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Dietary β-glucan Leads to Increased TNF-α Production in the Lung

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Abstract: Problem statement: Beta (β)-glucan is notable for its ability to stimulate the immune system and as such β-glucan and products containing β-glucan are used as dietary supplements for livestock and companion animals. β-glucan has been shown to activate macrophages and neutrophils and modulate the production of certain cytokines, including the pro-inflammatory cytokine, Tumor Necrosis Factor Alpha (TNF-α). Because TNF-α is a contributing factor in a number of chronic inflammatory diseases and is present at higher concentrations in bronchoalveolar fluid from patients with asthma a preliminary experiment was designed to determine if a diet supplemented with β-glucan leads to increased TNF-α production in response to chitin, an ubiquitous environmental antigen that is associated with airway inflammation. **Approach:** Mice were divided into two groups. One group was given normal rodent chow and water while the other group was given normal rodent chow and water containing β-glucan (1 mg mL^{-1}) for 14 days. After 14 days, two experimental protocols were conducted to evaluate TNF-α production. In experimental protocol 1, mice were injected intraperitoneally with 4% thioglycollate broth and TNF-α production from the immune cells elicited into the peritoneal cavity was evaluated. In experimental protocol 2, mice were exposed to either chitin or PBS (as a control) via intranasal administration for two consecutive days. Six hours post secondary exposure, Bronchoalveolar Lavage Fluid (BALF) was collected and ELISA for TNF-α performed. **Results:** TNF-α expression by thioglycollate-elicited cells isolated from animals that consumed β-glucan was greater (27 fold) than controls. Similarly, dietary β-glucan was also associated with increased TNF-α expression (four fold) in the lung, after chitin exposure *in vivo*. **Conclusion:** These preliminary results suggest that dietary β-glucan may promote inflammatory responses after exposure to chitin and therefore could be contributing factor to lung inflammation particularly in animals prone to airway inflammatory diseases.

Key words: Inflammation, allergy, prebiotics, nutrition

INTRODUCTION

Beta-glucans are naturally occurring polysaccharides that have been scientifically shown to modify biological defenses by nutritionally potentiating immune responses (Volman *et al*., 2008). Found in mushrooms, yeast and grains, β-glucans promote resistance to diseases caused by microorganisms through enhancement of both innate and adaptive immune responses (Vetvicka *et al*., 2008; Vetvicka and Vetvickova, 2012) and therefore are used as a dietary feed supplement to promote the health of humans and animals.

In vertebrates, the recognition of and response to β-glucan is mediated primarily by cell surface receptors that have been found on a number of cell types including innate inflammatory cells like macrophages and neutrophils (Tsoni and Brown, 2008). Expression of TNF-α occurs predominantly in response to activation of membrane bound pattern recognition molecules (Nathan, 1987) but activation through β-glucan receptors has also been shown to induce its expression (Tsoni and Brown, 2008; Sonck *et al*., 2010).

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TNF-α is a contributing factor in a number of chronic inflammatory diseases and has been found to be present in higher concentrations in bronchoalveolar fluid from patients with asthma (Mukhopadhyay *et al*., 2006). Moreover, inhalation of TNF-alpha triggers bronchial hyperresponsiveness and promotes airway neutrophilia (Kips *et al*., 1992; Thomas, 2001; Thomas and Heywood, 2002). Furthermore, mouse models of asthma that lack or block the expression TNF-alpha or TNF-alpha receptors show attenuation in airway inflammation after antigenic stimulation (Kips *et al*., 1992; Zuany-Amorim *et al*., 2004).

Allergic-like lung inflammation can affect many domestic animals (pets as well as food and fiber animals). Farm animals such as cattle, equine and companion animals, particularly cats can develop respiratory problems brought on by exposure to common allergens such as pollen, animal dander and chitin containing insect fragments, fungal molds and bacteria. Heaves, for example is a condition suffered by horses that shares many similarities with human asthma, including lower airway inflammation, reversible airway obstruction and bronchial hyper-responsiveness (Leclere *et al*., 2011). In a study examining the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Gucht *et al*. (2003) reported that TNF-α can cause bronchial hyperreactivity and bronchoconstriction leading to asthma-like symptoms in pigs (Gucht *et al*., 2003).

