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Immunomodulatory effects of dietary IMUNO-2865 in mice, pre-and post-vaccine challenge with parainfluenza virus 5

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ABSTRACT

Herbal remedies and nutraceuticals continue to be used as treatments for a variety of maladies ranging from joint disease to obesity. IMUNO-2865 is a natural nutraceutical supplement that has been advertised to modulate inflammation, boost cytokine activity promoting a robust immunity, but has yet to be evaluated as an adjuvant. In the present study, 4-week-old C57BL/6 female mice (n = 45) were fed 0, 5 or 50 mg/5 g tablet IMUNO-2865 (I-2865) in a tablet formulated feed. One group of mice (n = 15, 5 mice/diet) were placed on a feed diet for 14 days, while the other group of 30 mice (10 mice/diet) were placed on the diet for 28 days. Five mice from each diet group in the 28-day feeding trial were vaccinated on day 7 with a mouse recombinant parainfluenza virus to mimic viral challenge. On days 0, 14 and 28 blood samples were collected. Mice were humanely euthanized on days 14 and 28. Spleens were collected to analyze organ weight/body weight ratios, cell recovery, T cell and B cell phenotype, cell proliferation, antibody titers and cytokine production. Administration of dietary I-2865 for 14 days had no effect on murine immunity. In the 28-day dietary vaccine trial, I-2865 supplementation did not enhance vaccine response, based on vaccine antigen-specific IgG titers, nor did it alter T cell and B cell phenotype, function or cytokine response, but it did decrease splenocyte numbers in the vaccinated mice.

1. Introduction

Herbal medicine is a popular subfield in human and veterinary medicine with herbal remedies and nutraceuticals used for a variety of maladies ranging from obesity to arthritis [1–4]. In a 2007 survey by the U.S. Centers for Disease Control and Prevention, approximately 40% of Americans (~52 million people) reported using complementary and alternative medicine, especially botanical dietary supplements [5]. American consumers used dietary supplements mainly to feel better, improve energy levels, and boost their immune system [6]. Nearly all cultures known to science have, throughout history, used a variety of plants or materials derived from plants for the prevention and treatment of disease [7]. Even more interesting is the field of zoopharmacognosy, which can be described as a self-medicating behavioral phenomenon in which people mostly ingest or topically apply a range of non-commercial environmental products (i.e. plants, and soil components) aimed at preventing or reducing the harmful effects of pathogens and toxins [8]. Numerous scientific reports that highlight this phenomenon in the animal world exist to date [9,10]. Surprisingly, regulations for herbal supplements in the United States do not require surveillance or the reporting of adverse events by the manufacturer to the FDA. At the same time, very little has been done to investigate potential adverse effects that may be associated with extended or high-dose use of medicinal herbs [7]. However, these supplements are not without risks. Hepatotoxicity has been documented with a number of nutraceuticals and drug interactions have also been reported in a variety of products [11,12].

An American made herbal compound, IMUNO-2865 (I-2865), has been reported to have immune stimulating qualities in humans and animals. It is a hemicellulose compound comprised of *Graminiae* and *Dioscoreaceae* plant families, and several species of mushrooms. Results from a human trial of 3 g of I-2865 daily for two weeks indicated that cancer killing natural killer (NK) cell activity significantly increased from 10.1% to 27.2% [13]. Another study showed that the nutraceutical significantly increased proliferation, phagocytosis, and nitric oxide production in a murine monocyte/macrophage cell line

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(J774A.1) compared to control cells [14]. Further, the authors reported that the nutraceutical I-2865 increased human circulating lymphocytes by 18% although the individual lymphocyte subtypes were not significantly increased. Results from these two studies suggest that I-2865 can stimulate both the innate and adaptive immune systems.

The current study investigated whether dietary administration of this nutraceutical I-2865 in the feed modulated the immune system in a murine model or could function as an adjuvant as well as ascertain whether there would be any detrimental effects on the animal at high doses. The study was divided in to two stages. The first stage of the study, using mice as an in vivo animal model, was to determine whether dietary I-2865 upregulated immunity in the absence of challenge. The second stage of the study evaluated whether I-2865 could enhance the host active immunity as an adjuvant following challenge with a conjugate vaccine. Immune response was measured through a number of endpoints including splenic/body weight ratio, splenocyte proliferation, splenocyte cell subsets, and splenocyte cytokine production. Any kind of effect of the nutraceutical, positive or negative would be recorded and reported.

2. Materials and methods

2.1. Animals

Forty-five female C57BL/6 mice (4-weeks-of-age) were obtained from Charles River Laboratories (Portage, MA) and housed in the Animal Resource Facility of the University of Georgia College of Veterinary Medicine (Athens, GA). The mice were housed under climate controlled conditions of 23 ± 2.0 °C. 40–60% humidity, and 12:12 light:dark cycle. All mice received a commercial diet (Rodent BLT's™, BioServ, Flemmington, NJ) supplemented by the diet manufacturer with or without I-2865 (Animal Necessity, New York, NY) and water ad libitum. The three treatment diets (0 mg I-2865/5 g tablet, 5 mg I-2865/5 g tablet and 50 mg I-2865/5 g tablet) were packaged, shipped, and stored at 23 °C in a climate-controlled room. After a one-week acclimation period, each mouse/cage received an ear notch to allow for identification and individual tracking throughout the study. Care and maintenance were in accordance with and approved by the Institutional Care and Use Committee (IACUC) at the University of Georgia.

2.2. Experimental design

The study was divided into two parallel dietary treatment trials. The first trial was a 14-day feed study. Fifteen female C57BL/6 mice housed in three cages (5 mice/cage) were randomly selected and assigned to one of the three treatments: 0 mg I-2865, 5 mg I-2865, and 50 mg I-2865. Blood samples were collected, via the retroorbital sinus following topical anesthesia with Proparacaine hydrochloride, from all mice on day 0 and day 14. Following completion of the 14-day study, the mice were euthanized and the spleens were harvested. The second trial was a 28-day vaccine study involving 30 female C57BL/6 mice housed in 6 cages (5 mice/cage). Two cages were randomly assigned to each treatment group. On day 1, mice in one of the two diet treatment cages were vaccinated with murine H5N1 HA and the other diet treatment cage of mice was vaccinated with vehicle-PBS. This resulted in 6 different treatment groups: 0 mg I-2865-PBS, 5 mg I-2865-PBS, 50 mg I-2865-PBS, 0 mg I-2865-vaccine, 5 mg I-2865-vaccine and 50 mg I-2865-vaccine group. These mice remained on their specific commercial diet for 28-days. At the end of the 28-days, blood was collected, the mice were humanely euthanized and the spleens were harvested.

