Orally administered β-glucan attenuates the Th2 response in a model of airway hypersensitivity.

Running Title: β-glucan attenuates allergic Th2 response.

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Abstract

β-glucan is a polysaccharide that is extracted from fungal cell walls. Wellmune WGP®, a preparation of β-1,3/1,6-glucans, is a dietary supplement that has immunomodulating properties. Here we asked what affect WGP had on a mouse model of asthma. OVA-induced asthma in mice is characterized by infiltration of eosinophils into the lung, production of Th2 cytokines and IgE production. WGP (400μg) or PBS was orally administered to OVA-challenged mice daily throughout the experiment. Administration of WGP significantly reduced the influx of eosinophils into the lungs of OVA-challenged mice compared to control mice. In addition, daily administration of WGP inhibited pulmonary production of Th2 cytokines (IL-4, IL-5, IL13), however serum IgE levels in the bronchoalveolar lavage fluid (BALF) were unaffected by WGP treatment. These data indicate that WGP could be useful as an oral supplement for some asthma patients, however, it would need to be combined with other therapies that target other aspects of the disease such as IgE levels. Additionally, WGP would need to be studied in combination with other therapies before administration to patients.
**Introduction**

Asthma is a chronic inflammatory airway disease that is caused by an allergic response in many cases. It affects 5-10% of the population and is associated with significant morbidity and mortality (1). It is characterized by airway inflammation, airway hyperresponsiveness (AHR) and mucus production (2). Lung inflammation is caused by infiltration of eosinophils and T cells secreting Th2-cytokines (IL-4, IL-5, IL-13) into the lung. IL-4, IL-5 and IL-13 are important for IgE production, eosinophil recruitment and survival, mucus secretion and AHR development (3). In addition to eosinophilic Th2-associated asthma, other asthma phenotypes occur such as neutrophilic non-Th2 associated asthma. Currently the main treatment for asthma is corticosteroid administration, however some asthma phenotypes are refractory to corticosteroid therapy (4). There is currently a significant level of research into the development of immunotherapies for asthma. These involve the targeting of IgE or cytokines such as IL-4, IL-5, IL-13 or others. While many of these therapies are ineffective individually, researchers are currently examining the potential for combination therapies (5).

β-glucan, a fungal cell wall extract, is marketed as a dietary supplement that can promote immune balance (6). β-glucans are found in the cell walls of fungi, plants and some bacteria. β-D-glucans or β-1,6-glucans are recognized by the Dectin-1 receptor. Dectin 1 is a type II transmembrane C-type lectin-like receptor, which recognizes both soluble and particulate β-glucans (7-9). While Dectin-1 can bind both soluble and particulate β-glucans, only particulate β-glucans activate Dectin-1 signaling
and responses (10). β-glucan-induced signaling through Dectin-1 promotes Th1 and Th17 responses (11).

β-glucan has been shown to exert beneficial therapeutic effects against various diseases. It has been shown to stimulate tumoricidal activity when co-administered with anti-tumor antibodies (12-17). However, divergent results regarding the effect of β-glucan on respiratory health have been reported. It has been suggested that exposure to microbial products or mould can adversely affect the development of asthma (18), however recent data suggest that β-glucans and other microbial signals may play a protective role for the development of asthma (19). β-glucan administration has been shown to prevent/improve symptoms of allergic rhinitis and upper respiratory tract infections (20, 21). However, there is minimal data on the effect of β-glucan on asthma severity (22). In this study, we therefore examined the effect of oral administration of particulate β-glucan (WGP) in a mouse model of ovalbumin (OVA)-induced asthma. Here we demonstrate that oral administration of WGP reduces eosinophil influx and the production of Th2 cytokines (IL-4, IL-5, IL-13) in the lungs of OVA-challenged mice.