Because β-glucan is known to induce TNF-α production by innate immune cells we sought to investigate the effect of dietary β-glucan on the inflammatory response in the lung to inhaled allergens. In this preliminary study, mice were given β-glucan in their water for 14 days and innate TNF-α production after antigen exposure was measured. Chitin, a ubiquitous substance that has been shown to promote allergic inflammation and has been associated with asthma (Reese *et al*., 2007; Burton and Zaccone, 2007), was used as the antigen. We find that consumption of β-glucan caused increases in TNF-α expression by cells stimulated with chitin ex vivo and *in vivo*.

MATERIALS AND METHODS

Animals: Female wild-type Balb/c mice, purchased from Harlan Laboratory, Indianapolis Indiana, between 5-8 weeks of were used in this study. Mice were randomly assigned to one of two treatment groups; water and water with β-glucan. All mice were maintained in the Laboratory Animal Resource Unit of North Carolina A&T State University and all experiments were approval by the Institutional Animal Care and Use Committee.

β-Glucan administration and measurement of water consumption: 1-3, 1-6 β-glucan tablets [200 mg] (The Vitamin Shoppe (Greensboro, NC) were crushed with a sterile mortar and pestle. The β-glucan powder was added to drinking water at 1mg mL^{-1} and mice were allowed to drink *ad libitum* for 14 days. Water with and without β-glucan was replenished every two days. At each replenishment period, total water consumption was determined by measuring the volume of water that remained and subtracting it from the initial volume that was placed in each cage's water bottle. The average daily water consumption per mouse in ml was calculated by dividing the total water consumption in ml by the number of mice in the cage. All mice were given normal rodent chow *ad libitum* for the duration of the 14-day study.

Thioglycollate induced peritonitis, cell culture and differential cell analysis. Two ml of sterile 4% thioglycollate broth dissolved in PBS was injected into the peritoneal cavity (i.p.) of mice that had been consuming water or β-glucan for 14 days. After four days, mice were sacrificed and the peritoneal cavity was flushed with 5 mL of cold sterile PBS. The peritoneal washings were centrifuged at 1200 rpm for 5 min, resuspended in complete Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 0.1% penicillin-Streptomycin). Equal numbers of viable cells as determined by trypan blue exclusion were seeded into 48 well tissue culture plates for 24 h. Cell supernatants were collected after 24 h and assayed by ELISA for TNF-α. In addition cells from the peritoneal washings were affixed to glass slides using a Shandon cytospin 4 (Thermo Fisher Scientific Waltham, Massachusetts) at 700 rpm for 5 min. Slides were dried, fixed and then stained using HEMA-3 stain (Fisher Scientific, Pittsburg, Pennsylvania). Using a compound light microscope with a 100x oil immersion lens 100 cells per slide were identified and counted based on color and morphology.

Chitin Preparation and Intranasal Aspiration: Chitin (Sigma Aldrich, St. Louis, Missouri) was prepared as follows: chitin was suspended in sterile Phosphate Buffered Saline (PBS) [10 mg mL^{-1}] rocked for 1 h at room temperature, sonicated 6×for 5 min and centrifuged at 100g for 1min. The supernatant, free of large particles was removed and aliquoted into microcentrifuge tubes at 1.0 mL per tube. The chitin aliquots were autoclaved and stored at 4°C. For intranasal aspiration, a total of 0.5 mg of chitin in a 50 µL volume (or 50 µL of sterile PBS was administered to the nose of anaesthetized mice (on day 14 after the start of β-glucan treatment) to allow for aspiration during normal respiration. Mice were exposed to chitin once a day for two consecutive days.

Collection of bronchioalveolar lavage fluid: Bronchio Alveolar Lavage Fluid (BALF) was collected 6 h post second intranasal exposure of chitin. Generally, the procedure was as follows. Mice were euthanized with CO_2 and the bronchioaveolar lavage fluid was collected by instilling and gently retrieving 1ml of sterile Phosphate Buffered Saline (PBS) supplemented with 5% fetal calf serum into the lung via the trachea using a sterile syringe and canulla. The cell free supernatant was removed and stored at-20°C until analyzed by ELISA.

