Nutritional Formula Improved Immune Profiles of Seniors Living in Nursing Homes

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OBJECTIVES: To assess whether an experimental nutritional formula (EXP) supports immune function in seniors living in long-term care facilities.

DESIGN: Prospective, randomized, double-blind, controlled trial conducted September 2002 through January 2003.

SETTING: North central Florida nursing homes.

PARTICIPANTS: Subjects aged 65 and older (n = 157).

INTERVENTION: Subjects received 240 mL/d of EXP or standard liquid nutrition (CON) for 4 weeks before and 6 weeks after an influenza vaccination.

MEASUREMENTS: Influenza vaccine antibody responses, immunophenotyping, lymphocyte activation, cytokines, and clinical measures (fever, number of prescribed antibiotics).

RESULTS: Ninety-two subjects (n = 40, CON; n = 52, EXP) completed the study. Geometric mean antibody titers were similar between groups, yet the percentage of subjects with H1N1 antibody titers greater than 100 postvaccination was higher in the EXP group than in the CON group (43% vs 23%, $P = .047$). Similar trends were found for the percentage of subjects (intent to treat) with fourfold increases against the B/Hong Kong component (64% vs 46%, $P = .09$) or with H3N2 antibody titers of 40 or more (97% vs 89%, $P = .06$). EXP subjects had higher levels of influenza-activated lymphocytes ($CD69^+$and $CD25^+$). Cytokine production after mitogen activation was lower in EXP than CON subjects (interleukin (IL)-6: 20 ± 3 vs 29 ± 3 ng/mL, $P = .045$; IL-10: 310 ± 60 vs 603 ± 140 pg/mL, $P = .06$). Fewer EXP subjects were treated for fever (5% vs 16%, $P = .02$) or prescribed antibiotics (7 vs 11 new antibiotics/100 days of study, $P = .06$).

CONCLUSION: Seniors consuming the EXP formula demonstrated enhanced immune function, indicated by increased influenza vaccine response and lymphocyte activation, less fever, and fewer newly prescribed antibiotics than those consuming a standard ready-to-drink nutritional supplement. **J Am Geriatr Soc 54:1861–1870, 2006.**

Key words: nutrition; seniors; frail older people; immunity; antibody response; antibiotics; influenza

Aging, frailty, and chronic diseases are associated with impaired immune function and are compounded by immune dysregulation from malnutrition.[1,2] Several immune parameters decline with age: lymphocyte activation/proliferative responses to mitogens, Fas-mediated apoptosis, helper T-lymphocyte function, B-lymphocyte numbers, delayed-type skin hypersensitivity, ability to generate high-affinity antibodies, and NK-cell cytotoxicity.[3–5] By contrast, other aspects of immune function increase with age: memory T-lymphocytes, Th2 cytokine profiles, and concentrations of autoantibodies.[4,6] These age-related immune changes contribute to greater risk of infection in frail seniors.

Residents of long-term care (LTC) facilities or nursing homes are particularly vulnerable to infection. In addition, LTC facilities provide environments that promote infectious outbreaks (i.e., influenza).[7–9] More importantly, residents who are infected experience high rates of mortality.[10–12] Because immune changes from aging and malnutrition are similar, nutrient supplementation may improve immune status and clinical outcome in frail seniors.[13,14]

In a relatively healthy population of seniors, it was previously found that a nutritional formula enhanced immune function as measured by antibody response to the influenza vaccine and reduced the number of days of symptoms of upper respiratory tract infections.[15] This formula contained vitamins, minerals, and nutrients important for immune function (protein, antioxidants (vitamins E, C, β-carotene), selenium, zinc, fructo-oligosaccharides (FOS), and structured triacylglycerol). In the present study, various

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markers of immune cell function were examined from a more-frail (potentially more-nutritionally deficient and immune dysregulated) population to explore mechanisms of nutrition-mediated immune benefit. Unlike the previous study, smokers and diabetics were included, which represented a more-heterogeneous population. Clinical outcomes examined included fever and newly prescribed antibiotics. Tolerance of the immune formula (EXP) was compared with that of a commercially available liquid nutritional formula (CON).

METHODS

Subjects and Design

The institutional review board of the University of Florida (Gainesville, FL) approved this randomized, double-blind, controlled, parallel study of frail seniors residing in seven LTC facilities in north central Florida fed EXP or an isonitrogenous/isoenergetic standard liquid nutritional formula (CON; EnsurePlus®, Abbott Laboratories, Columbus, OH). The study was conducted in accordance with the Helsinki Declaration of 1975 and 1983 revision.

Informed consent was obtained from men and women aged 65 and older (n = 157) with a body mass index (BMI) less than 30 kg/m^2. Subjects agreed to discontinue consumption of vitamin, mineral, herbal, or dietary supplements with the exception of calcium, vitamin D, iron, fiber supplements, or vitamin B_{12} injections. Inclusion also required eligibility to receive the U.S. Public Health Service influenza vaccine (2002–03) containing 15 μg of each of the following hemagglutinin antigens: A/Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Hong Kong/1434/2002 (Fluzone, Aventis Pasteur, Swiftwater, PA). Exclusion criteria included known intolerance to study formulas or vaccine components, immune system insufficiency or disease, severe swallowing disorder, dietary protein restriction, dialysis, current cancer radiation or chemotherapy treatment, and prednisone or prednisolone therapy greater than 10 mg/d. Subjects who refused the influenza vaccination at Week 4 were not included in the analysis.