**Material and Methods**

**Animals**

C57BL/6 mice were maintained under specific pathogen-free conditions at the NCI–Frederick, MD. Animal care was provided in accordance with the procedures in, “A Guide for the Care and Use of Laboratory Animals”. Ethical approval for the animal
experiments detailed in this manuscript was received from the Institutional Animal Care and Use Committee at the NCI-Frederick.

**Oral administration of PBS or WGP**

Mice were treated daily with 25-100μl PBS containing 400μg Wellmune WGP from Biothera (Eagen, MN) or PBS alone by oral gavage or alternatively by feeding by pipet as needed. Treatment was started seven days prior to beginning OVA sensitization, and was continued for the duration of the study.

**Sensitization and airway challenge**

Allergic airway hypersensitivity to ovalbumin (OVA) was induced using grade V chicken egg OVA (Sigma, St. Louis, MO). Sensitization was initiated by an intraperitoneal (i.p.) injection of 100μg OVA in 200μl of 11-13mg/ml aluminum hydroxide colloidal suspension (Alum), followed by a second i.p. injection two weeks later. Ten days after the last sensitization, airway hypersensitivity was induced by 20-minute nebulization sessions challenging with 1% (w/v) OVA in PBS for four consecutive days. Mice were sacrificed, and tissues harvested 24h after the last nebulization. A schematic of the treatment schedule is shown in Fig. 1.

**Bronchoalveolar lavage fluid collection**

Prior to BALF collection, mice were given a ketamine/xylazine mixture to induce deep anesthesia and analgesia. Once sedated, as determined by toe-pinches, the thoracic cavity was opened to reveal the lungs and trachea. Using a cannula and leur lock syringe, the
lungs were expanded using 1mL of PBS and recollected as BALF, for a total of 2-3 washes. The mice were euthanized by heart perfusion using 5-10mL PBS, and the lungs and other tissues were collected.

**BALF cytospin and differential cell count analysis**

Cells from the BAL were collected, and the BALF was saved for cytokine analysis. Red blood cell lysis was performed if necessary, cells were washed in PBS, and then counted using a hemacytometer. 100µL containing approximately 5x10⁴ cells were loaded onto a cytospin column and centrifuged onto a microscope slide. Following Diffquick cell staining, differential cell counts were blindly assessed for each sample, counting at least 500 cells per slide.

**BALF Assays**

BALF was clarified by centrifugation, aliquoted and stored at -80°C for later analysis. Cytokine levels were measured in BALF using ELISA kits from eBioscience (San Diego, CA) and R&D Systems (Minneapolis, MN). Myeloperoxidase (MPO) activity in BALF samples was measured using the Myeloperoxidase activity fluorometric assay kit from BioVision (Mountain View, CA). Eosinophil peroxidase (EPO) in BALF samples was measured using the Eosinophil peroxidase ELISA kit from antibodies-online.com (Atlanta, GA). Soluble collagen levels in BALF were measured using the previously described protocol in Current Protocols in Immunology. Briefly 200µl of 0.1% Direct Red 80 in picric acid (Sigma Aldrich, St. Louis, MO) was added to 50µl of lavage fluid and incubated for 60min at 37°C. The samples were centrifuged and the pellet was
washed with 100% ethanol. The pellet was resuspended in 200µl 0.5M NaOH, incubated for 30min at 37°C and the absorbance was read at 540nm.

**Lung and spleen cell isolation and flow cytometry**

Lung cells were isolated by collagenase and DNase digestion (Sigma Aldrich). Splenocytes were mashed through 100µm nylon cell strainers and erythrocytes were lysed in ACK buffer. Lung cells were washed with HBSS and splenocytes were washed with PBS. Lung cells and splenocytes were pre-incubated with 2.4G2 to block Fc receptor binding, followed by incubation with various cell surface Abs: CD3 (17-A2), NK1.1 (PK136), B220 (RA3-6B2), F4/80 (BM8), MHCII (M5/114.15.2), (eBioscience, San Diego, CA), Siglec F (E50-2440), CD11c (N418), CD11b (M1/70), Gr-1 (RB6-8C5) and Ly6G (1A8) (BD Biosciences, San Jose, CA). Cells were fixed with BD Cytofix and analysed on BD LSRII.