2.3. Feed and mouse weight

At the beginning of each week, 200 g of feed was weighed and administered to each corresponding cage. Feed consumption was monitored daily throughout the study. During any given time in which the amount of feed was deemed to be insufficient to finish out the week, the remaining feed in the cage was weighed and new feed was added to bring the total weight to 200 g. At the start of each week, any remaining feed in the cage was weighed, discarded, and new feed was administered. Mice were also weighed weekly throughout the study to monitor weight change. A separate sterile glass beaker was used for each cage to ensure no cross contamination of feed or vaccine (during vaccine segment) occurred among the cages. All weights for feed and mice were recorded in grams.

2.4. Blood collection

Peripheral blood was collected via the retro-orbital sinus. Approximately 150–200 μL were collected into a sterile 1.5 mL microcentrifuge tube without anticoagulant. Immediately post-bleeding, 7 μL were used to make a blood smear. Blood was incubated for approximately 1 h at 23 °C and then centrifuged at 8000 ×*g* for 3 min. Serum from each sample was then collected and split evenly into two sterile 0.5 mL microcentrifuge tubes. All serum samples were stored at −80 °C until analyzed.

2.5. Vaccine

The H5N1 HA vaccine (generously supplied by Dr. Biao He, Department of Infectious Disease) was administered via a single 100 μL injection in the right hind limb using a 1 mL syringe fitted with a sterile 27-g 1/2 in. needle. One cage per diet treatment received either PBS or vaccine.

2.6. Organ collection

At the end of each dietary trial, mice were humanely euthanized. The spleens were collected into individual sterile petri dishes and weighed. Each spleen was dissociated using a 60 μm sieve screen and collected in a 15 mL conical tube (Fisher Scientific) in incomplete RPMI media. The tubes were centrifuged at 394 ×*g*, 23 °C for 7 min. The supernatant was discarded and cells were resuspended in 1 mL incomplete RPMI media and 2 mL 1× RBC lysis buffer (Affymetrix eBioscience, San Diego, CA) and incubated for 5 min at 23 °C. The pellet was resuspended in 5 mL incomplete RPMI media and centrifuged at 394 ×*g*, 23 °C for 7 min. The supernatant was discarded and the cells were resuspended in 5 mL complete RPMI media (10% fetal bovine serum, 1% penicillin-streptomycin). A 100 μL aliquot of the cell suspension was collected into a 0.5 mL microcentrifuge tube for cell enumeration using a Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA). Cell viability was assessed with Trypan Blue exclusion dye (Fisher Scientific). All samples were diluted to 5.0×10^6/mL in complete RPMI media.

2.7. Flow cytometry

Splenocytes (5×10^5 cells/100 μL) were aliquoted into individual wells of a round bottom 96-well Costar culture plate (Corning, Corning, MA) and stained with 100 μL of anti-mouse PE-CD4, FITC-CD8, FITC-MHCII and PE-CD45RB220 (Thermo Fisher Scientific, Waltham.MA). The plate was then placed on an orbital shaker (Thomas Scientific, Swedesboro, NJ) for 30 min on ice in the dark. The plate was then washed with 100 μL/well PBS and centrifuged at 394 ×*g*, 7 °C for 7 min. The supernatant was aspirated and the cells were resuspended in 100 μL PBS and transferred to individual 5 mL polystyrene round-bottom culture tubes (Becton Dickinson, Labware 3, Franklin Lakes, NJ) containing 0.5 mL PBS. The tubes were kept on ice and read using an LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ).

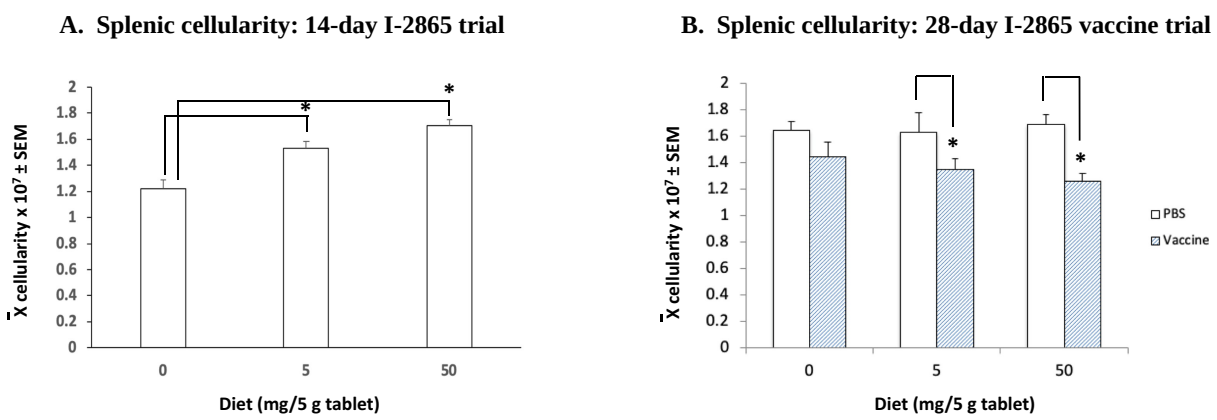

Fig. 1. Splenic cellularity.
Spleens were aseptically removed, dissociated over a sieves screen and subjected to lysis buffer and a number of washed steps to obtain an enriched splenic leukocyte population. Cells were resuspended in incomplete medium and enumerated on a Nexcelom Cellometer Auto T4. Enriched splenic cell recovery for the 14-day I-2865 dietary trial (Fig. 1A) and 28-day I-2865 dietary vaccine trial (Fig. 1B) were quantified. Values are expressed as mean cellularity ± SEM × 10^7/mL, $^*p \leq 0.05$, Unpaired *t*-test with Welch's correction.

Table 1
Splenic T cell subsets: 14-day dietary trial.

A.