Before randomization, a nurse practitioner examined subjects and recorded medical histories, current medications, and dietary supplement intake. Body weight was obtained using calibrated scales; height was measured or obtained from medical records. Group assignments for each facility were prepared based on stratifications (BMI (<22 kg/m^2 or 22–30 kg/m^2), diabetes mellitus (yes/no), and smoking status (smoker/nonsmoker) and were balanced between EXP and CON. Upon randomization via sealed envelope, each subject was given a study number and group assignment. At baseline and for the duration of the 10-week study, subjects were instructed to consume one can per day (240 mL) of CON or EXP. Study coordinators distributed formulas, encouraged consumption, and recorded daily intake to the nearest quarter can. The formulas, manufactured under good manufacturing practices (Abbott Laboratories), were formulated to be isonitrogenous/isoenergetic, with similar appearance, aroma, and flavor. CON was EnsurePlus, whereas EXP contained higher levels of antioxidants (vitamins E, C, and β-carotene), B vitamins, selenium, and zinc than CON. EXP also contained structured triacylglycerol and a prebiotic (FOS). The nutrient content of EXP has been previously described.[15] Both formulas were labeled similarly to prevent subjects, staff, or others from identifying them. In addition to study formula intake records, compliance was assessed by measuring serum α-tocopherol and β-carotene concentrations (Craft Technologies, Inc., Wilson, NC) as described previously.[15] It was anticipated that subjects might not be able to consume all of the formula (240 mL) daily, so in an effort to evaluate the effect of formulas, an evaluable subject was defined as consuming an average daily intake of formula of 75% of the 240 mL (180 mL) or more. Intent-to-treat subjects were those who were randomized, vaccinated, and compared to make sure that the groups were comparable in study variables at baseline.

Nonfasting blood samples were obtained at baseline, vaccination (Week 4), and study end (Week 10). At baseline and study end, blood was drawn for a comprehensive metabolic profile, hemoglobin A1c, complete blood cell count (CBC) with differential, cytokine studies, lymphocyte activation markers, immune cell phenotypes, and compliance variables (serum α-tocopherol and β-carotene concentrations).

Antibody Response

Response to influenza vaccine was used as a marker of immune function. Peak responsiveness to the influenza vaccine was targeted at 6 weeks postvaccination, because some seniors seroconvert after the typical time point of 4 weeks.[16,17] Blood samples for vaccine responses were collected at vaccination and study end. Serum was separated and stored at –20°C. Influenza antibody titers from all time points for a single subject were measured simultaneously using a modified hemagglutination inhibition procedure[15] and vaccine antigens from the World Health Organization Collaborating Center for Influenza, Centers for Disease Control and Prevention (Atlanta, GA).[18]

Mitogen and Antigen Responsiveness

Influenza Antigen Preparation for Cellular Stimulation Assays

Influenza antigen for cellular stimulation assays was derived from influenza vaccine (same lot number and manufacturer) used to vaccinate the subjects. Before use in cell culture, the vaccine was dialyzed to remove preservative, potentially toxic to in vitro cell cultures, using Pyroclean (AlerChek Inc., Portland, ME) depyrogenated Amicon Ultra-15 10,000-molecular-weight-cutoff diafiltration units (Millipore Corp., Bedford, MA). Volume of dialyzed vaccine was brought to original volume with Dulbecco's phosphate-buffered saline (DPBS; BioWhittaker, Walkersville, MD). Hemagglutination of chicken erythrocytes (Colorado Serum, Denver, CO) indicated that influenza antigen activity of dialyzed vaccine was comparable with that of native vaccine.

Preparation of Samples for Determination of Lymphocyte Activation Markers

Blood samples for immunophenotyping and cytokine production were collected at baseline, vaccination, and study end. Samples, packaged to maintain room tempera-

ture (i.e., packed in an insulated container within a Polyfoam shipping container), were transported to Abbott Laboratories for processing within 24 hours of collection. For cellular stimulation, whole blood samples were cultured for 48 hours at 37°C and 5% carbon dioxide (CO_2) with phytohemagglutinin (PHA-L, Sigma-Aldrich, St. Louis, MO, 10 μg/mL), influenza antigen (final dilution 1:50 in complete culture medium (RPMI 1640; BioWhittaker) with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT)) or complete-culture medium alone in a humidified incubator. After the stimulation period, samples were processed and analyzed using standard methods for flow cytometry.[19] Monoclonal antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC) and appropriate isotype control antisera combinations were used to identify CD25-, CD69-, CD8-, and CD2-positive or -negative cells. After staining, erythrocytes were lysed with a 0.15 M ammonium chloride solution; remaining leukocytes were washed using DPBS with 5% fetal bovine serum and fixed with 1% paraformaldehyde solution. Samples were evaluated, using a Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA) FACSCalibur™ or a BDIS FACSort™ flow cytometer; data were analyzed using BDIS Attractors software. Approximately 10,000 lymphocytes were counted for each determination. Lymphocyte subsets were expressed as percentages of total lymphocytes and absolute cell numbers.

Cytokine Production After Mitogen and Antigen Stimulation

Peripheral blood polymorphonuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma-Aldrich). PBMCs were adjusted to a concentration of 1×10^6 cells per mL with complete-culture medium and cultured with 10 μg/mL PHA-L, influenza antigen (final dilution 1:50 as above) or complete-culture medium alone for 48 hours in a humidified 37°C, 5% CO_2 incubator. After the stimulation period, cell cultures were centrifuged; supernatants were removed and stored at −80°C.