Measurement of TNF-α: To determine the levels TNF-α within the BALF and culture supernatants Enzyme-Linked Immuno Sorbent Assay (ELISA) (Biolegend, San Diego, California) was performed per manufacturer's instructions. Plates were read at 405nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis: GraphPad Prism (La Jolla, CA) was used to create graphs and conduct all statistical analysis. The statistical analysis applied to data was the student t-test. P values less than 0.05 were considered significant.

RESULTS

β-glucan consumption: To be assured that mice would consume β-glucan added to their water we performed an experiment whereby consumption of water with and without β-glucan was monitored during a 14-day time period. We found no significant differences in water intake (Fig. 1A) nor were there differences in feed intake (data not shown) or weight gain (Fig. 1B).

β-glucan consumption leads to increased TNF-α expression: β-glucans are able to activate leukocytes directly, stimulating phagocytic, cytotoxic and anti-microbial activities that include the production of proinflammatory mediators such as TNF-α (Vetvicka *et al*., 2008). We report that the expression of TNF-α by leukocytes isolated from the peritoneal cavity was greater (27 fold) in mice that consumed β-glucan as compared to mice that consumed water only (Fig. 2A). Interperitoneal injection with thioglycollate broth results in recruitment of high numbers of macrophages and has been shown to cause influx of other white blood cells (Konat, 2008). Furthermore, β-glucan has been shown to activate and mobilize neutrophils into tissues (LeBlanc *et al*., 2006). Therefore to be assured that the difference in TNF-α was not due to differences in the cell types that are capable of producing TNF-α within the cell culture a differential analysis on the cells collected from the peritoneum was performed. Figure 2B demonstrates that in our experiment i.p. injection of thioglycollate resulted in recruitment of macrophages as well as other types of white blood cells and that similar cell types were recruited and collected from both the control and β-glucan groups (Fig. 2B). Taken together these data suggest that the difference in TNF-α production was due to the stimulatory affects of β-glucan and not differences in the number of TNF-α producing cells in culture (Fig. 2B).

Because we observed increased expression of the pro-inflammatory cytokine TNF-α from immune cells isolated from animals that consumed β-glucan, we sought to determine if there was a physiological consequence, focusing on TNF-α production in the lung, after exposure to the ubiquitous allergen chitin. To that end, mice were allowed to consume regular water or water supplemented with β-glucan. After 14 days, mice were exposed to chitin intranasally for two consecutive days. Bronchoalveolar lavage fluid was collected 6 h post the second chitin exposure and assayed for levels of TNF-α. Whereas chitin induced TNF-α production from both the water and β-glucan groups, the TNF-α level was significantly greater (p value<0.05) in the mice fed β-glucan (Fig. 3).

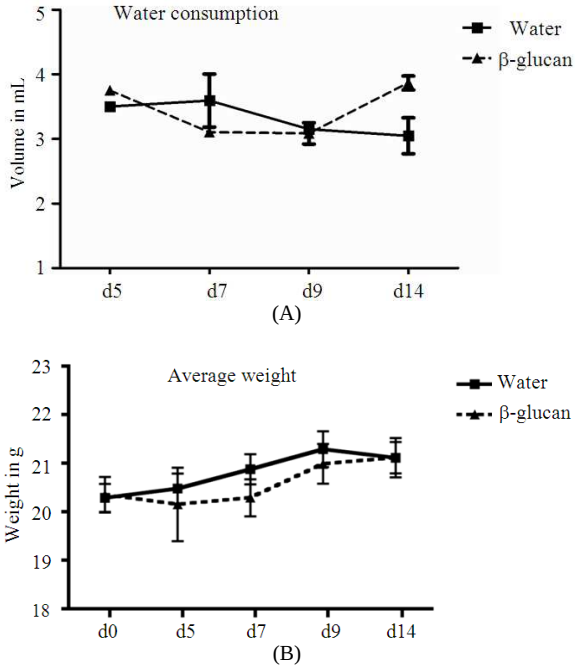

Fig. 1: Analysis of Weight and Water Consumption. (A) Water consumption was measured every other day utilizing a graduated cylinder. The amount left was subtracted to obtain the amount consumed. Total consumption was calculated to find average daily consumption per mouse. (B) For determination of weight, mice were weighed on the days indicted using an Arbor 1605 electronic balance