Treatment	%CD45RB220$^-$CD90$^-$	%CD45RB220$^+$ CD90$^-$	%CD45RB220$^-$CD90$^+$
0 mg	43.2 ± 3.3	39.5 ± 1.98	14.2 ± 1.26
5 mg	53.3 ± 4.4	34.5 ± 3.28	9.2 ± 1.90
50 mg	52.3 ± 2.3	34.5 ± 1.48	10.6 ± 1.00

B.

Treatment	%CD4$^-$CD8$^-$	%CD4$^-$CD8$^+$	%CD4$^+$CD8$^-$
0 mg	70.4 ± 1.6	14.7 ± 1.1	13.2 ± 0.8
5 mg	81.8 ± 2.4	7.8 ± 1.7*	8.5 ± 1.3**
50 mg	78.5 ± 2.1	9.0 ± 1.2	10.4 ± 0.9**

$n = 5$ mice/treatment, mean ± SEM, $^*p \leq 0.05$, Unpaired *t*-test with Welch's correction, CD4 0 mg vs 5 mg.
$^{**}p = 0.0014$; CD4 0 mg vs 50 mg $^{**}p = 0.006$; CD8 0 mg vs 5 mg $^*p = 0.01$.

2.8. Splenic lymphocyte proliferation

Splenocytes (5×10^5 cells/100 μL) were added to triplicate wells of a Costar 96-well tissue culture plate (Corning) containing either 100 μL media, 0.1, 0.5, or 1.0 μg Con A (T cell mitogen) in complete media and incubated at 37 °C, 5% CO2 to assess spontaneous and T cell proliferation. Following 64 h incubation, 20 μL of AlamarBlue™ (Thermo Fisher Scientific) were added to each individual well and the plate was incubated for an additional 8 h at 37 °C, 5% CO_2. The plate was read on a BioTek Synergy 4 (BioTek, Winooski VT) plate reader and results generated were delta optical density (Δ OD) of absorbance 570 nm and 600 nm.

2.9. Splenic supernatant for cytokine analysis

Splenocytes (2.5×10^6 cells/0.5 mL) were added to duplicate wells of a Costar 24 well flat-bottom tissue culture plate (Corning) containing 0.5 mL of complete media or 0.5 mL of 1.0 μL Con A in complete media and incubated at 37 °C, 5% CO2 for 48 h. Following the incubation, supernatant was collected into 1.5 mL microcentrifuge tubes and sub-aliquoted at equal volumes into two 1.5 mL microcentrifuge tubes. All samples were frozen at −80 °C until use.

Table 2
Splenic leukocytes: 28-day vaccine dietary trial.

A.

Treatment	CD4$^+$ CD25$^-$	CD4$^-$CD25$^+$	CD4$^+$ CD25$^+$
0 mg PBS	19.7 ± 1.89	1.14 ± 0.14	1.36 ± 0.12
0 mg vaccine	20.7 ± 2.33	1.44 ± 0.21	1.66 ± 0.14
5 mg PBS	16.3 ± 0.77	1.40 ± 0.12	1.50 ± 0.13
5 mg vaccine	17.1 ± 0.99	1.28 ± 0.05	1.40 ± 1.30
50 mg PBS	20.6 ± 1.37	1.24 ± 0.11	1.38 ± 0.16
50 mg vaccine	19.9 ± 0.87	1.16 ± 0.11	1.50 ± 0.09

B.

Treatment	CD8$^+$ CD25-	CD8$^-$ CD25$^+$	CD8$^+$ CD25$^+$
0 mg PBS	8.20 ± 0.74	2.04 ± 0.17	0.06 ± 0.02
0 mg vaccine	8.84 ± 0.71	2.34 ± 0.19	0.10 ± 0.03
5 mg PBS	8.60 ± 0.92	2.08 ± 0.20	0.08 ± 0.02
5 mg vaccine	8.84 ± 0.71	2.34 ± 0.19	0.10 ± 0.03
50 mg PBS	8.84 ± 0.34	2.16 ± 0.05	0.10 ± 0.00
50 mg vaccine	9.66 ± 0.49	2.42 ± 0.20	0.18 ± 0.13

C.

Treatment	CD45RB$_{220}$$^+$ MHCII$^-$	CD45RB$_{220}$$^-$MHCII$^+$	CD45RB$_{220}$$^+$ MHCII$^+$
0 mg PBS	12.0 ± 0.52	2.50 ± 0.31	35.1 ± 1.63
0 mg vaccine	15.2 ± 2.62	2.04 ± 0.10	35.9 ± 2.62
5 mg PBS	18.3 ± 0.94***	1.90 ± 0.11	35.0 ± 2.37
5 mg vaccine	13.7 ± 0.67**	1.78 ± 0.26	34.9 ± 3.92
50 mg PBS	12.6 ± 0.77***	2.26 ± 0.39	35.9 ± 2.62
50 mg vaccine	11.3 ± 0.77	2.28 ± 0.12	39.9 ± 1.72

$n = 5$ mice/treatment, mean ± SEM, Unpaired *t*-test with Welch's correction: 5 mg PBS vs 5 mg Vaccine $^{**}p = 0.005$, 0 mg PBS vs 5 mg PBS $^{***}p = 0.0002$, 5 mg PBS vs 50 mg PBS $^{***}p = 0.0006$.

2.10. Splenic impression smear cytologic analysis

Prior to tissue dissociation, each spleen was bilaterally sectioned and one sectioned piece was gently applied to a sterile frosted microscope slide (Corning). The slides were air dried and then manually stained with modified Wrights stain (Sigma-Aldrich) using a 10 min stain/10 min dilute stain protocol. The slides were evaluated by a board-certified clinical pathologist (KM), who was blinded to the treatment groups. The magnification was 500×.