Cytokine analyses were performed using the Cytometric Bead Array (BD-Pharmingen, San Jose, CA) assay, which allows for simultaneous quantification of six cytokines (interferon-gamma (IFN-γ), tumor necrosis-α (TNF-α), interleukin (IL)-6, IL-10, IL-4, and IL-2) from one sample. Supernatants from all time points for a single subject were assayed on the same day to eliminate day-to-day assay variation.

Immune Cell Phenotypes

Blood samples were processed and analyzed using standard methods for flow cytometry.[19] Optimally titrated monoclonal antibodies, labeled with FITC, PE, PerCP or APC fluorochromes (BDIS), and appropriate isotype control antisera were mixed in three or four fluor combinations to identify CD3-, CD14-, and CD45-; CD56-/CD57-, CD28-, CD8-, and CD3-; CD45RA-, CD45RO-, CD4-, and CD8-; or CD56-, CD16-, CD19-, and CD3-positive and -negative cells in plasma-free blood. Approximately 10,000 events per sample were evaluated and expressed as a percentage of lymphocytes and absolute cell numbers.

Safety Variables and Clinical Outcomes

Blood was drawn at baseline and study completion for determination of safety variables (comprehensive metabolic profile, complete blood count (CBC) with differential, and hemoglobin A1c) and processed by Covenant Healthcare Laboratory, Inc. (Mayo, FL). Adverse events and changes in clinical status were noted at time of incidence and followed for 1 week after the final visit. Study coordinators recorded medications, including newly prescribed antibiotics, daily. Body weight was measured at each time point.

Statistics

All randomized and influenza-vaccinated subjects were included in an intent-to-treat analysis. A priori it was determined that subjects who were vaccinated, consumed a daily average intake of 180 mL or more, and completed at least 60 of the 70 days of study were considered protocol compliant and evaluable. Results are presented for intent to treat, but in an attempt to understand the effects of the experimental formulation, there was a focus on the compliant group (the group that consumed at least 75% of the products on a daily basis). Primary variables (response to influenza vaccine) were analyzed using repeated measures analysis implemented with the SAS procedure PROC MIXED (SAS Institute, Inc., Cary, NC). Comparisons were made between groups from subjects who achieved a >180 antibody (H1N1) or ≥ 40 antibody (H3N2 and B component) titer or percentage of subjects who experienced more than fourfold increase in antibody level. Analyses of lymphocyte populations were made using analysis of covariance using prevaccination levels as covariates with main effect for treatment. Factors in the model included feeding, visit, feeding-by-visit interaction, blocking for site, and covariate antibody level at baseline. Lymphocyte subsets, activated lymphocytes, CBC with differential, metabolic profile, and cytokines were compared between the two groups using the same model and cofactors as the primary variables. During the conduct of the study, a few blood samples at the final time point were inadvertently shipped to another location or delayed because of severe weather in transport and then shipped to Abbott Laboratories for analysis, resulting in samples being processed more than 24 hours after final blood draw. Because cytokine expression and activation may occur with holding blood, an analysis was performed with all samples (intent-to-treat and evaluable) and without the samples held for more than 24 hours. Pattern of activation was similar, but extended holding time influenced cytokines. Serum nutrient levels were compared. Clinical outcomes (fever and number of newly prescribed antibiotics) between groups were evaluated using the Cochran-Mantel-Haenszel comparison. *P*-values between .05 and .1 indicate marginally significant differences (clinically important), whereas *P*-values less than .05 indicate significant differences between the EXP and CON groups. Data are reported as mean ± standard error of the mean unless otherwise noted.

RESULTS

Subjects

One hundred fifty-seven seniors (CON = 76; EXP = 81) consented or assented (legally authorized representative

consented for subject) to participate from September 2002 through January 2003. One hundred forty-eight subjects were randomized, vaccinated, and included in intent-to-treat analysis (CON = 72; EXP = 76). Randomization yielded no differences in stratifications or sex between groups, although the mean age of subjects in the CON group was 2.6 years older than subjects randomized to EXP ($P = .03$), and more subjects in the EXP group had a history of cancer ($P = .03$, Table 1). There was a trend toward more subjects in the evaluable group of EXP who had experienced a stroke ($P = .06$). Ninety-two subjects CON = 40; EXP = 52) were considered compliant and evaluable. Reasons for noncompliance were inadequate intake (< 180 mL average daily consumption of formula, CON = 27; EXP = 17), discharge from LTC (CON = 1; EXP = 0), consent withdrawal (CON = 2; EXP = 5), death (CON = 2, EXP = 2). Serum α-tocopherol and β-carotene increased significantly from baseline to final blood draws in evaluable and intent-to-treat cohorts of EXP but not CON subjects, $P < .01$ (Table 2).

Reasons for study discontinuation did not differ between groups. More subjects with a BMI less than 22 kg/m^2 (n = 17 vs n = 6, $P = .05$) and with diabetes mellitus (n = 13 vs n = 6) remained in the evaluable EXP group than in the evaluable CON group (Table 1). Also, at the screening visit, more subjects in the evaluable EXP group reported consuming nutritional/herbal supplements.

