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Dietary whole glucan particles do not affect antibody or cell-mediated immune responses to influenza virus vaccination in mice

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Influenza virus is a serious health concern. β-glucans derived from plants, bacteria, and fungi have been shown to potentiate immune system responses including those elicited by vaccination. However, in these studies β-glucan was administered as an adjuvant in the vaccine preparation. We hypothesized that addition of a commercially-available whole glucan particle supplement to the diet would improve immune response to primary and secondary influenza vaccination in mice. β-glucan was added to pelleted diet and fed to mice at concentrations designed to deliver 0 (control), 1.8 or 90 $mg \cdot kg^{-1} \cdot day^{-1}$ to each mouse. Influenza vaccine was given intramuscularly in the left hindlimb and primary and secondary responses were assessed. Supplementation with β-glucan was not effective in boosting immune responses to the vaccine, either in the primary or secondary vaccination experiments. Surprisingly, addition of particulate β-glucan to the vaccine itself also failed to elicit a greater antibody response. These observations suggest that this particular form of β-glucan is ineffective in boosting immune response to intramuscular influenza vaccination. Further study is warranted to determine if the use of different mouse models, different vaccine delivery systems, or β-glucans purified from different strains of bacteria, fungi, or plants could improve outcomes using this or similar protocols.

Key words: Beta-glucan, influenza, vaccine, immunity

INTRODUCTION

The influenza family of viruses is the most common cause of respiratory tract infections in all age groups. Antigenic drift in the hemagglutinin and neuraminidase surface proteins of influenza A and B is responsible for recurrent epidemics that affect millions of persons annually (Cox and Subbarao, 1999). As a result, yearly vaccinations against the mostly likely strains of influenza are recommended, especially for those at high risk of contracting the disease. Influenza epidemics between 1990-1999 accounted for more than 36,000 deaths annually with a further 226,000 hospitalizations each year (Fiore et al., 2008). Populations at the highest risk for influenza-associated morbidity and mortality include adults ≥ 65 years of age and children < 2 years of age, with older adults accounting for the vast majority of deaths due to influenza infection each year (Thompson et al., 2003). Notably, the efficacy of the most common inactivated vaccines in persons aged 65 years and older is less than in younger adults (Cox and Subbarao, 1999). Other populations with increased risk of influenza-related deaths include individuals with chronic cardiovascular and/or pulmonary diseases (Barker and Mullooly, 1982), cancer patients (Pollyea et al.), and individuals with diabetes (Valdez et al., 1999). Therefore, adjuvant and/or dietary therapies with a potential to increase the efficacy of the influenza vaccine in at-risk populations are desirable to reduce illness

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rates and decrease healthcare costs during the yearly influenza season.

β-glucans are polysaccharide cell wall components of various types of plants, bacteria, and fungi and constitute approximately half of the cell wall mass of many pathogenic fungi (Klis et al., 2001). β-glucan molecules have been purified from both yeast and oats for use as immune stimulators (Murphy et al., 2010). β-glucans have long been known to be immunostimulatory and have been studied for their ability to potentiate immune responses since the early 1960s (Fitzpatrick et al, 1964). β-(1→3)-D-glucans are potent stimulators of both innate (Goodridge et al., 2009) and adaptive immune responses (Berner et al., 2005; Inoue et al., 2002; Miura et al., 1999). Particles containing β-glucans have been shown to be powerful stimulators of phagocytosis and can activate macrophages (Brown et al., 2002) and natural killer (NK) cells (Garcia-Lora et al., 2001) to attack virally-infected and cancerous cells as well as bacterial cells. Purified forms of β-(1→3)-D-glucans have also been shown *in vitro* to stimulate macrophage production of reactive oxygen species (ROS) and pro-inflammatory cytokines such as interleukin (IL)-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α through signaling by the β-glucan receptor, Dectin-1 (Saijo et al., 2007). Particles containing β-glucans can also potentiate the adaptive immune system by activating dendritic cells (Munz et al., 2005), allowing for more effective antigen processing and presentation when the host organism is infected. Several excellent recent reviews describe the effects of β-glucans on immune function more fully than is possible here (Goodridge et al., 2009; Murphy et al., 2010; Volman et al., 2008), and we refer the reader to these sources for further information on this subject.