**Blood cell measurement**

Blood samples were taken prior to the start of the experiment, and again on the day of harvest. Cell composition was measured using the Hemavet 950 automated blood cell counter, (Drew Scientific, Waterbury, CT), according to the manufacturer’s protocol.

**RNA isolation and analysis**

A portion of the lung was excised and placed in Trizol for RNA isolation. The lung tissue in Trizol was homogenized using a mini-beadbeater. RNA was extracted using the Trizol protocol and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was
synthesized using Taqman Reverse Transcription Reagents for RT-PCR (Applied Biosystems). Quantitative RT-PCR was performed using ABI Taqman Primer and Probe sets and normalization was performed against Hprt1.

**Measurement of IgE**

Total IgE levels in serum were measured using the IgE ELISA from BD Biosciences (San Jose, CA). OVA specific IgE was measured using the Legend Max Mouse OVA Specific IgE ELISA kit from Biolegend (San Diego, CA).

**Lung Histology**

The lungs were placed in 10% formalin, embedded in paraffin wax blocks and 4μm sections cut and stained for either haematoxylin and eosin (H&E) or periodic acid Schiff (PAS) to determine airway inflammation and goblet cell metaplasia respectively. All histological sections were assessed blind by a pathologist and the total number of bronchiole and perivascular aggregates of inflammatory cells were counted in each lung. Mucous cell metaplasia within the bronchioles was compared to normal control tissue and graded as 0 = no difference, 1 = scattered mucous cells, 2 = aggregates of mucous cells, 3 = monolayer layer of mucous cells and 4 = multilayered mucous cells.

**Statistical analysis**

Data are expressed as means +/- s.e.m. Statistical significance was determined using Student’s t-test or 1-way ANOVA followed by Bonferroni’s post-test. Statistical significance was set at *p<0.05, **p<0.01, ***p<0.001.
Results

**WGP reduces total BALF cell numbers and eosinophil accumulation.** To examine the effect of orally administered WGP on allergic airway inflammation, we sensitized and challenged WT mice with OVA. Oral administration of WGP affected the composition of cells recovered from the BALF. The total number of cells, total number of eosinophils and the percentage of macrophages were significantly reduced while the percentage of neutrophils was increased in the WGP-treated mice (Fig. 2A-B). MPO activity and EPO levels correspond to the number of neutrophils and eosinophils, respectively in a sample. While the percentage of neutrophils was increased in WGP-treated mice, MPO activity in the BALF was similar between PBS and WGP-treated mice (Fig. 2C). This is likely due to the similar total number of neutrophils in the BALF from PBS and WGP-treated mice. EPO levels were reduced in WGP-treated mice (Fig. 2D), which is consistent with the reduced eosinophil numbers in the BALF from these mice. These data indicate that oral administration of WGP significantly inhibits the recruitment of inflammatory cells, in particular eosinophils and to a lesser extent macrophages, to the lungs in this model of allergic inflammation.

**WGP modulates lung cellular composition.** As we observed differences in BALF cellular composition, we examined the effect of WGP on lung cellular composition. Similar to our findings in BALF, eosinophils and macrophages (percentage and total cell number) were reduced, while the percentage of neutrophils was increased in the lungs of WGP-treated mice compared to PBS-treated mice (Fig. 3A-B). Taken together these data
indicate that WGP significantly inhibits the recruitment of eosinophils and macrophages to the lungs of these mice while it promotes a modest increase in neutrophil recruitment.