Antibody Response

Although there were no significant differences in geometric mean titers observed between groups, values for evaluable and intent-to-treat subjects for all three vaccine components tended to be higher in the EXP group (Table 3). The percentage of evaluable subjects with antibody titers greater than 100 for H1N1 (A/Caledonia) at 42 days postvaccination was higher in the EXP than the CON group ($P = .047$; Table 3), with a similar trend for subjects in the intent-to-treat analysis ($P = .09$). Trends were also observed in the percentage of evaluable subjects achieving fourfold increase or more over vaccination levels (response to vaccine) in antibody titer against the B/Hong Kong component ($P = .09$) in the EXP vs CON group. A higher percentage of intent-to-treat subjects achieving an antibody titer of 40 or more against H3N2 (A/Panama) component favoring the EXP group ($P = .06$) was also observed.

Mitogen and Antigen Responsiveness

Lymphocyte Activation Markers

White blood cell count (EXP: 6,880 ± 245 cells/μL, baseline; 6,900 ± 279 cells/μL, study end vs CON: 7,430 ± 380 cells/μL, baseline; 7,130 ± 367 cells/μL, study end), percentage of lymphocytes (EXP: 24.4 ± 0.9%, baseline; 24.2 ± 1.0%, study end, vs CON: 25.6 ± 1.5%, baseline; 26.0 ± 1.4%, study end), and T-lymphocyte subsets ($CD4^+$or $CD8^+$) did not differ between groups of evaluable subjects, but cell-activation-marker (CD69 and CD25) expression specific to influenza antigens was markedly higher in evaluable subjects in the EXP group (Figure 1A). T-lymphocyte populations contributing to these activation differences were $CD2^-$ (T-lymphocytes, Figure 1B) and $CD2^+CD8^-$ subsets (helper T-cells, Figure 1C). Expression of activation markers in influenza-activated lymphocytes from subjects in the intent-to-treat group were not different between study groups with the exception of a higher trend in non-T-cells expressing CD69 and CD25 in the EXP group (73 ± 7 vs 57 ± 7, $P = .10$) and similarly in the number of total lymphocytes expressing CD69 and CD25 (162 ± 11) versus the CON group (135 ± 12; $P = .09$). No differences were seen between the EXP and CON groups in cellular activation to the mitogen PHA for the evaluable or intent-to-treat subjects (data not shown).

Cytokine Production

Because length of time from blood draw influences cytokine expression, samples were analyzed by groups (< 24 hours and all subjects). Upon PBMC stimulation with PHA, significantly lower IL-6 ($P = .045$) accompanied by a similar trend for IL-10 ($P = .06$) was noted in the EXP group at study end for evaluable subjects (Table 4). In mitogen-stimulated PBMCs from intent-to-treat subjects, IL-10 was lower ($P = .04$) at the final visit in the EXP group than in the CON group in samples processed within 24 hours. No other differences in cytokines were noted between groups in the intent-to-treat subjects. Influenza-specific cytokine secretion was low compared with PHA; no significant differences were noted between groups (data not shown).

Immune Cell Phenotypes

Differences were observed at the final time point with respect to lymphocyte phenotyping; in the intent-to-treat groups, a higher percentage of cytotoxic T-lymphocytes (14% vs 13%; $P = .06$) and fewer memory cytotoxic T-lymphocytes (9.5% vs 10.4%, $P = .03$) were found in the EXP group than in the CON group. In the evaluable groups, there were more natural killer T (NKT)-lymphocytes (% and numbers) expressing $CD56^+/CD57^+$, $CD28^+$, and $CD3^+$ in the EXP group than in the CON group (1.07% ± 0.06% vs 0.91% ± 0.07%, $P = .08$; 17 ± 1 vs 15 ± 1 cells/μL, $P = .09$) and more B-lymphocytes (148 ± 7 vs 122 ± 8 cells/μL, $P = .02$), as well as a higher percentage of naïve T-helper lymphocytes (11.3% vs 10.5%, $P = .06$) and a lower percentage of memory cytotoxic T-lymphocytes (8.9% vs 9.8%; $P = .01$).

Safety Variables and Clinical Outcomes: Intent to Treat

A comprehensive metabolic profile, CBC with differential, and hemoglobin A1c were safety measurements, and no mean differences were found between the EXP and CON groups with the exception of hemoglobin A1c and serum glucose levels. Hemoglobin A1c was slightly lower in subjects receiving EXP (5.4% ± 0.08% vs 5.3% ± 0.08%; $P = .06$) at the final blood draw. A similar trend for serum glucose levels was observed in EXP at the final time point (134.3 ± 7.0 mg/dL vs 118.9 ± 4.9 mg/dL; $P = .07$). No differences in serum albumin levels between groups were noted, although there was a slight increase in serum albumin levels from baseline to final time point (Table 2). There were similar numbers of subjects hospitalized during the study (CON = 5, EXP = 5). Body weights were not different between groups, nor were there differences in weight gains over the study (Table 1).

Table 1. Characteristics of Seniors Residing in North Central Florida Nursing Homes for Evaluable* and Intent-to-Treat (ITT)† Analyses