Because of their reported *in vitro* immunostimulatory effects, β-glucans have been used as primary and adjuvant treatments for infectious pathogens and vaccinations *in vivo*. β-(1→3)-D-glucans increased the innate immune response to bacterial infections such as *Escherichia coli* (Rasmussen Busund et al., 1992). β-glucans also decreased susceptibility to post-exercise upper-respiratory viral infection (Davis et al., 2004), and zymosan, a particle containing β-glucans, increased the effectiveness of a DNA vaccine against human immunodeficiency virus-1 in mice when used as an adjuvant (Ara et al., 2001). Few studies have examined the effect of β-glucans on the immune response to influenza virus infection, but it was shown that β-glucan purified from *S. cerevisiae* increased the IFN-γ and nitric oxide-mediated antiviral response to swine influenza virus (Jung et al., 2004). Additionally, the β-glucan lentinan, given intranasally and intravenously in mice infected with an lethal dose $(LD)_{75}$ dose of influenza virus, resulted in complete protection against mortality (Irinoda et al., 1992). Although no studies have examined the effect of adjuvant β-glucan given with an anti-influenza vaccine, it has previously been shown that β-glucans can be used as an adjuvant when given with a vaccine against bovine serum albumin (BSA) resulting in an enhanced anti-BSA IgG response (Berner et al., 2008).

To our knowledge, no studies have examined whether enteral β-glucan supplementation can enhance immune responses to vaccination. Thus, we hypothesized that a purified β-glucan powder, when combined with a standard mouse diet, would increase antibody responses to both primary and secondary injections of the influenza vaccine in mice. In accordance with this hypothesis, we tested the anti-influenza Immunoglobulin (Ig)G and IgM responses to vaccination in Balb/cAnHsd mice. We also tested the *ex vivo* cell-mediated immune (CMI) response in isolated splenocytes. We hypothesized that β-glucan supplementation would increase *in vitro* influenza vaccine-induced IFN-□ and reduce IL-10 production when compared to controls.

We used a >75% purity yeast-derived whole-glucan powder (WGP) as we believe studies researching nutritional supplementation with commercially-available products are highly important in informing future human trials. The goal of this research was to provide initial evidence for the effect of a commercially-available nutritional supplement on influenza vaccine responses rather than to test for the pharmacological effects of β-glucans as most

previous studies in the domain have done.

MATERIALS AND METHODS

Materials

Yeast β-(1→3, 1→6)-D-glucan derived from *Saccharomyces cerevisiae* (Wellmune WGP®, Biothera, Eagan, MN) was supplied by the manufacturer as powder to be incorporated into the diet. The powder was certified as endotoxin-free and 77% pure by the manufacturer. Composition of the β-glucan supplement is given in Table 1.

Animals

Six-week-old Balb/cAnNHsd (Harlan, Indianapolis, IN, n = 107) were maintained in standard housing in individual cages in an AAALAC-accredited animal care facility for at least 1 week prior to the start of experiments. All experimentation was approved by our institution's IACUC. Mice were allowed *ad libitum* access to water and to experimental diets. The mice were housed on a 12 hour reverse light-dark cycle with the dark period from 10:00 to 22:00 hrs.

Experimental Diets

Two experimental β-glucan-enriched diets (low and high concentration β-glucan) were custom produced by Harlan Teklad (Madison, WI) by addition of β-glucan powder to Teklad 7001 4% fat diet. The experimental diets were thoroughly mixed and pelleted by Harlan Teklad and were visually identical to standard Teklad 7001 pellets. Concentrations were chosen to give, respectively, a dosage of β-glucan equal to $1.8 mg \cdot kg^{-1} \cdot day^{-1}$ (low) or $90 mg \cdot kg^{-1} \cdot day^{-1}$ (high) for a 25g mouse ingesting 4g of diet per day. The $1.8 mg \cdot kg^{-1} \cdot day^{-1}$ dose is consistent with the manufacturer's recommended dose of 2 mg/kg BW. The $90 mg \cdot kg^{-1} \cdot day^{-1}$ dose is close to the recommended upper level of β-glucan intake *per food serving* based on supporting information in the generally recognized as safe (GRAS) dossier (200 mg). Furthermore, these levels are within the range that has been shown to result in no observed adverse effects (NOAEL) in toxicological testing (2-100 mg/kg) (Babicek et al. 2007). The Teklad 7001 diet (Table 2) was chosen because it is free of Baker's Yeast (*S. cerevisiae*), the organism from which the β-glucan powder used in this experiment was derived. Standard Teklad 7001 diet was used as the control diet in all experiments, and all mice were maintained on this diet during the 1 wk housing acclimation phase in order to wash out any effects of yeast ingestion that may have occurred during the time before the mice were delivered to our facility.