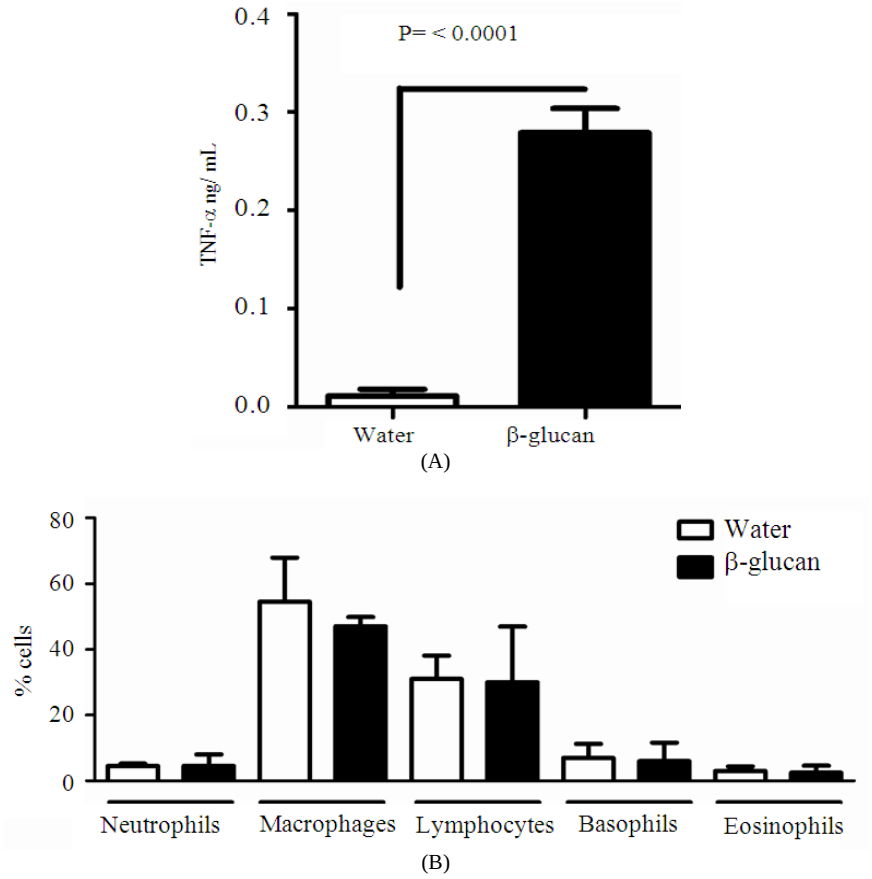

Fig. 2: The effect of β-glucan on TNF-α expression after thioglycollate induced peritonitis. Mice were given water (n = 4) or β-glucan (n = 4) for 14 days. On day14 mice were injected i.p. with 4% thioglycollate broth. After 5 days, influxed cells were collected from the peritoneal cavity. (A) Equal numbers of viable cells were plated for 24 h. TNF-α was measured in the culture supernatants by ELISA. (B) Differential cell counts of cells collected from the peritoneal washings of mice injected i.p. with 4% thioglycollate broth. Cells were affixed to slides, stained and differential cell counts were performed. Data are expressed as means ± SD and were compared using a two-tailed unpaired Student t test with a 95% confidence level.

DISCUSSION

β-glucan is one of the most studied immune enhancing substances in existence. In mice and humans, beta-glucans have been shown to activate macrophages and neutrophils affect the action of T and B-lymphocytes and modulate the production of certain cytokines including IFN-γ and TNF-α.