**WGP modulates systemic cellular composition.** As WGP was administered orally to the mice and modulated cellular composition in the lung, we next wanted to determine whether it had any systemic effect on cellular composition in the blood or other organs. Similar to the BALF and lung findings, WGP administration did not significantly affect the total number of neutrophils (Fig. 4A & C), however it modestly increased the percentage of neutrophils in both the spleen and blood (Fig. 4B &D). The WBC count was higher in WGP-treated mice, while a small decrease in the percentage of lymphocytes, corresponding to the increase in the percentage of neutrophils, was observed in these mice (Fig. 4C-D). Interestingly, similar total cell numbers/percentages of eosinophils and monocytes/macrophages were observed in the spleen and the blood. These data indicate that the increase in BALF/lung neutrophils is due to a systemic increase in neutrophils while the BALF/lung decrease in eosinophils and macrophages is localized suggesting reduced recruitment.

**WGP does not effect OVA-induced histopathological changes in the lung.** To determine the effect of WGP on OVA-induced histopathological changes in the lung, lung sections were stained with H&E and scored. Variable changes were seen in different areas of the lung with a mixed inflammatory cell infiltrate predominantly observed in peribronchial and perivascular areas in OVA-challenged mice; however, WGP had no effect on these changes (Fig. 5A-B, C-D & G). Similar features were seen in bronchioles
of both PBS and WGP-treated mice with folded epithelium and narrowing of lumens and no differences in goblet cell metaplasia or mucous production (Fig. 5C, F & H). Lung fibrosis/airway remodeling is characterized by enhanced collagen deposition (23). Soluble collagen levels in the lung were similar between PBS and WGP-treated mice (Fig. 5I), demonstrating that some aspects of the airway hyperresponsiveness model are unaffected by WGP treatment.

**WGP reduces Th2 cytokine levels in the lung.** Th2 cells secreting IL-4, IL-5 and IL-13 are important for eosinophil recruitment and survival and for IgE production (24). To further investigate the effect of WGP on allergic inflammation, cytokine levels (Th1, Th2 and Th17) in the lungs of PBS and OVA-challenged mice were measured. Consistent with previously published data from this model, OVA challenged mice displayed increased levels of these cytokines in the lungs compared to PBS challenged mice (data not shown). Interestingly, the levels of Il4 and Il5 mRNA, which are important for eosinophil recruitment, along with Il13, were reduced in the lungs of WGP-treated OVA-challenged mice (Fig. 6A). Th1 (Ifng) and Th17 (Il17) cytokine mRNA levels were similar between PBS and WGP-treated OVA-challenged mice. In addition Foxp3 mRNA levels, found in Tregs, were also measured and were similar between PBS and WGP-treated mice (Fig. 6A). Consistent with the mRNA data, IL-4, IL-5 and IL-13 protein levels were significantly reduced in WGP-treated mice while IFN-γ and IL-17 levels were mostly not detected (Fig. 6B and data not shown). As IgE production is an important aspect of the OVA-challenge asthma model, we next examined IgE production in the serum of these mice. Total IgE levels and OVA-specific IgE levels (Fig. 6C) were
similar between PBS and WGP-treated mice. Together, these data demonstrate reduced Th2 cytokine production in WGP-treated mice compared to PBS-treated mice while Th1 and Th17 cytokine levels are similar between the two groups.

**Discussion**

Several natural products are advertised as immunotherapeutics and many claims are made about their effects on a wide range of diseases including asthma. Here we tested the effect of Wellmune WGP β-1,3/1,6-glucan during a mouse model of OVA-induced asthma. We demonstrated that daily oral administration of WGP reduces eosinophil influx into the lungs and the production of Th2 cytokines (IL-4, IL-5, IL-13) compared to control mice. Serum IgE levels and OVA-specific IgE levels were unaffected by administration of WGP. Together these data indicate that WGP administration would not be sufficient as a single treatment for asthma however it may prove useful as a combination therapy.