Experimental Design

Responses to both primary and secondary injections of influenza vaccine were tested. Briefly, 50µl of Fluzone (Aventis Pasteur, Swiftwater, PA) influenza vaccine (2008-2009 formulation, containing 45µg of hemagglutinin of each of strains A/H1N1/Brisbane/59/2007, A/H3N2/Uruguay/ 716/2007, B/Florida/04/2008) was injected intramuscularly (i.m.) into the left hindlimb of each mouse using a 25 gauge needle. All mice were maintained on their respective diets for at least one week prior to vaccination in order to wash out the effects of any previous yeast consumption by the mice before their arrival at our facility. For the analysis of primary vaccine response, mice (n = 48, 16 per group) were maintained on their experimental diets for four weeks after injections, and blood was collected as described below.

Table 1: Composition of Wellmune WGP®.

	Percent of Total Weight
Carbohydrates	83.81%
Protein	2.31%
Fat	6.58%
Ash	0.94%
Moisture	6.36%
Beta Glucan	77%

Table 2: Nutritional composition of Teklad 7001 diet.

	Percent of Total Weight	Percent of Total Kcal
Protein	10.1	34.0
Fat	25.0	12.0
Fiber	4.3	54.0
Ash	4.7	

For the analysis of the secondary vaccine response, all mice (n = 48, 16 per group) were maintained on the control diet (no β-glucan) for 5 weeks after the primary injection. Mice were then randomized to one of the three experimental diets. After one week on the experimental diet, the secondary booster vaccination was given and mice were maintained on these diets for 4 weeks. Blood was collected during this portion of the study as described below. The study designs for the primary and secondary vaccine studies are included in Figure 1.

For the adjuvant experiment, mice (n = 11, 6 HI, 5 LO) were housed and vaccine was injected as in the primary response study as above. Ten $mg \cdot ml^{-1}$ of particulate β-glucan was mixed with the influenza vaccine and delivered with the injection to mimic the concentration of BSA-conjugated β-glucan used in the Berner study (Berner et al., 2008). For this experiment, β-glucan was administered only at the time of injection.

Blood Collection and Tissue Collection Procedures

Blood was collected in the primary vaccine response experiment prior to vaccine injection (pre-immune) and at 1, 2 , and 4 weeks post-vaccination. Blood was collected in the secondary vaccine response experiment prior to the primary vaccination, prior to the secondary vaccination, and at 1 , 2 , and 4 weeks post-booster vaccination. At all except the final time point in both experiments, approximately 200µl of blood was drawn from the retro-orbital sinus vein via a Pasteur pipette under anesthesia with isoflurane. Isoflurane was administered in oxygen at a rate of 2-3 $L \cdot min^{-1}$. Blood was dispensed into heparinized tubes and centrifuged at 1100 × *g* at 4°C for 15 min. Plasma was harvested and frozen at -20°C until analysis. At week 4 after the final vaccine injection in both experiments, blood was drawn after sacrifice from the inferior vena cava and processed as above.

At week 4 after the final injection in both experiments, mice were sacrificed by rapid CO_2 asphyxiation followed by cervical dislocation. Spleens were harvested under sterile conditions and immediately placed on ice in sterile media containing RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin-glutamine, and 0.05% 2-mercaptoethanol. Spleens from 2-3 mice were pooled from each group in order to isolate a sufficient number of cells for the cell culture experiments described herein.