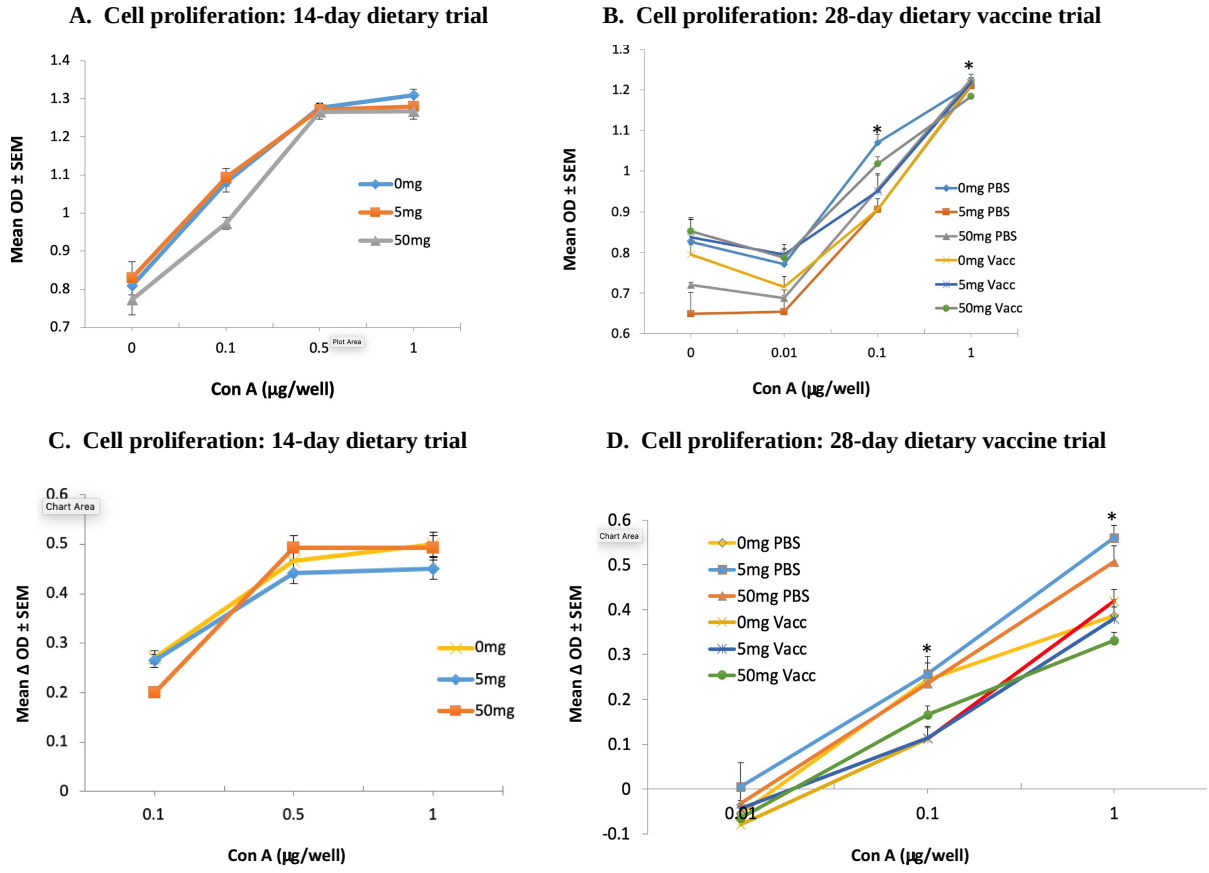

Fig. 2. Splenic proliferation.
Enriched murine splenic cells were incubated at 5×10^5 in 200 µL/well complete medium containing 0, 0.1, 0.5 and 1.0 µg/well Con A for the 14-day I-2865 dietary trial or 0, 0.01, 0.1 and 1.0 mg/well Con A for the 28-day I-2865 vaccine trial for 72 h. AlamarBlue™ was added 8 h prior to the 72 h reading with the reduction in the dye measured spectrophotometrically (570–600 nm). Values were expressed as mean OD ± SEM (Fig. 2A and B) and mean delta (Δ) OD ± SEM (Fig. 2C and D) from 5 replicates/treatment/trial, $^*p \leq 0.05$, Unpaired *t*-test with Welch's correction: Con A 0.1 0 mg PBS vs 0 mg Vacc * $p = 0.03$; Con A 0.1 5 mg PBS vs 5 mg Vacc* $p = 0.01$; Con A 1.0 5 mg PBS vs 5 mg Vacc* $p = 0.04$; Con A 1.0 50 mg PBS vs 50 mg Vac** $p = 0.004$.

2.11. Meso Scale Discovery cytokine assay

A V-PLEX Proinflammatory Panel 1 Mouse Kit (Catalog # K15048D) was purchased from Meso Scale Discovery (MSD, Rockville, MD). Murine serum samples and splenocyte supernatants were analyzed to determine the concentration of the ten cytokines: IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, and TNF-α. All steps and procedures were followed in accordance with the kit manual provided by MSD. The plate was read using the MSD QuickPlex SQ120 and data were analyzed using Workbench 4.0 software.

2.12. Total anti-IgG titers

Murine HA (H5N1 HA)-specific serum antibody titers were measured using an IgG enzyme-linked immunosorbent assay (ELISA). Nunc MaxiSorp high protein-binding capacity 96 well ELISA plates (Thermo Fisher Scientific) were coated with 2 µg/mL recombinant H5N1 HA protein/inactivated H5N1 virus and incubated overnight at 4 °C. Following incubation, plates were washed thrice with 250 µL/well KPL wash solution (KPL, Inc.). Then, 200 µL blocking buffer (5% nonfat dried powdered milk and 0.5% BSA in KPL wash solution (SeraCare, Milford, MA)) were added to each well and incubated for 1 h at 23 °C. The plate was then washed thrice and serial dilutions of serum samples in blocking buffer were added and incubated for 1 h at 23 °C. Following another wash, 100 µL of a 1:1000 dilution of alkaline phosphatase-labeled goat anti- mouse IgG (SeraCare) was added to each well and the plate was incubated for 1 h at 23 °C. Each well then received 100 µL of pNPP phosphatase substrate (KPL, Inc.), and was incubated for 1 h at 23 °C in the dark. For the final step, 100 µL stop solution (5% EDTA in ultrapure water) was added to each well and optical density of 405 nm was determined using a Biotek Synergy 4 plate reader. The IgG titer was determined to be the lowest serum serial dilution with an OD greater than that of the mean of the "no serum" OD plus 3× standard deviations above the mean "no serum" OD.

