IMPRIME PGG to WB was studied at 10 µg/mL and 100 µg/mL, and at both 30 minutes and 120 minutes. After binding, cells were washed twice with PBS and then incubated with CD62L-PB (BioLegend) for 30 minutes at room temperature. RBC were lysed by incubating with 2 mL of FACS/Lyse (eBiosciences, San Diego, CA) at room temperature for 15 minutes. Cells were washed twice with PBS, fixed with 1% paraformaldehyde and analyzed on LSRII flow cytometer. FACS data analyzed by FlowJo software.

Results are shown in FIG. 19.

Example 7

Whole blood (WB) was collected from healthy volunteers in heparin containing tubes as described in Example 3. Aliquots of WB were incubated with IMPRIME PGG at final concentration of 10 µg/mL or 100 µg/mL, or treated with PBS or Citrate Buffer as a baseline control, or with 100 ng/mL of TLR4 agonist LPS (*E. coli* strain 0127:B8, Sigma, St. Louis, MO)

as a positive control. The cultures were incubated at 37°C in a humidified 5% CO₂ incubator. After 20-24 hours, the WB was centrifuged for 10 min at 1600 *x rpm*, and the plasma supernatant was collected. Samples were stored in a 96-well Matrix storage plate (Matrix Technologies, Hudson, NH) at -80°C until ready to use. The presence of IL-8 in the plasma samples of IMPRIME PGG-, or control-treated WB were determined by performing a Human CXCL8/IL-8 ELISA (R&D Systems, Catalog# D8000C, Minneapolis, MN) per the manufacturer's instructions.

Results are shown in FIG. 20.

Example 8

Sc5b-9 modulation study. The MicroVue SC5b-9 Plus EIA kit (Quidel Corp., San Diego, CA) was used to measure the amount of the SC5b-9 complex present in plasma specimens according to the vendor's instruction. Briefly, Standards, Controls, and 1:20 diluted test specimens (untreated or various treated plasma preparations) were added to microassay wells pre-coated with a specific anti-SC5b-9 monoclonal antibody. The plate was incubated at room temperature for 60 minutes followed by five washes. The plate was then incubated at room temperature for 30 minutes with the provided SC5b-9 Plus Conjugate that contained a horseradish peroxidase-conjugated murine anti-human Ab specific for SC5b-9. The plate was then washed five times, incubated with a chromogenic enzyme substrate TMB for 60 minutes at room temperature to initiate the enzymatic reaction and subsequently quenched with the stop solution. OD₄₅₀ was measured.

Results were calculated from the generated standard curve using liner regression analysis. Results are shown in FIG. 21.

Example 9

ROC curve analysis was carried out using GraphPad Prism software. Results are shown in FIGS. 27 and 28.

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 comprising:
 - analyzing a biological sample obtained from a subject for a biomarker anti- β -glucan antibody compared to a reference standard;
 - computing a Relative Antibody Unit (RAU) value for anti- β -glucan antibody in the sample; and
 - identifying the subject as biomarker negative for β -glucan immunotherapy if the RAU value is less than a predetermined RAU value for the biomarker anti- β -glucan antibody, wherein the predetermined RAU value is selected based on a correlation of specificity and sensitivity to at least one endpoint that stratifies biomarker-positive subjects and biomarker-negative subjects, and
 - wherein anti- β -glucan IgG₂ is to be administered to the biomarker-negative subject.
2. A method comprising:
 - analyzing a biological sample obtained from a subject for a biomarker anti- β -glucan antibody compared to a reference standard;
 - identifying the subject as biomarker negative for β -glucan immunotherapy if the sample contains an amount of the anti- β -glucan antibody that is less than a predetermined cutoff value for the biomarker anti- β -glucan antibody, wherein the predetermined cutoff value is selected based on a correlation of specificity and sensitivity to at least one endpoint that stratifies biomarker-positive subjects from biomarker-negative subjects, and
 - wherein anti- β -glucan IgG₂ is to be administered to the biomarker-negative subject.
3. The method of claim 1 wherein the biomarker anti- β -glucan antibody comprises IgG.
4. The method of claim 1 wherein the biomarker anti- β -glucan antibody comprises IgG and the predetermined RAU value is 200.
5. The method of claim 1 wherein the biomarker anti- β -glucan antibody comprises IgM.

6. The method of claim 1 wherein the biomarker anti- β -glucan antibody comprises IgM and the predetermined RAU value is 300.

7. The method of claim 1 wherein the β -glucan is derived from yeast.
8. The method of claim 1 wherein the β -glucan comprises a β -1,3/1,6 glucan.
- 5 9. The method of claim 1 wherein the β -glucan comprises $\beta(1,6)$ -[poly-(1,3)-D-glucopyranosyl]-poly- $\beta(1,3)$ -D-glucopyranose.
- 10 10. The method of claim 1 wherein the sample is analyzed for a biomarker anti- β -glucan antibody using an enzyme-linked immunosorbent assay (ELISA).
11. The method of claim 1 wherein the endpoint comprises clinical outcomes, binding to immune cells, activation of complement components, modulation of immune cell surface marker expression or induction of cytokines.
- 15 12. The method of claim 1 wherein the predetermined cutoff value is about 20 $\mu\text{g/ml}$ anti- β -glucan IgG.
13. The method of claim 2 wherein the biomarker anti- β -glucan antibody comprises IgG.
- 20 14. The method of claim 2 wherein the biomarker anti- β -glucan antibody comprises IgM.
15. The method of claim 2 wherein the β -glucan is derived from yeast.
- 25 16. The method of claim 2 wherein the β -glucan comprises a β -1,3/1,6 glucan.
17. The method of claim 2 wherein the β -glucan comprises $\beta(1,6)$ -[poly-(1,3)-D-glucopyranosyl]-poly- $\beta(1,3)$ -D-glucopyranose.
- 30 18. The method of claim 2 wherein the sample is analyzed for a biomarker anti β -glucan antibody using an enzyme-linked immunosorbent assay (ELISA).

19. The method of claim 2 wherein the endpoint comprises clinical outcomes, binding to immune cells, activation of complement components, modulation of immune cell surface marker expression or induction of cytokines.

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20. The method of claim 2 wherein the predetermined cutoff value is about 20 µg/ml anti-β-glucan IgG.