Characteristic	Control	Experimental
Subjects, n		
Evaluable	40	52
ITT	72	76
Sex, male/female, n		
Evaluable	15/25	14/38
ITT	22/50	20/56
Age, mean ± SEM		
Evaluable	85.4 ± 1.0	81.4 ± 1.0‡
ITT	84.7 ± 0.8	82.1 ± 0.8§
Number of medications per subject at baseline, mean ± SEM		
Evaluable	9.7 ± 0.7	10.5 ± 0.7
ITT	10.8 ± 0.5	11.3 ± 0.6
BMI at baseline, kg/m^2, mean ± SEM		
Evaluable	24.2 ± 0.5	23.7 ± 0.5
ITT	22.5 ± 0.5	23.5 ± 0.4
Body weight, kg, mean ± SEM		
Baseline		
Evaluable	65.1 ± 1.9	63.8 ± 1.5
ITT	60.9 ± 1.5	62.3 ± 1.3
Final		
Evaluable	65.4 ± 1.8	64.3 ± 1.6
ITT	60.2 ± 1.6	62.3 ± 1.4
Stratification at baseline, n		
BMI <22 kg/m^2	6‖	17
Evaluable	26	25
ITT		
BMI 22–30 kg/m^2		
Evaluable	34	35
ITT	46	51
Smoker		
Evaluable	1	5
ITT	2	7
Diabetes mellitus		
Evaluable	6	13
ITT	15	18
Consuming nutritional or herbal supplements before study, n (%)		
Evaluable	31 (78)	48 (92)¶
ITT	59 (82)	69 (91)
Comorbidities at screening, n (%)		
Arthritis		
Evaluable	22 (55)	17 (33)#
ITT	36 (50)	29 (38)
Hypertension		
Evaluable	22 (55)	36 (69)
ITT	47 (65)	56 (74)
History of stroke		
Evaluable	12 (30)	27 (52)**
ITT	28 (39)	34 (45)
History of cancer		
Evaluable	4 (10)	11 (21)
ITT	7 (10)	18 (24)††
History of depression		
Evaluable	23 (58)	29 (56)
ITT	48 (67)	48 (63)
Emphysema/asthma/chronic obstructive pulmonary disease		
Evaluable	10 (25)	11 (21)
ITT	21 (29)	22 (29)

* Subjects who consumed a daily average intake of 180 mL or more of formula over the 70-day study were considered to be compliant to the protocol and evaluable.
† Data are not reported from subjects who were randomized but were later excluded for not receiving the influenza vaccine (n = 10).
P = ‡.01, § .03, and ‖ .05, between experimental (EXP) and control (CON) within each column.
¶ P = .25, number of subjects consuming nutritional, herbal, or vitamin/mineral supplements (multi- or single vitamin/mineral supplements) before baseline.
P = #.04, ** .06, †† .03 between EXP and CON within each column.
SEM = standard error of the mean; BMI = body mass index.

Clinical Outcomes

The number of subjects who dropped out of the study or experienced adverse events was similar between groups with the exception of fever. Fewer subjects consuming EXP experienced fever during the study (4/76, 5% vs 12/72, 16%; P = .02).

EXP subjects had 36 courses of newly prescribed antibiotics, accounting for 315 total days of prescribed antibiotics for 76 subjects (7 new antibiotics/1,000 days of study), whereas CON subjects had 55 courses of antibiotics, accounting for 478 total days for 72 subjects (11 new antibiotics/1000 days of study; P = .06). Similarly, there was a trend toward more days of antibiotic use in the CON group (10 days of antibiotics/100 days of study) than in the EXP group (6 days of antibiotics/100 days of study; P = .09). The reasons, for which physicians prescribed antibiotics were urinary tract (CON = 29, EXP = 14), respiratory tract (CON = 18, EXP = 6), and other infections (CON = 7, EXP = 11).

DISCUSSION

This study showed improvement in markers of immune function in a frail, elderly population consuming EXP. Potential mechanisms for the nutritional benefit were examined, and immune improvement was found to be associated with lymphocyte function. Furthermore, evidence of clinical benefit (less fever and antibiotic use) was observed in subjects consuming EXP. This study supports previous findings in less frail seniors and demonstrates that the combination of nutrients affects immune function in older people.[15]

Residents of LTC facilities are at high risk of malnutrition;[2,20,21] therefore, many seniors are given nutritional supplements in pill, liquid, or food form, but little evidence of immunological or clinical benefit of such strategies exists. A few studies have demonstrated improved immunity with nutritional supplementation; for example, one found that dietary supplementation of institutionalized older people (n = 725) with minerals (zinc and selenium) increased antibody titer to the H3N2 component of the

Table 2. Compliance and Serum Concentrations

Variable	CON	EXP
	Mean ± Standard Error of the Mean	
Formula intake per day, mL*		
Evaluable	219 ± 2	220 ± 3
ITT	177 ± 7	184 ± 7
Serum alpha-tocopherol, μg/mL		
Baseline		
Evaluable	14.2 ± 0.8	14.4 ± 0.7
ITT	14.5 ± 0.7	14.2 ± 0.5
Final		
Evaluable	13.0 ± 0.7	19.8 ± 0.7†
ITT	13.3 ± 0.6	18.3 ± 0.7†
Serum beta-carotene, μg/mL		
Baseline		
Evaluable	0.14 ± 0.01	0.15 ± 0.01
ITT	0.14 ± 0.01	0.14 ± 0.01
Final		
Evaluable	0.15 ± 0.01	1.58 ± 0.12†
ITT	0.16 ± 0.01	1.31 ± 0.10†
Serum lipids, mg/dL		
Baseline		
Evaluable	275 ± 13	279 ± 11
ITT	271 ± 11	270 ± 9
Final		
Evaluable	283 ± 16	284 ± 10
ITT	280 ± 15	275 ± 9
Serum albumin, g/dL		
Baseline		
Evaluable	3.6 ± 0.1	3.8 ± 0.1
ITT	3.6 ± 0.1	3.7 ± 0.1
Final		
Evaluable	3.9 ± 0.1	4.0 ± 0.1
ITT	3.9 ± 0.1	4.0 ± 0.1

Note: Subjects who consumed a daily average intake of formula of 180 mL or more were considered compliant to the protocol and evaluable. Data are not reported from subjects who were randomized but were later excluded for not receiving the influenza vaccine (n = 10).