Inflammation is the body's first line of defense occurring commonly in tissues and organs that have been injured or exposed to microbial pathogens. However, inflammation can also cause damage to host tissues and in severe cases lead to sepsis, organ failure and death (Barton, 2008). TNF-α is a pro-inflammatory cytokine that is important to innate immune responses. It is a key cytokine with a role in many inflammatory conditions and is commonly associated with the deleterious effects of the inflammatory immune response (Minor *et al*., 2010; Schett *et al*., 2011). In asthma, TNF-α contributes to increases in vascular permeability, acts is a chemoattractant for neutrophils and eosinophils and is involved in the activation of cytokine release by T-cells (Berry *et al*., 2007).

The goal of this preliminary study was to investigate the effect of dietary β-glucan on TNF-α production. Utilizing a mouse model, we evaluated TNF-α production of mice given β-glucan for 14 days. We report that leukocytes from mice that consumed β-glucan expressed higher levels of TNF-α. This is consistent with the fact that interperitoneal injection with thioglycollate broth has been shown to induce an inflammatory response that includes not only recruitment of inflammatory cells but also increases in the level of inflammatory cytokines such as TNF-α (Wan *et al*., 2009).

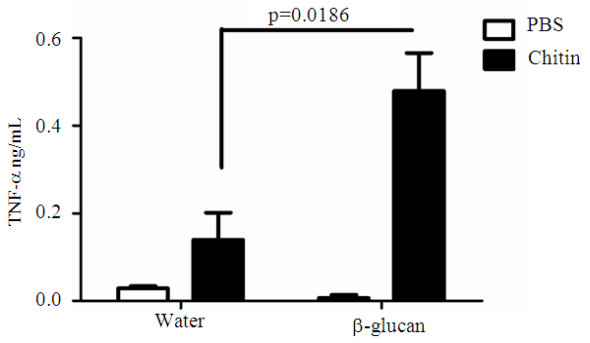

Fig. 3: The effect of β-glucan on TNF-α expression in the lungs after chitin exposure. Mice were given water or β-glucan for 14 days. After 14 days mice were exposed to chitin (n=6) or PBS (n=6) intranasally for twoconsecutive days. BAL fluid was collected 6 hours post the second chitin exposure and assayed for levels of TNF-α by ELISA. Data are expressed as means ± SD and were compared using a two-tailed unpaired Student t test with a 95% confidence level

As β-glucan consumption leads to increased TNF-α production we sought to evaluate the consequences of this on the lung after antigen exposure. While there are many inhaled allergens that can lead to or exacerbate the immune response and lead to allergic asthma; one of the most prevalent in nature is chitin. Chitin is a ubiquitous naturally occurring polysaccharide that is found in the cell walls of fungi, exoskeleton of crustaceans and insects and the micro-filarial sheath of parasitic nematodes. Chitin has been shown to promote allergic inflammation and has been associated with asthma (Reese *et al*., 2007; Burton and Zaccone, 2007). We report that TNF-α production was significantly higher in the lungs of mice that consumed β-glucan and were exposed to chitin intranasally. However, our experiments do not address whether the increase in TNF-α is specific to chitin inhalation or if it is true for other environmental inhaled antigens or respiratory infections. Further experiments evaluating the role of dietary β-glucan on inflammation in other organs and tissues as well as future investigations assessing the affect of β-glucan consumption on the expression of other pro-inflammatory cytokines and inflammatory cell recruitment are necessary.

CONCLUSION

Whereas inhalation of beta-glucan has been shown to trigger or act as an adjuvant in the development of airway inflammation to inhaled antigens (Inoue *et al*., 2009; Neveu *et al*., 2011), to our knowledge this is the first data presented to suggest that consumption of β-glucan may also lead to hightened airway inflammation. Furthermore, dietary β-glucan has been associated with the development of Th1 responses (Baran *et al*., 2007) and thought to be protective against allergy and asthma (Wichers, 2009). These preliminary findings suggest however, that that usage of dietary supplements containing β-glucan may lead to increased TNF-α production and may cause inflammation or exacerbate existing inflammatory conditions and it use is potentially problematic in animals that are prone to or have airway inflammatory conditions.

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