Plasma Antibody and Ex vivo Cytokine Measures

Plasma total anti-influenza IgG and IgM were measured by enzyme-linked immunosorbant assay (ELISA). Briefly, 96-well ELISA plates were incubated overnight with 50µl of Fluzone diluted 1:80 in carbonate coating buffer. Non-specific binding was blocked with PBS supplemented with 10% FBS (IgG) or 10% dry milk (IgM) and incubated for one hour at 37°C. After washing, 50µl of plasma samples were added at a dilution of 1:50 (based upon prior titration experiments) for IgG and 1:300 for IgM in a diluting buffer of phosphate buffered saline (PBS)/10% FBS (IgG) or PBS/1% dry milk (IgM) and incubated for one hour at 37°C. Plates were washed again, and 50µl of rabbit anti-mouse IgG or rabbit anti-mouse IgM (Invitrogen, Carlsbad, CA) diluted 1:400 in diluting buffer was added. Plates were incubated again at 37°C for one hr and then washed. Plates were then incubated for 20 minutes in 50µl of a 1:1 mixture of 3,3', 5,5'

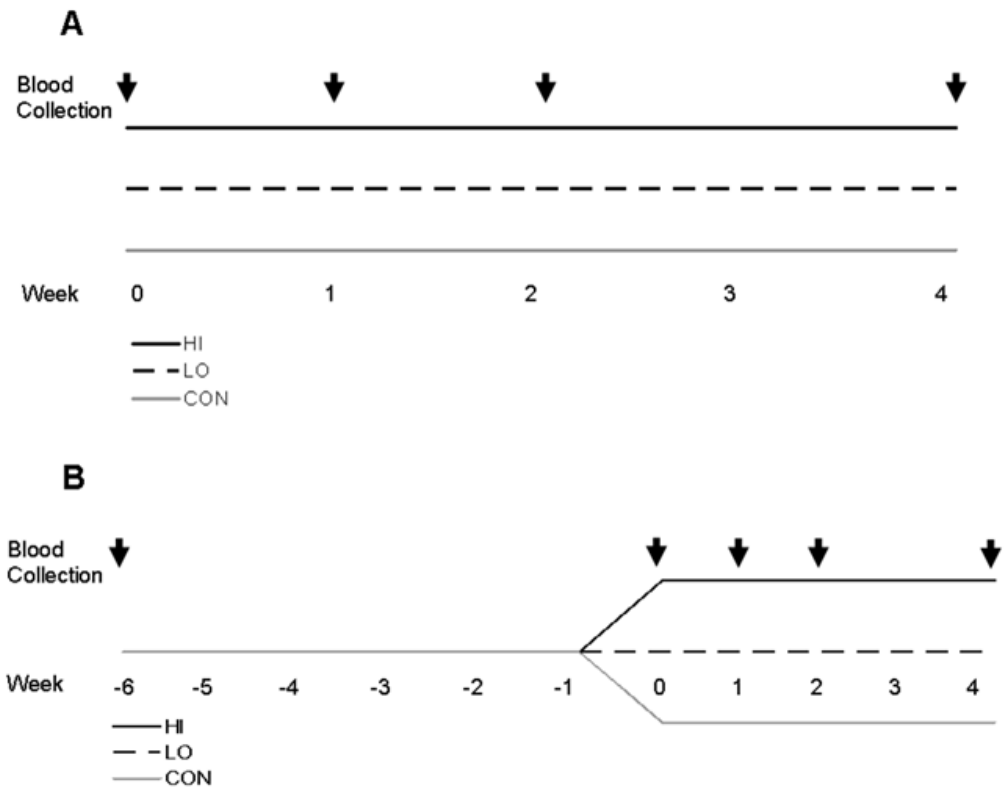

Figure 1: Study design. **A)** Primary vaccine study. Influenza vaccine injection was given at week 0 and sacrifice was performed at week 4. **B)** Secondary vaccine study. Initial influenza vaccine was given at week (-6) and was boosted with a second injection at week 0. Sacrifice was performed at week 4. HI=90 mg β-glucan/kg body weight/day; LO=1.8 mg β-glucan/kg body weight/day; CON=0 mg β-glucan/kg body weight/day.

tetramethylbenzidine and hydrogen peroxide (TMB Substrate Reagent Set, BD Biosciences, San Jose, CA) and read at 405nm on a spectrophotometric plate reader (Labsystems Multiskan, Fisher Scientific, Pittsburgh, PA). Plasma Ig is expressed relative to an inter-plate control and quantified as the difference in optical density (OD) at 405nm from the pre-injection time point. That is, the formula for calculation of plasma Ig is

$$\text{Corrected Ig Expression} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{pre-injection}}}{\text{OD}_{\text{platecontrol}}}$$

Splenocytes were added to 96-well cell culture plates (TPP, Zurich, Switzerland) at a concentration of 2×10^5 cells/well and incubated with Fluzone at a concentration of 10 hemagglutinating units (HAU)·ml^{-1}, 5 HAU·ml^{-1}, or 0 HAU·ml^{-1} for 48 hr. Supernatants were removed and stored at -20°C until analysis. Interferon-gamma (IFN-γ) and interleukin-10 (IL-10) production were assessed by ELISA (R&D Systems, Minneapolis, MN) following manufacturer's recommendations. Supernatant samples were diluted 1:4 for analysis.