*Formula consumed is based on days in the study for intent-to-treat (ITT) subjects and 70 days for evaluable subjects.

†$P < .001$ between experimental (EXP) and control (CON) within each column.

influenza vaccine, whereas vitamin supplementation (vitamin C, E, and β-carotene) offered no immunological benefit.[22] A study of 96 independently living individuals found improved nutritional status and decreased infection-related illnesses after 1 year of vitamin and trace element supplementation,[23] but questions regarding validity of this research have been posed.[24,25] Research regarding individual nutrient or vitamin supplementation have provided conflicting results; for example, one study supplemented diets of LTC residents with a multinutrient capsule and 200 IU vitamin E or placebo.[13] Over a 1-year period, there were fewer respiratory tract infections, primarily colds.[13] This is in contrast to a study of noninstitutionalized seniors supplemented with 200 mg α-tocopherol, a multivitamin supplement, both or a placebo and showed no effect on incidence of respiratory tract infections, although subjects experienced increased severity of respiratory tract infections with α-tocopherol supplementation.[26] Similarly, another study provided a multivitamin-mineral supplement to noninstitutionalized seniors and found no difference in incidence of infection over a 4-month period.[27]

Researchers have used vaccine response and antibody production to influenza to measure immune responsiveness after nutrient supplementation. One study supplemented the diets of older people in LTC with a multivitamin and mineral tablet and, despite significant increases in serum nutrient concentrations, found no difference between the supplemented and placebo groups with respect to antibody levels after influenza vaccination.[28]

The current study showed immunological benefit (improved lymphocyte responsiveness and antibody titers to the H1N1 component of the influenza vaccine) and clinical benefit (less fever and fewer prescribed antibiotics) in seniors taking a formula containing antioxidants, selenium, and zinc in combination with structured triacylglycerol and FOS.

Possible mechanisms for immune benefit from EXP were examined, and no difference was found in white blood cell populations or lymphocyte population subsets, with the exception of a few important subsets. For example, subjects in the group consuming EXP tended to have more cells expressing CD28 (costimulatory molecule), indicating potential for improved responsiveness. CD28 is a costimulatory molecule expressed on T-cells and NKT cells. Ligation of CD28 is necessary for T-cell receptor-mediated activation. Without CD28 ligation, naive T-cells activated though the T-cell receptor will become anergic (nonresponsive) or apoptotic. Although the role of CD28 with regard to NKT cells in humans has yet to be completely understood, animal studies have shown that CD28 costimulation of NKT cells results in increased cytokine production, increased antimetastatic capabilities, and increased cytotoxicity.[29] Also, there was a shift away from a more aged-like lymphocyte profile in the EXP group. Those in the EXP group had more naïve T-helper cells and fewer memory cytotoxic lymphocytes than those in the CON group.

A key finding of this study was that EXP consumption increased specific activation (i.e., influenza-mediated activation) of the T-lymphocyte populations. Reports consistently indicate that lymphocyte activation and surface activation markers CD69 and CD25 typically decline with age.[10] CD69 is expressed on the surface of activated cells within 4 hours and remains elevated for up to 48 hours. CD25, a subunit of the IL-2 receptor, is expressed on the surface of activated cells within 24 hours and continues to increase expression for 120 hours or longer. The main differences between treatment groups of the current study were within specific influenza-activated T-lymphocyte populations expressing CD69 or CD25 or coexpressing CD69 and CD25. By evaluating these surface activation markers on lymphocytes exposed in vitro to the influenza vaccine antigens, it was found that subjects consuming EXP had more activated T-lymphocyte subsets (numbers and percentages), specifically, the subset of T-lymphocytes expressing CD2, CD69, and CD25 and lacking CD8 markers or putative helper T-cells (Figure 1). Because CD4 and CD3 expression downregulates upon activation, CD2 and CD8 were used in activation studies. $CD8^{-}CD2^{+}$ cells are assumed to be helper T-cells, although some NK cells express

Table 3. Antibody Responses to Components of the Influenza Vaccine

		Study End		
Vaccine Component	Before Vaccination GMT Mean (95% Confidence Interval)	GMT	Subjects with Titer > 100 (H1N1 Component) or ≥ 40 (H3N2 and B Components)	Subjects with ≥ Fourfold Increase in Titer
			%	
A/Caledonia/20/99 (H1N1)				
CON				
Evaluable	15 (12–20)	60 (44–82)	23	43
ITT	19 (15–24)	63 (47–83)	29	42
EXP				
Evaluable	24 (18–31)	79 (59–105)	43*	46
ITT	23 (18–29)	76 (59–98)	44†	44
A/Panama/2007/99 (H3N2)				
CON				
Evaluable	81 (52–127)	344 (234–504)	95	30
ITT	86 (59–123)	336 (230–490)	89	40
EXP				
Evaluable	141 (96–206)	398 (279–568)	98	41
ITT	132 (92–224)	428 (311–589)	97‡	33
B/Hong Kong/1434/2002				
CON				
Evaluable	51 (34–78)	279 (187–415)	95	46
ITT	54 (40–74)	290 (210–400)	67	52
EXP				
Evaluable	59 (42–83)	336 (235–481)	94	64‖
ITT	54 (41–72)	381 (288–503)	60	64

$P =$ *.047, †.09, ‡.06, ‖.09 between experimental (EXP) and control (CON) subjects within each column.
GMT = geometric mean HI antibody titer; ITT = intent to treat.