2.13. Statistics

All data were analyzed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA). A Tukey-Kramer one-way ANOVA was used to determine statistical differences in cellular response in the presence of mitogen stimuli compared to medium alone. Unpaired student *t*-test with a Welch's correction for unequal standard deviations was used to determine the statistical differences between non-vaccine and respective vaccine groups. Significance was determined by a $p \leq 0.05$.

3. Results

3.1. Feed consumption and mouse weight gain

Feed consumption varied throughout the 14-day I-2865 dietary trial study and the 28-day dietary I-2865-vaccine trial study by cage, but not by treatment, which may have been attributed in part, by the composition of the feed tablets. For example, after week 1, mice housed in the 0 mg I-2865 cage, which received vehicle-PBS injection, consumed

Table 3
Serum cytokine concentrations.

A. Serum cytokine/chemokine levels: 14-day feeding trial

Serum (pg/mL)	Baseline	0 mg	5 mg	50 mg
IFNγ	0.23 ± 0.04	0.39 ± 0.07	0.27 ± 0.07	0.40 ± 0.10
IL-10	13.29 ± 2.20	13.57 ± 1.07	14.9 ± 1.27	15.5 ± 2.04
IL-12p.70	< 7.59	< 7.59	< 7.59	< 7.59
IL-1β	0.63 ± 0.22	1.82 ± 1.51	0.50 ± 0.14	8.64 ± 7.93
IL-2	< 0.701	< 0.701	< 0.701	< 0.701
IL-4	0.15 ± 0.02	0.14 ± 0.06	0.11 ± 0.03	0.12 ± 0.02
IL-5	4.02 ± 0.68	3.16 ± 0.52	4.54 ± 0.56	5.83 ± 1.98
IL-6	3.36 ± 0.37	6.24 ± 1.45	8.20 ± 4.15	28.79 ± 15.39
KC/GRO	94.86 ± 10.0	78.32 ± 5.21	79.88 ± 4.98	129.9 ± 17.04**
TNF-α	9.11 ± 1.35	6.90 ± 0.43	8.19 ± 0.42	8.89 ± 1.10

B. Serum cytokine/chemokine levels: 28-day vaccine-feeding trial

Serum (pg/mL)	0 mg PBS	0 mg Vac	5 mg PBS	5 mg Vac	50 mg PBS	50 mg Vac
IFNγ	0.40 ± 0.01	0.32 ± 0.08	0.32 ± 0.04	0.67 ± 0.12	0.34 ± 0.03	0.47 ± 0.08
IL-10	16.2 ± 2.97	13.2 ± 0.92	11.5 ± 0.62	15.1 ± 1.23*	10.8 ± 1.09	17.5 ± 1.38*
IL-12p.70	< 7.59	< 7.59	8.18 ± 1.34	< 7.59	8.88 ± 1.24	< 7.59
IL-1β	0.53 ± 0.07	0.29 ± 0.04	0.59 ± 0.07	0.31 ± 0.04	0.58 ± 0.11	0.61 ± 0.14
IL-2	< 0.701	< 0.701	< 0.701	< 0.701	< 0.701	< 0.701
IL-4	0.17 ± 0.02	< 0.100	0.10 ± 0.03	0.19 ± 0.06	0.10 ± 0.03	0.11 ± 0.04
IL-5	4.13 ± 0.52	3.41 ± 0.26	4.10 ± 0.83	5.02 ± 0.79	4.68 ± 1.02	3.94 ± 0.73
IL-6	4.64 ± 0.80	3.77 ± 1.35	2.76 ± 0.37	4.77 ± 0.67*	2.43 ± 0.29	4.03 ± 0.69
KC/GRO	86.0 ± 13.2	62.6 ± 7.45	70.1 ± 7.04	72.74 ± 4.70	59.1 ± 4.26	90.6 ± 12.6
TNF-α	7.26 ± 0.73	5.88 ± 0.57	8.05 ± 0.79	8.59 ± 1.05	6.29 ± 0.95	8.46 ± 0.94

$n = 5$ mice/treatment, mean ± SEM, $^{*}p \leq 0.05$, Unpaired *t*-test with Welch's correction.

323.6 g of feed/5 mice and the next week the same cage consumed 580.32 g of feed/5 mice. During these same two weeks, the 0 mg vaccine cage consumed 509.74 g and 461.12 g of feed/5 mice, respectively. Closer inspection of the cages revealed that there was a large amount of feed particles mixed in with the litter in the bottom of each cage. To account for this, the change in cage weight was monitored the following week. The 0 mg I-2865-PBS cage consumed 577.55 g of feed however the cage weight changed by 556.04 g suggesting a measurable amount of feed had been wasted by the mice.

There was no significant difference between average mouse weight gain/cage throughout the 14-day I-2865 dietary trial study or the 28-day I-2865 dietary vaccine trial study. The baseline average mouse weight ± SEM across all nine cages at the start of the study was 16.6 ± 0.1 g. At the end of the two-week feed study, there was no significant difference across the three treatment cages with an average mouse weight ± SEM of 18.1 ± 0.3 g. At the conclusion of the 28-day I-2865-vaccine trial, the average weight ± SEM per mouse was 20.8 ± 0.3 g for the 6 cages inferring that regardless of diet or vaccine administration, all mice experienced a positive weight gain.

3.2. Organ/body weight ratio and cellularity

Analysis of the largest murine secondary lymphoid organ revealed that there was no significant difference in spleen/body weight ratios for the mice in the 14-day dietary trial (0 g: 0.006 ± 0.001, 5 g: 0.007 ± 0.001, 50 g: 0.007 ± 0.001) or in the 28-day vaccine dietary trial. Post dissociation and enrichment, splenic cellularity was significantly increased in both the 5 mg and 50 mg diets during the 14-day dietary trail (Fig. 1A.) For the 28-day vaccine dietary trial, diet had no effect on splenic cellularity in the non-vaccinated groups. Surprisingly, in the vaccine groups, splenic cellularity tended to be lower than the non-vaccine groups reaching significance at the 5 mg and 50 mg I-2865-vaccine groups inferring that the vaccine mobilized the lymphocytes out of the spleen (Fig. 1B). Cell viability post dissociation was > 85%.