Statistics

All statistical tests were performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Plasma IgG and body weight differences were assessed between groups by repeated-measures analysis of variance (ANOVA). Supernatant cytokine differences were analyzed using a 3 (group) × 3 (Fluzone concentration) ANOVA. Food intake data was analyzed across groups by one-way ANOVA. All analyses were followed by post-hoc Bonferroni tests in the event of a significant main effect or interaction. Statistical significance was set at P ≤

Table 3: Body weights and mean daily food intake.

Study:	Primary Vaccination			Secondary Vaccination		
Group	Pre Weight (g)	Post Weight (g)	Daily Food (g)	Pre Weight (g)	Post Weight (g)	Daily Food (g)
HI	25.3 ± 0.3	26.1 ± 0.3	4.4 ± 0.1	27.1 ± 0.6	28.5 ± 0.4	3.9 ± 0.1
LO	25.0 ± 0.4	26.2 ± 0.4	4.1 ± 0.2	26.8 ± 0.5	27.5 ± 0.5	4.0 ± 0.1
CON	24.8 ± 0.6	25.9 ± 0.4	4.3 ± 0.1	27.2 ± 0.5	28.7 ± 0.4	3.9 ± 0.1
p	*0.747*	*0.851*	*0.175*	*0.816*	*0.109*	*0.537*

Mean ± SEM HI=90 mg β-glucan/kg body weight/day; LO=1.8 mg β-glucan/kg body weight/day; CON=0 mg β-glucan/kg body weight/day; Pre=pre-intervention; Post=post-intervention.

0.05 for all tests.

RESULTS

Body Weight and Food Intake

Body weights were not different between any groups in the primary or secondary vaccination trials either pre- or post-intervention, as shown in Table 3. Mice gained very little weight during the study (1.0 ± 0.1g in the primary trial, 1.1 ± 0.3g in the secondary trial), and there were no differences between groups in magnitude of body weight change. Average daily food intake did not differ between groups in either study (Table 2), suggesting that addition of the β-glucan powder to the diet did not have any effect on food intake in either group.

Plasma Antibody Response

In the primary vaccine response portion of the study, no differences were found between groups for either plasma IgM (Figure 2A) or IgG (Figure 2C). There were also no differences between groups in plasma IgM (Figure 2B) or IgG (Figure 2D) levels at any time point in the secondary vaccine trial. Finally, there were no differences in anti-influenza IgM or IgG primary vaccine responses between groups injected either with vaccine alone or vaccine + β-glucan as adjuvant (Figure 3).

Splenocyte Cytokine Production

There were no differences between groups in influenza vaccine-stimulated IFN-γ production at Fluzone concentrations of 10 HAU/ml, 5 HAU/ml, and 0 HAU/ml in either the primary vaccine trial (Figure 4A) or secondary vaccine trial (Figure 4B). Vaccine-stimulated IL-10 production was below the detection limits for the ELISA (data not shown) in both trials, suggesting that the splenic immune response to influenza in these mice is primarily driven by T helper (Th)1 and not Th2 cells.

DISCUSSION

Several studies have indicated that both natural and derivative forms of β-glucans can be effective in reducing symptom severity in response to several types of viruses including human immunodeficiency virus (HIV)-1 (Wang et al., 2008) and herpes simplex virus (HSV)-1 (Davis et al., 2004). β-glucans have been shown to increase anti-viral responses and protect against mortality in animals infected with influenza virus (Irinoda et al., 1992; Jung et al., 2004), leading us to hypothesize that nutritional supplementation with a yeast-derived β-(1→3, 1→6)-D-glucan powder would increase the antibody and/or cell-mediated immune responses to influenza virus vaccination in mice. A dietary supplement that boosts immune responses to vaccination could be envisioned as an inexpensive, safe, and potentially easy-to-administer treatment to individuals that exhibit suboptimal responses to current vaccines (e.g. elderly, kidney patients, diabetics).