Asthma is a heterogeneous disease and patients present with various distinct clinical phenotypes. Different mouse models are being developed to model the different phenotypes found in asthma patients (3, 25, 26). The OVA-induced airway hypersensitivity model of asthma used here results in eosinophilic inflammation in the lung, pulmonary production of Th2 cytokines (IL-4, IL-5 and IL-13) and serum IgE production (3, 26). The role of Th2 cells in patients with asthma is well established and T cell activation has been related to asthma severity in some studies (27, 28). IL-4, IL-5 and IL-13 have been shown to have roles in IgE production, eosinophil survival, AHR
development and tissue remodeling (29). Here we observed a significant reduction in production of Th2 cytokines and eosinophil influx, however IgE production and soluble collagen levels were unaffected by the administration of WGP. Other factors in addition to Th2 cells/cytokines are involved in the pathogenesis of asthma (3) however it appears that WGP is specific in targeting the production of IL-4, IL-5, IL-13 and resulting eosinophilia in this model while it has no effect against other aspects of the disease.

β-glucan has immunomodulating properties and it has been shown to exert beneficial therapeutic effects against various diseases including allergic diseases. One study demonstrated that administration of WGP to ragweed allergy sufferers reduced allergy symptoms however similar to our findings it had no effect on serum IgE levels (30). Another study showed that β-glucan administration to subjects with seasonal allergic rhinitis resulted in reduced IL-4 and IL-5 levels (31). Our data demonstrating reduced IL-4 and IL-5 levels in WGP-treated mice in an OVA-induced asthma model is in agreement with these findings. In addition a study demonstrated a reduction in asthmatic symptoms following β-glucan administration and increased IL-10 levels (22). Our findings suggest that WGP could be used to target some aspects of asthma (Th2 cytokines and eosinophilia). While WGP alone would not be sufficient to treat asthma, it could be used in combination with other therapies that target different aspects of the disease such as IgE levels. As WGP is an oral supplement, it could be considerably more cost effective and may have fewer side effects than immunotherapies. In addition, as IL-4, IL-5 and IL-13 are all reduced in response to WGP, the broader effects of WGP could
be more beneficial than immunotherapies that target one specific cytokine such as IL-4 or IL-5. Further studies would need to be conducted to determine the effect of WGP in combination with other therapies for specific subsets of asthma patients.

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Figure Legends

**Figure 1.** Schematic for induction of airway inflammation and treatment with WGP. PBS or 400μg WGP in PBS was orally administered to mice daily via oral gavage or pipette feeding. Oral administration of PBS or WGP began 7 days prior to sensitization with ovalbumin (OVA)/Alum and continued daily for the duration of the experiment. Mice were sensitized via i.p. injection of 100μg OVA in 1.8-2.6mg Alum on Days 0 and 14. Mice were challenged with OVA (1% w/v in PBS) for 20min using a nebulizer on Days 24, 25, 26 and 27. Mice were euthanized 24h following the last challenge.

**Figure 2.** Effect of oral administration of WGP on BALF cells in OVA-challenged mice. Cell numbers (A) and percentages (B) in BALFs from OVA-challenged mice fed with PBS or WGP. Graphs display mean +/- s.e.m. from 4 independent experiments,
n=19/group. (C) MPO Activity in BALF. Graph displays mean +/- s.e.m. from 5 independent experiments (n=21-22/group). (D) EPO levels in BALF. Graph displays mean +/- s.e.m. from 3 independent experiments (n=15/group).

**Figure 3.** Effect of oral administration of WGP on lung cellular composition in OVA-challenged mice. Cell numbers (A) and percentages (B) in lungs from OVA-challenged mice fed with PBS or WGP. Graphs display mean +/- s.e.m. from 3 independent experiments, n=13-14/group.

**Figure 4.** Effect of oral administration of WGP on spleen and blood cellular composition in OVA-challenged mice. Cell numbers (A) and percentages (B) in spleens from OVA-challenged mice fed with PBS or WGP. Graph displays mean +/- s.e.m. from 3-4 independent experiments, n=14-19/group. Cell numbers (C) and percentages (D) in blood from OVA-challenged mice fed with PBS or WGP. Graph displays mean +/- s.e.m. from 2 independent experiments, n=8-10/group.