3.3. Flow cytometry

B cell and T cell flow data from the 14-day dietary trial showed that mice fed an I-2865 supplemented diet yielded different findings based on cell phenotype. The splenic CD45RB220^{+} B cell populations were comparable (5 mg I-2865: 34.5 ± 3.28% and 50 mg I-2865: 34.5 ± 1.48%) to the 0 mg I-2865 control diet (39.5 ± 1.98%). Interestingly, the splenic CD90^{+} pan T cell populations were numerically declined for both the 5 mg and 50 mg I-2865 groups, but was not significant ($p = 0.066$) (Table 1A). Analysis of the T cell subsets revealed that the CD4+ T cells were significantly decreased in both the 5 mg and 50 mg I-2865 dietary groups plus the CD8+ T cells were also significantly decreased in the 5 mg I-2865 group (Table 1B).

In the dietary I-2865-vaccine study with an expanded cell surface marker panel, the splenic CD4^{+} cell (Table 2A) and CD8^{+} cell populations (Table 2B), activated and non-activated, were numerically similar with no significant differences across treatment. The 5 mg I-2865-PBS CD45RB220^{+}MHCII^{-} cell population was significantly higher than 0 mg I-2865 -PBS group while the 50 mg I-2865 -PBS group had approximately the same percent expression of CD45RB220^{+} MHCII^{-} cells as 0 mg I-2865-PBS, but was significantly < 5 mg I-2865-PBS group. The 5 mg I-2865 -PBS group was also significantly higher in CD45RB220^{+} MHCII^{-} cells compared to the 5 mg I-2865-vaccine group (Table 2C).

3.4. Splenic proliferation

Con A-induced splenocyte proliferation was statistically analyzed after subtracting the basal cell spontaneous proliferation (cells cultured in media alone) from the corresponding Con A treatments resulting in a Δ mean absorbance. The 14-day feeding trial yielded no significant differences among the groups receiving I-2865 in their diets compared to the 0 mg I-2865 diet (Fig. 2A and C). However, there were significant differences seen in the vaccine study. The three feed treatment PBS groups' Δ mean absorbances were all numerically higher than their corresponding vaccine groups. The 0 mg I-2865-PBS and 5 mg I-2865-

Table 4
Cultured splenic cell secretory cytokine concentrations.

A. Splenic supernatant cytokine levels: 14-day feeding trial

Supernatant-12 day (pg/mL)		0 mg	5 mg	50 mg
IFNγ	Media	29.4 ± 10.3	23.3 ± 6.59	27.6 ± 1.16
	Con A	3250 ± 111	3360 ± 36.4	3330 ± 64.4
IL-10	Media	280 ± 7.35	321 ± 32.50	287 ± 31.0
	Con A	1270 ± 328	1330 ± 270	938 ± 190
IL-12p.70	Media	< 7.59	< 7.59	< 7.59
	Con A	15.3 ± 4.45	11.7 ± 3.20	9.11 ± 3.40
IL-1β	Media	4.70 ± 0.55	5.90 ± 0.54	7.03 ± 1.11
	Con A	24.9 ± 2.74	19.3 ± 1.31	19.8 ± 2.17
IL-2	Media	28.8 ± 1.88	29.7 ± 3.08	26.8 ± 1.39
	Con A	2800 ± 279	2600 ± 222	3330 ± 279
IL-4	Media	1.37 ± 0.53	1.35 ± 0.42	2.18 ± 0.84
	Con A	20.6 ± 1.30	31.7 ± 4.75	31.2 ± 4.16
IL-5	Media	< 0.237	< 0.237	< 0.237
	Con A	17.4 ± 2.57	18.9 ± 3.98	18.4 ± 6.01
IL-6	Media	308 ± 81.4	460 ± 38.4	445 ± 69.8
	Con A	4060 ± 398	4400 ± 537	3920 ± 689
KC/GRO	Media	50.2 ± 10.3	68.4 ± 6.67	60.3 ± 8.61
	Con A	179 ± 25.3	225 ± 30.7	181 ± 45.5
TNF-α	Media	141 ± 16.3	153 ± 8.97	149 ± 12.6
	Con A	1430 ± 98.9	1150 ± 67.9*	981 ± 36.1*

B. Splenic supernatant cytokine levels: 28-day feeding vaccine trial

Supernatant-28 day (pg/mL)		0 mg PBS	0 mg Vac	5 mg PBS	5 mg Vac	50 mg PBS	50 mg Vac
IFNγ	Media	8.73 ± 2.14	31.1 ± 9.07	4.11 ± 1.52	113 ± 38.2	13.4 ± 2.59	69.6 ± 35.7
	Con A	7250 ± 780	7250 ± 218	5220 ± 924	7520 ± 698	8890 ± 2290	7000 ± 610
IL-10	Media	595 ± 50.4	828 ± 95.3	526 ± 31.1	1130 ± 89.9	497 ± 33.1	1020 ± 120
	Con A	1370 ± 338	1300 ± 95.9	873 ± 131	1270 ± 124	1010 ± 148	1200 ± 123
IL-12p.70	Media	< 7.59	< 7.59	< 7.59	< 7.59	< 7.59	< 7.59
	Con A	< 7.59	> 7.59	< 7.59	< 7.59	< 7.59	> 7.59
IL-1β	Media	11.5 ± 1.80	10.5 ± 1.40	14.4 ± 2.61	19.0 ± 3.72	13.1 ± 2.64	16.8 ± 3.62
	Con A	18.8 ± 1.13	15.6 ± 1.19	16.7 ± 2.94	17.8 ± 2.18	19.2 ± 2.85	19.3 ± 1.78
IL-2	Media	23.5 ± 3.99	77.9 ± 10.8	15.7 ± 2.20	106 ± 8.48	20.7 ± 2.42	82.0 ± 6.79
	Con A	4920 ± 359	4240 ± 491	3610 ± 409	3390 ± 264	4070 ± 467	4200 ± 182
IL-4	Media	1.76 ± 0.60	3.49 ± 0.26	0.68 ± 0.09	3.04 ± 0.24	1.33 ± 0.13	3.11 ± 0.49
	Con A	44.3 ± 5.58	59.2 ± 2.64	37.9 ± 5.51	57.2 ± 5.15	43.8 ± 4.79	49.4 ± 3.68
IL-5	Media	0.11 ± 0.01	6.64 ± 1.52	0.12 ± 0.04	19.9 ± 3.34*	0.18 ± 0.04	11.5 ± 2.17
	Con A	19.4 ± 2.57	36.4 ± 7.57	39.1 ± 10.2	64.0 ± 9.93	33.6 ± 10.8	37.1 ± 7.97
IL-6	Media	266 ± 42.2	621 ± 37.1	204 ± 34.1	911 ± 80.3	316 ± 22.2	796 ± 152
	Con A	3530 ± 590	3250 ± 234	2360 ± 272	2960 ± 205	3030 ± 292	3270 ± 284
KC/GRO	Media	26.6 ± 2.19	49.7 ± 5.71	35.5 ± 7.04	65.5 ± 12.6	36.2 ± 6.86	65.4 ± 8.35
	Con A	36.8 ± 2.69	54.7 ± 7.93	37.5 ± 3.89	54.6 ± 6.44	45.8 ± 9.32	58.1 ± 2.45
TNF-α	Media	157 ± 4.89	182 ± 8.59	138 ± 13.5	218 ± 14.2	171 ± 14.5	231 ± 27.5
	Con A	1220 ± 83.1	1200 ± 73.4	999 ± 71.0	1070 ± 43.6	1250 ± 151	1260 ± 39.0