Results from our experiments indicate that provision of a commercially-available β-(1→3)-D-glucan supplement in the diet did not increase plasma anti-influenza IgG or IgM antibody levels after either primary or secondary intramuscular

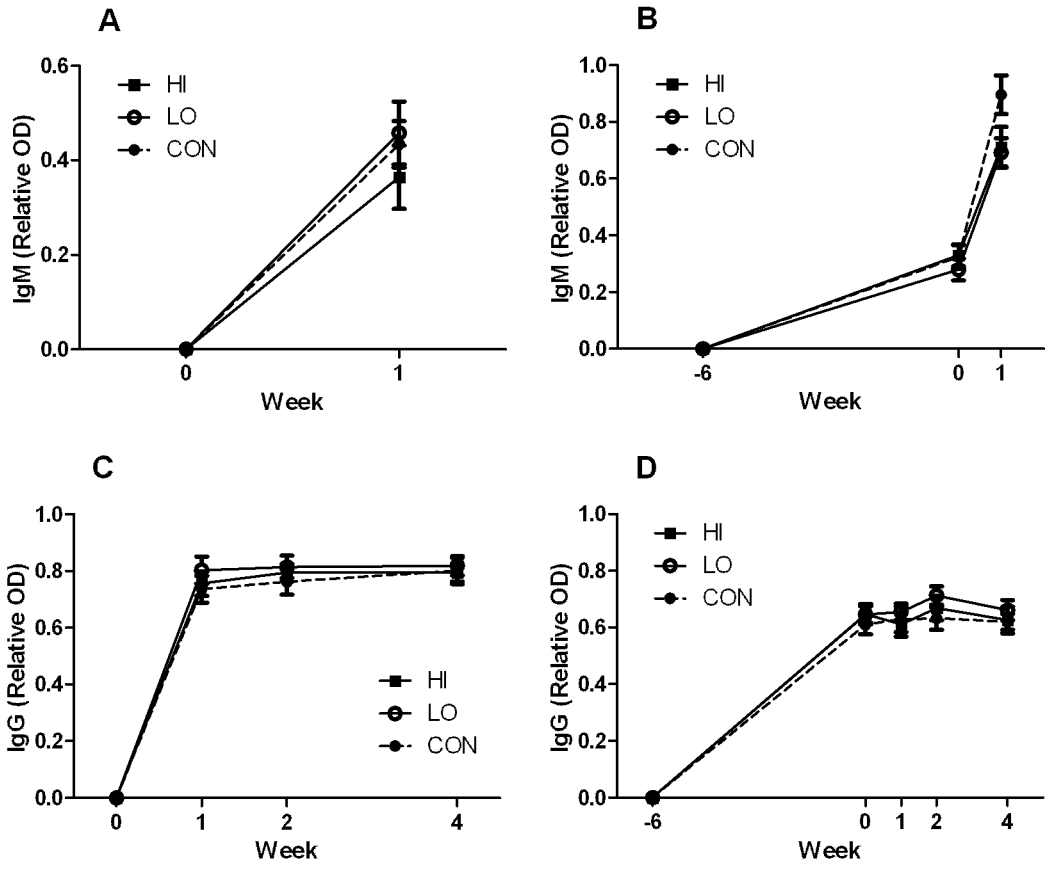

Figure 2: Plasma anti-influenza antibody responses for primary and secondary vaccine studies (Mean ± SEM). **A)** Anti-influenza IgM response during the primary vaccine study. There was no significant interaction ($F_{2,39}$=0.634, p=0.563). **B)** Anti-influenza IgM response during the secondary vaccine trial. There was no significant interaction ($F_{2,\ 23}$=1.794, p=0.183). **C)** Anti-influenza IgG response during the primary vaccine study. There was no significant interaction ($F_{3,68}$=0.496, p=0.706). **D)** Anti-influenza IgG response during the secondary vaccine study. There was no significant interaction ($F_{5,92}$=0.763, p=0.585). HI=90 mg β-glucan/kg body weight/day; LO=1.8 mg β-glucan/kg body weight/day; CON=0 mg β-glucan/kg body weight/day.