CD2. Cellular responses or activation differences may have been more pronounced if the EXP group had been compared with a group not receiving nutritional supplementation.

In vitro activation results support the in vivo antibody response data as markers of improved immune function. Increases in helper T-cell antigen activation may indicate one mechanism of enhanced vaccine-specific antibody production. Approximately twice as many subjects (43% vs 23%, $P = .047$) consuming EXP as consuming CON achieved an H1N1 antibody titer greater than 100 (protective titer in older people).[30] Similar trends were detected with the other two components of the vaccine with respect to a fourfold increase or more (B component) or an antibody level of 40 or more (H3N2 component) in intent-to-treat analysis (Table 3).

Cytokine profiles were different between the two groups. Cytokines regulate immune function in a paracrine and/or autocrine fashion to dampen or stimulate cellular responses. IL-2 and IFN-γ typically decline with age,[8,14,23] whereas TNF-α, IL-6 (cytokines that influence inflammation), and IL-10 (immunosuppressive) typically increase with age.[2,31–33] In this study, EXP subjects produced lower levels of the proinflammatory IL-6 ($P = .045$, evaluable subjects) and immunosuppressive IL-10 ($P = .06$, evaluable subjects; $P = .04$, intent-to-treat subjects) upon stimulation than CON subjects. Increases in inflammatory and suppressive cytokines have been implicated in several chronic disease states; the observed differences in cytokine profile would be considered beneficial for seniors. Furthermore, decreases in IL-6 may reflect the smaller number of EXP subjects who experienced fever.

Subjects were stratified according to BMI, smoking status, and diabetes mellitus, yet more subjects who were diabetic or had a BMI less than 22 kg/m^2 in the EXP group completed the study. This suggests that frailer individuals with potentially the greatest nutritional needs completed the study as a result of consuming EXP rather than CON (Table 1). Even though the EXP group (evaluable) had more subjects in the "at risk" population, there were significantly greater improvements in immune outcomes than in the CON group.

Immune benefit by nutritional supplementation has recently been noted in a study of free-living subjects (aged <40). After vitamin and mineral supplementation, a subgroup of subjects with type 2 diabetes mellitus had fewer days of illness and less absenteeism from work.[34] Subjects with diabetes mellitus may receive the greatest benefit from nutritional supplementation, as in the current study, where more than twice as many subjects with diabetes mellitus in the EXP group then in the CON group completed the study.

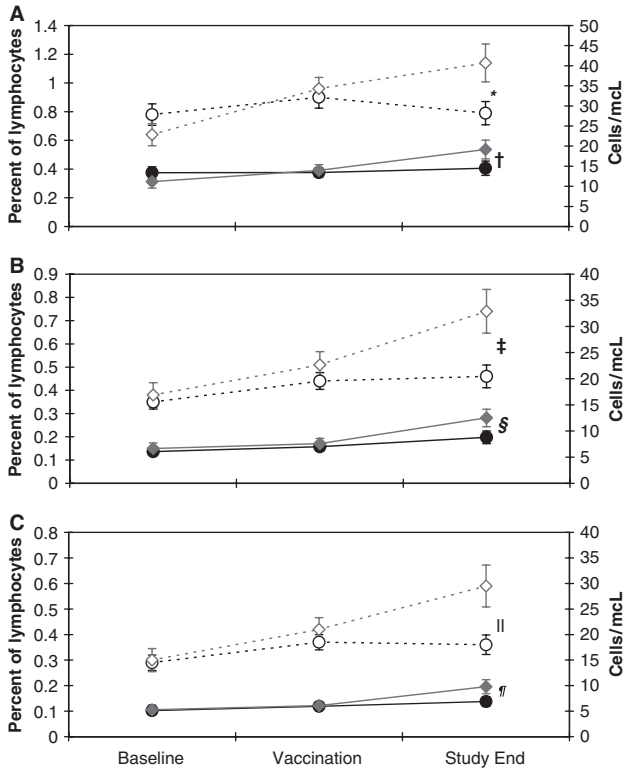

Figure 1. Activation of lymphocytes from evaluable subjects in response to influenza antigens. Percentages and numbers of lymphocyte populations expressing CD69 and CD25 after activation with influenza vaccine antigens. (A) Total lymphocytes expressing CD69 and CD25. (B) T-lymphocytes ($CD2^+$) expressing CD69 and CD25. (C) Helper T-lymphocytes ($CD2^+$ and $CD8^-$) expressing CD69 and CD25. Diamonds represent experimental group; circles represent the control group. Open symbols are percentage of lymphocytes; closed symbols are cell numbers. Time points reflect the baseline, vaccination, and final visits, respectively, from left to right. Significant differences between groups: P = *.008, †.02, ‡.03, §.08, ‖.01, ¶.02.

The average body weight and BMI of the groups (intent to treat or evaluable) between baseline and final time points were not different in the current study. For many subjects, study formulas replaced between-meal liquid or high-calorie supplements administered with medications, yet immune differences were not simply the result of extra energy and protein. The CON and EXP formulas were both well tolerated, and no differences in formula-related complaints were noted. Serum α-tocopherol and β-carotene concentrations rose in the EXP group, indicating good compliance (Tables 2, $P < .01$).