**Figure 5.** Effect of oral administration of WGP on lung histology and soluble collagen. (A-F) Representative sections from both treatment groups demonstrate the varied morphological changes seen within the lung. H&E sections from a representative mouse treated with PBS (A - B) demonstrate more prominent epithelial infolding and lumen reduction related to mucous cell metaplasia. The surrounding tissue shows peribronchiolar and perivascular inflammatory infiltrates comprising lymphocytes with scattered eosinophils. Similar features were seen in a representative mouse treated with WGP (D -
E) although in this section the changes within the bronchiole are subtle and the lymphocytic infiltrate denser. PAS stains from different areas reveal mucous cell metaplasia in each group (C and F). (G-H) Graphs display mean +/- s.e.m. Inflammatory Focus Score (G) and Mucus Cell Score (H). Graphs are the cumulative result of 2 independent experiments (n=10). (I) Graph displays mean +/- s.e.m. of soluble collagen levels in BALF. Graph is the cumulative result of 5 independent experiments (n=22-23).

**Figure 6.** Cytokine levels and IgE levels in OVA-challenged mice. (A) RNA was extracted from lung tissue from OVA-challenged mice fed with PBS or WGP. Il4, Il5, Il13, Ifng, Il17 and Foxp3 mRNA levels were analyzed by Real Time qPCR. Graphs display mean +/- s.e.m. from 3 independent experiments, n=14-15/group. (B) Cytokine levels were measured in BALF by ELISA. Graphs display mean +/- s.e.m. from 3 independent experiments, n=12-15/group. (C) Total IgE and OVA-specific IgE levels were measured in the serum by ELISA. Graph displays mean +/- s.e.m. from 5 independent experiments, n=23-25/group.

References

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Figure 1.

Day: -7  
Begin daily oral administration of PBS or 400μg WGP  

0  
Sensitization Ovalbumin/Alum i.p.  

14  
Sensitization Ovalbumin/Alum i.p.  

24 25 26 27 28  
Ovalbumin (Nebulization challenge)  
Harvest
Figure 2.

A

- **Lymphocytes**
- **Eosinophils**
- **Neutrophils**
- **Macrophages**
- **Total Cells**

B

- **Lymphocytes**
- **Eosinophils**
- **Neutrophils**
- **Macrophages**

C

MPO Activity (pU/ml)

D

EPO (pg/ml)

* and ** indicate significant differences between PBS and WGP groups.
Figure 3.

A) Lung Cells (x10^6)

Macrophages
Lymphocytes
Eosinophils
Neutrophils
Total Cells

B) % Lung Cells

PBS | WGP
---|---
Lymphocytes | Eosinophils | Neutrophils | Macrophages
---|---|---|---
0 | 0 | 0 | 0
10 | 10 | 10 | 10
20 | 20 | 20 | 20
30 | 30 | 30 | 30

*significance level
Figure 4.

(A) Spleen Cells (x10^6)

(B) % Spleen Cells

(C) Cell Count in Blood (K/μl)

(D) % Blood Cells
Figure 5.

Soluble Collagen (pg/ml) vs. PBS and WGP

Mucus Cell Score vs. PBS and WGP

Inflammatory Focus Score vs. PBS and WGP

Soluble Collagen (ug/ml) vs. PBS and WGP
Figure 6.

A. Bar graphs showing the expression levels of IL-4, IL-5, IL-13, IL-17, Foxp3, mRNA/Control, and their comparisons between PBS and WGP treatments.

B. Bar graphs showing the concentration levels of IL-4, IL-5, IL-13, and their comparisons between PBS and WGP treatments.

C. Bar graphs showing the concentration levels of IgE and Anti-OVA IgE between Pre and Post treatments with PBS and WGP.