injections of Fluzone influenza vaccine in mice. Additionally, influenza vaccine-stimulated splenocyte IFN-γ production was not different between β-glucan-fed mice and mice fed a normal chow diet in either primary or secondary vaccination studies. Influenza vaccine-stimulated IL-10 production was below detectable limits, suggesting that the cell-mediated response in this model is primarily Th1-driven. Body weight and food intake in mice were not different between groups in either study, indicating that our randomization procedures were effective and, more importantly, that dietary β-glucan did not affect normal feed intake behavior. Moreover, addition of β-glucan as an adjuvant in the influenza vaccine was ineffective in boosting antibody response to primary injection, suggesting that this type of β-glucan supplement is not efficacious in improving immune response to influenza vaccine regardless of the method of administration. This was surprising because a number of studies have used β-glucan supplementation as a method of improving immune responses. Many studies demonstrating positive effects of β-glucans on immunity have used Balb/c mice (Berner et al., 2008; Harnack et al., 2009; Torello et al., 2010; Zhou et al., 2009), thus we are confident that this mouse model is appropriate for studies such as the one described here.

There are several other potential explanations as to why our interventions were ineffective in modulating immune response to influenza

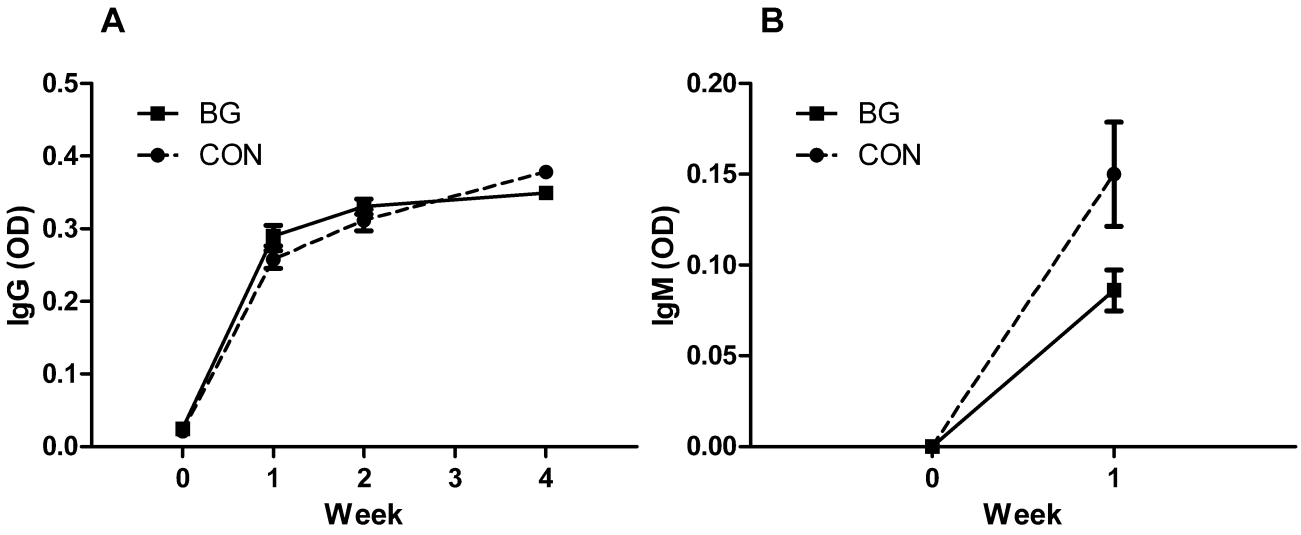

Figure 3: Plasma antibody responses for adjuvant study (Mean ± SEM). **A)** Anti-influenza IgG response. There was a significant interaction ($F_{3,27}$=3.884, p=0.020). However, there was no significant main effect of adjuvant ($F_{1,9}$=0.601, p=0.458). **B)** Anti-influenza IgM response. There was no significant interaction ($F_{1,7}$=5.172, p=0.057). BG=vaccine + β-glucan; CON=vaccine only.