The consistent increase in responsiveness to the influenza vaccine, T-cell activation, altered cytokine production, and cellular changes point toward an immunological benefit of EXP that is reflected by the improved clinical outcomes. There was significantly less fever requiring intervention in the EXP group and fewer newly prescribed antibiotics for upper respiratory and urinary tract infections. Changes were consistent and support previous findings.[15] It is unlikely, and probably undesirable given the complexity of the immune system, that nutritional supplementation would mediate large changes in immune function in older people. However, the nutritional approach of this study demonstrated a collection of improvements that presumably could reduce the risk of infection and improve quality of life. Others have identified that quality of life is correlated with immune function,[35] although this was not the focus of the current study. Therefore, the study of nutritional supplementation on improved immune function, which ultimately affects quality of life, is warranted.

In summary, EXP containing a structured triacylglycerol, protein, antioxidants, selenium, zinc, and FOS provided immunological benefit beyond that provided by a nutritional formula typically used in LTC environments. Furthermore, immunological changes provide evidence of a mechanism for the observed clinical outcomes. This study and previous work[15] provide evidence that EXP formula improves the quality of life of seniors, as demonstrated by fewer days of symptoms of upper respiratory tract infections, less fever, fewer antibiotics, improved lymphocyte responsiveness and vaccine response to influenza vaccine.

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Table 4. Cytokine Concentration After Mitogen Stimulation Before Feeding (Baseline Visit) and 6 Weeks Postvaccination (Final Visit) in Evaluable and Intent-to-Treat (ITT) Subjects

Cytokine	Control		Experimental		*P*-value Between Groups at Final Time Point
	Baseline	Final	Baseline	Final	
Subjects at each time point, n					
Evaluable	40	40	52	52	
*Samples held <24 hours**		28		35	
ITT	69	69	74	74	
*Samples held <24 hours**		43		50	
IL-2, pg/mL, mean ± SEM					
Evaluable	45 ± 10	102 ± 32	90 ± 25	148 ± 32	.28
*Samples held <24 hours**		109 ± 40		169 ± 44	.43
ITT	43 ± 7	120 ± 27	84 ± 17	132 ± 25	.75
*Samples held <24 hours**		138 ± 37		146 ± 34	.91
IL-4, pg/mL, mean ± SEM					
Evaluable	54 ± 13	35 ± 8	63 ± 17	46 ± 11	.28
*Samples held <24 hours**		42 ± 11		56 ± 15	.50
ITT	47 ± 8	34 ± 6	81 ± 18	41 ± 8	.67
*Samples held <24 hours**		42 ± 9		50 ± 11	.68
IL-6, ng/mL, mean ± SEM					
Evaluable	30 ± 6	26 ± 3	31 ± 6	21 ± 2	.10
*Samples held <24 hours**		29 ± 3		20 ± 3	.045
ITT	27 ± 3	26 ± 4	30 ± 4	22 ± 2	.25
*Samples held <24 hours**		29 ± 5		21 ± 2	.16
IL-10, pg/mL, mean ± SEM					
Evaluable	299 ± 67	524 ± 110	225 ± 43	338 ± 52	.19
*Samples held <24 hours**		603 ± 140		310 ± 60	.06
ITT	229 ± 39	470 ± 82	241 ± 34	342 ± 41	.25
*Samples held <24 hours**		573 ± 110		318 ± 47	.04
Tumor necrosis factor α pg/mL, mean ± SEM					
Evaluable	749 ± 79	311 ± 34	831 ± 100	395 ± 45	.15
*Samples held <24 hours**		346 ± 45		395 ± 56	.37
ITT	731 ± 65	365 ± 52	846 ± 84	383 ± 35	.91
*Samples held <24 hours**		414 ± 71		385 ± 42	.75
Interferon gamma, pg/mL, mean ± SEM					
Evaluable	1,040 ± 369	1,599 ± 521	878 ± 198	1,660 ± 374	.43
*Samples held <24 hours**		1,782 ± 626		1,738 ± 489	.78
ITT	786 ± 211	1,497 ± 382	970 ± 183	1,516 ± 297	.53
*Samples held <24 hours**		1,762 ± 495		1,591 ± 383	.96

* During the conduct of the study, a few blood samples at the final time point were inadvertently shipped to another location or delayed because of severe weather in transport and then shipped to Abbott Laboratories for analysis. This error resulted in samples being processed more than 24 hours after the final blood draw. Because cytokine expression may occur with holding samples, an analysis was performed with all samples and without the samples held for more than 24 hours.
SEM = standard error of the mean; IL = interleukin.

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Author Contributions: Bobbi Langkamp-Henken was principle investigator and oversaw the protocol development, protocol implementation, acquisition of subjects, data analysis, interpretation of data, and preparation of manuscript. Steven M. Wood developed the study concept and worked closely with the principle investigator in manuscript preparation. Kelli A. Herlinger-Garcia assisted in protocol development and played a significant role in subject recruitment and preparation of manuscript. Debra J. Thomas assisted in design of laboratory strategy of phenotyping and cytokine analysis as well as interpretation. Joyce K. Stechmiller was coinvestigator and assisted in protocol development and acquisition of subjects and medical histories. Bradley S. Bender was coinvestigator, provided medical oversight, and assisted in study design and interpretation of results. Elizabeth M. Gardner was coinvestigator and assisted in the study design of cellular analysis and interpretation of results. Stephen J. DeMichele assisted in study design and review of manuscript. Joseph P. Schaller assisted in study design, antibody analysis, and interpretation of results. Donna M. Murasko assisted in study design.

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