n = 5 mice/treatment, mean ± SEM, $^{*}p \leq 0.05$, Unpaired *t*-test with Welch's correction.

PBS groups were significantly higher than the 0 mg Vaccine and 5 mg Vaccine groups, respectively. The 5 mg I-2865-PBS group was also significantly higher than the 5 mg Vaccine group stimulated at 0.1 μg/well and 1.0 μg/well Con A. The 50 mg I-2865-PBS group was significantly higher than the 50 mg I-2865-vaccine group under 1.0 μg Con A stimulation as well (Fig. 2B and D).

3.5. Spleen cytology

All smears from the 6 treatment groups showed similar cytologic findings. The smears were highly cellular and contained a mixture of small lymphocytes and intermediate-sized lymphocytes (when compared to the size of a murine neutrophil), admixed with low numbers of late-stage erythroid precursors (extramedullary hematopoiesis) and rare monocytes/macrophages. There were moderate numbers of erythrocytes in the background and neutrophils appeared proportionate to the degree of hemodilution.

3.6. Chemokine/cytokine levels

A multiplex cytokine/chemokine platform consisting of 10 analytes was employed to assess the impact of dietary I-2865 on systemic immunity as analyzed via serum or following immune activation via the use of a vaccine (H5N1 HA). Additionally, splenic lymphocytes were harvested at each trial termination (14-day and 28-day) and cultured in the presence or absence of a T cell mitogen (Con A) to assess whether dietary I-2865 modulated cytokine release. Serum 14-day I-2865 dietary trial cytokine levels were largely comparable across all 3 treatment groups as well as with the baseline samples (Table 3A). The only exception was KC/GRO, which was significantly increased in the 50 mg I-2865 group. In the 28-day I-2865 vaccine trial, eight of the ten serum cytokines were comparable to the 0 mg I-2865-PBS group. Serum IL-10 concentrations of the 5 mg and 50 mg I-2865-vaccine groups were increased when compared to the 5 mg and 50 mg I-2865-PBS groups, respectively. Further, serum IL-6 concentrations were increased in the 5 mg I-2865-vaccine group when compared to the 5 mg I-2865-PBS group (Table 3B).

Analysis of the splenic cell culture supernatants from the 14-day

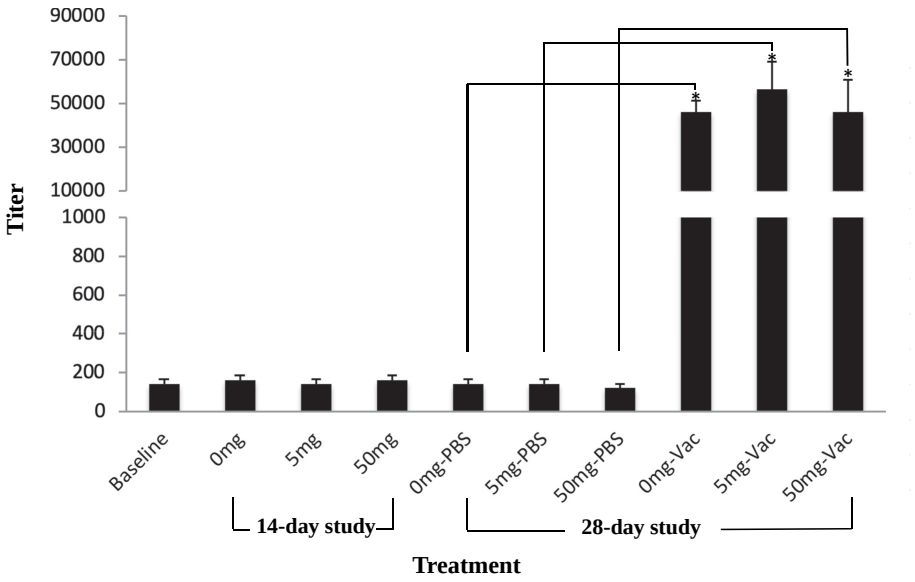

Fig. 3. H5N1 HA-specific anti-IgG titer.
Peripheral blood (~150–200 μL) was collected from the orbital sinus, allowed to clot for 60 min at 23 °C, centrifuged at 8000 ×*g* and the sera collected. An ELISA assay was performed to detect and measure IgG anti-H5N1 HA-specific antibodies (Fig. 3), $^{*}p \leq 0.05$, Unpaired *t*-test with Welch's correction.

feeding trial yielded the expected significant increases in cytokine levels between cells cultured in media alone versus cells cultured with Con A. When the cytokine levels were compared across diet, they were comparable to the 0 mg control diet with the exception of TNF-α for the Con A-stimulated cells, which showed a significant reduction in TNF-α with increasing I-2865 in the diet (Table 4A). Splenic cell culture supernatants from the 28-day vaccine trial showed comparable robust significant increases in cytokine levels between cells cultured in media alone compared to the cells cultured in Con A. Further, there were significant increases in select cytokines levels between non-vaccine and vaccine groups within the same diet. However, there were no significant differences in cytokine levels across the I-2865 diet groups with the exception of IL-5 in the 5 mg vaccine group which was significantly increased from the 0 mg vaccine group (Table 4B).