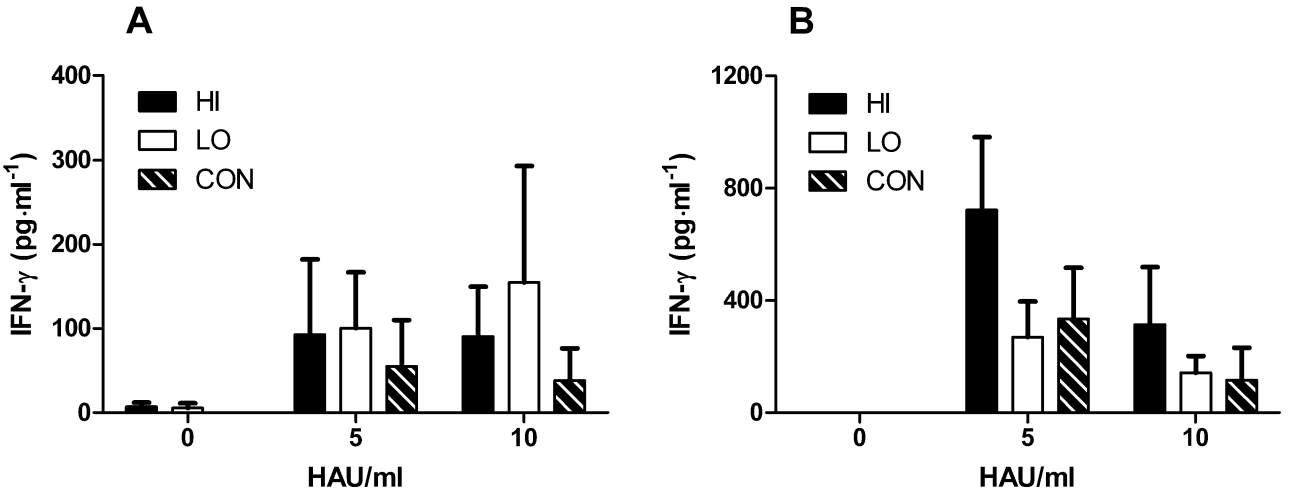

Figure 4: IFN-γ production. **A)** Antigen-stimulated splenocyte IFN-γ production in the primary vaccine trial. There was no significant interaction ($F_{6,24}$=0.423, p=0.857). **B)** Antigen-stimulated splenocyte IFN-γ production in the secondary vaccine trial. There was no significant interaction ($F_{4,36}$=0.785, p=0.543). Spleens from 2-3 mice were pooled in order to have sufficient cell numbers for cell culture. HI=90 mg β-glucan/kg body weight/day; LO=1.8 mg β-glucan/kg body weight/day; CON=0 mg β-glucan/kg body weight/day. HAU=influenza vaccine hemagglutinating units.

vaccination. The simplest explanation is that β-glucan supplementation may not be effective when given orally to boost immune responses to an intramuscular vaccine. Our findings, however, do not rule out the possibility that orally administered β-glucan affects vaccines when delivered by oral or intradermal routes. Along these lines, it would be interesting to test whether oral β-glucan supplementation affects immune responses to oral vaccines such as cholera or rotavirus. Macrophages and NK cells which have interacted with orally-ingested β-glucan may be more likely to

come into contact with vaccine components using these alternate strategies for vaccine delivery, thus increasing both the speed and strength of the immune response to the vaccine. Intradermal injection, on the other hand, has the added benefit of allowing for an *in vivo* measure of vaccine efficacy as it causes a local skin reaction upon successful vaccination in many instances, even in the absence of repeated exposures (Gelinck et al., 2009).

Additionally, our β-glucan formulation is of a rather low purity (77%) compared to many pharmaceutical-grade supplements. This formulation was chosen as we wished to study the effects of a commercially-available nutritional β-glucan supplement on the influenza vaccine response. Therefore, our results do not rule out the possibility that a similar study utilizing a higher-purity β-glucan may have a better outcome.

CONCLUSION

In this study, we were unable to show improvements in antibody or cell-mediated immune response to intramuscular influenza vaccine injection with a commercially-available dietary supplementation of yeast β-(1→3)-D-glucan in young mice. Sufficient responses to influenza vaccination are an important clinical outcome in both aged and immunocompromized individuals, and testing in these populations may be necessary to see significant results using this intervention. Use of a higher-purity, pharmaceutical-grade β-glucan preparation might also have a greater effect on immune responses to influenza vaccination. Further and more in depth study is needed to fully characterize the effects of β-(1→3)-D-glucans on adaptive immune responses to influenza and other vaccinations in clinically-relevant populations.

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