3.7. H5N1 HA-specific anti-IgG titer

The baseline anti-H5N1 HA serum IgG titers ranged from 1:100 to 1:200. The 0 mg, 5 mg, and 50 mg I-2865 treatment groups from the 2-week study and the 0 mg, 5 mg, and 50 mg I-2865-PBS treatment groups from the 28-day vaccine trial were also comparable to baseline values with a range of 1:100–1:200. Mice receiving the H5N1 HA vaccine had a significant increase in the anti-H5N1 HA serum IgG titers across all three treatment groups (0 mg I-2865-vaccine, 5 mg I-2865-vaccine and 50 mg I-2865-vaccine) compared to the non-vaccinated groups. In the 0 mg I-2865-vaccine treatment groups the IgG titers ranged from 1:25600 to 1:51200, whereas in the 5 mg I-2865-vaccine and 50 mg I-2865-vaccine treatment groups, the IgG titers ranged between 1:25600 to 1:102400. Interestingly, there was no significant difference in IgG titers between I-2865 dietary groups that were vaccinated (Fig. 3).

4. Discussion

As previously stated, I-2865 is made up of hemicellulose compounds with the arabinoxylans having the most immune stimulating properties [13]. In one study, wheat arabinoxylan hydrolyzates modulated nitric oxide production in the RAW macrophage cell line by increasing production in the presence of lipopolysaccharide [13]. Another study examining the effects of wheat arabinoxylans showed that the compound increased NK cell activity and had adjuvant like properties in mice injected with ovalbumin [15]. The largest area of research with arabinoxylans has focused on the beneficial effects with the gut microbiota, gut immunity, and control of diseases such as chronic diarrhea [16–18]. These data have shown that arabinoxylans have a wide degree of immunomodulatory properties.

I-2865 in the feed did not cause any significant or numerical differences in mouse weight throughout the study. It was uncertain at the start of the study if the compound would be more palatable than control diet alone, however the feed consumption and weight data indicated that this was not the case. The spleen/bodyweight ratio was also not altered by I-2865 in either study, however total leukocyte cellularity in the spleen was different, but cell viability was unaffected. Since the spleen is the largest secondary lymphoid organ in mammals, if circulating lymphocyte populations were modulated by I-2865 then corresponding changes should be observed in the spleen as well.

In the 14-day trial, splenic cellularity increased with increasing concentration of dietary I-2865, while cell viability was comparable across all 3 groups. Interestingly, flow cytometric data from the 14-day feed study showed that the percentage of splenic $CD4^+$ T-cell population in the 5 mg and 50 mg I-2865 groups were significantly lower than the 0 mg group. Further, $CD8^+$ T cells were numerically decreased in the 5 mg and 50 mg treatment group compared to the 0 mg group suggesting that I-2865 may enhance lymphocyte migration out of the spleen. This seems to support the study by Weeks and Perez (2009) who reported that I-2865 increased circulating lymphocyte populations by 18% [14]. In the 28-day vaccine study, splenic cellularity numerically decreased in the vaccine groups reaching significance in the 5 and 50 mg groups. Although the flow cytometric data from the 28-day vaccine study showed no significant differences in percentages of total T cell subset populations between the 0 mg, 5 mg, or 50 mg I-2865 treatment groups, when calculated against total cellularity, the absolute numbers of these T cell populations were decreased (data not shown). Thus, I-2865 appears to modulate the mobility of the splenic T cell populations and thus, warrants further investigation.

The pan murine B cell marker analysis showed that the percentage of B cell populations were not altered in the 14-day feed study as compared to the T cells. Additionally, the vaccine data revealed the B cell subset distribution was not affected except for a select subset in the 5 mg I-2865 PBS group. The $CD45R_B220^+MHCII^-$ subset in this group was significantly higher than either the 0 mg and 50 mg I-2865 PBS mice or the 5 mg I-2865 vaccine mice. Presently, there is no clear rationale for this observation and it will need to be explored in the future. Further, the combination of I-2865 and the vaccine did not appear to adversely impact the percentages of the B cell population in the spleen, which is comparable to results from our previous work [19]. Still, similar to the splenic T cell populations, absolute numbers of splenic B

cells were decreased in the I-2865 vaccinated mice.

As was predicted, serum IgG titers were significantly higher in mice that received the H5N1 HA vaccine compared to non-vaccinated mice [20]. Still, there were no significant differences among the three I-2865 diet vaccinated groups. This would suggest that I-2865 does not have adjuvant-like qualities since it did not enhance B cell antibody production when compared to the vaccine-alone group. These results differed from the previous work conducted in our lab where the homeopathic compound Engystol® significantly increased the serum IgG titer in mice that received 10 tablets of Engystol per liter [19]. It is worth noting that, in the present study, I-2865 delivery was via the feed and in the Engystol study, the homeopathic compound was dissolved and delivered via the water. This mode of delivery could have had an impact on the results.

In summary, dietary I-2865 did not overtly alter the murine immunity during the 14-day feed trial. These results are important because it demonstrates that I-2865 does not have immunomodulatory properties in the absence of antigenic stimulation as it would not be prudent to provide a supplement that constantly stimulates the immune system. Unfortunately, based on the 28-day vaccine trial, I-2865 did not enhance antigen-specific immune response when mice were challenged with the H5N1 vaccine. Spontaneous and T cell mitogen specific cellular proliferation remained unaltered and overall cytokine production was not significantly impacted. Further, splenocyte percentages of lymphocyte cell populations were not altered and IgG anti-H5N1 HA antibody titers were not increased by I-2865. The only noteworthy observation was that absolute numbers of the splenic lymphocytes were decreased suggesting altered migration. Collectively, these results indicate that I-2865, as delivered in formulated feed, does not significantly impact systemic immune response when challenged via a vaccine. One of the aspects to consider for this study is that the supplement was added into the diet prior to processing and it might be possible that the effects of processing the diet (extraction, heating, etc.) might have negatively impacted the efficacy of the herbal remedy or chemical compounds in the supplement. On the other hand, cytologic analysis of the spleen did not reveal any detrimental effect of the supplement even in the high dose group. Thus, it can be concluded that the supplement appears to be a safe regarding effects on the immune system. Different modes of delivery of this supplement need to be evaluated in future studies.

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