Clinical Nutrition 31 (2012) 337–344

Contents lists available at SciVerse ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Randomized control trials

Supplementation with aged garlic extract improves both NK and γδ-T cell function and reduces the severity of cold and flu symptoms: A randomized, double-blind, placebo-controlled nutrition intervention

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ARTICLE INFO

Article history:
Received 15 September 2011
Accepted 24 November 2011

Keywords:
Aged garlic extract
Human immunity
NK cell
γδ-T cell
Colds
Flu
Garlic

SUMMARY

Background & aims: Earlier studies show that dietary bioactive compounds can modify proliferation of γδ-T cells. Garlic contains numerous compounds that have this potential and, in addition, has been shown to influence NK cell function. Our primary aim was to demonstrate that aged garlic extract could modify these immune cells.

Methods: A randomized, double-blind, placebo-controlled parallel intervention study recruited 120 healthy subjects (60 per group) to determine the effect of aged garlic extract supplementation (2.56 g/d) on immune cell proliferation and cold and flu symptoms.

Results: After 45 d of consuming an encapsulated aged garlic extract, γδ-T cells ($p = 0.039$, $n = 56$) and NK cells ($p = 0.043$, $n = 56$) were shown to proliferate better compared to placebo. After 90 d of supplementation, illness diary entries showed that the incidence of colds and flu, a secondary outcome, were not statistically different; however, the group consuming the aged garlic extract appeared to have reduced severity as noted by a reduction in the number of symptoms reported (21% fewer, $p < 0.001$, z-test of proportions), a reduction in the number of days (61% fewer, $p < 0.001$, z-test) and incidences (58% fewer $p < 0.001$, z-test) where the subjects functioned sub-optimally and the number of work/school days missed due to illness (58% fewer, $p = 0.035$, z-test).

Conclusions: These results suggest that supplementation of the diet with aged garlic extract may enhance immune cell function and that this may be responsible, in part, for reduced severity of colds and flu.

1. Introduction

Garlic has a long history of providing health benefits. Daily consumption is associated with many cardiovascular and other benefits, but is also accompanied by strong breath and body odor. By a unique aging process, garlic can be rendered nearly odorless, due to the conversion of strong organosulfur compounds into water soluble compounds such as *S*-allyl-L-cysteine (SAC) and *S*-allyl-mercaptocysteine. Aged garlic extract (AGE) contains compounds with known immuno-modulating activity such as lectins and fructooligosaccharide[1,2] and some compounds that, in theory, might participate in modifying immunity such as apigenin, a flavonoid, N(alpha) fructosyl arginine, a Maillard reaction product[3] and tetrahydro-carbolines.[4] The purpose of this study was to ask whether AGE supplementation modifies immunity in humans, specifically regarding γδ-T cell and NK cell functions.

Innate immune cells, such as NK cells and monocyte/macrophages, respond to pathogen-associated molecular patterns (PAMP) via their pattern recognition receptors (PRR). While T cells are not considered part of the innate immune system, γδ-T cells are a unique T cell in that they have been shown to respond to PAMP,[5,6] they do not respond to typical protein antigens[7–9] and do not require major histocompatibility complex-1.[10] When a pathogen is present, the innate cell senses the molecular pattern and initiates a response. Our postulate is that dietary bioactive compounds resemble PAMP and weakly interact with PRR on innate cells. Recent research, from this[11] and other labs,[12–14] suggests that some dietary bioactive compounds prime certain cells to respond faster and stronger when they are subsequently stimulated by pathogens.

Bukowski identified alkylamines as antigens for γδ-T cells in vitro.[12] Tea contains a unique amino acid, L-theanine, which is hydrolyzed to glutamic acid and an alkylamine, ethylamine. In a study comparing tea drinkers to coffee drinkers, tea drinkers

Abbreviations: AGE, aged garlic extract; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptors; SAC, *S*-allyl-L-cysteine.

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0261-5614/$ – see front matter
doi:10.1016/j.clnu.2011.11.019

showed greater γδ-T cell proliferation *ex vivo*, presumably due to priming of the cells by ethylamine.[13] We followed up with a human study that confirmed the increased ability of γδ-T cells to proliferate after consumption of a standardized capsule containing the green tea components, L-theanine and catechins.[11] Jutila's group showed that proanthocyanidins from medicinal herbs also primed γδ-T cells in vitro, and suggested it was through a weak interaction with PRR.[14–16] Thus, γδ-T cells appear to be modified by dietary compounds. An NCI workshop[17] suggested that NK cells may also be modified by diet.[18–20] NK cells interact with tumor cells and virally infected cells[18,21] and are known to have PRR.[22,23] Some clinical and preclinical studies have indicated that AGE improves NK cell activity,[24,25] but this type of research has not been done with γδ-T cells. We hypothesized that the function of γδ-T cells and NK cells would be modified after consuming AGE and, that if AGE supplementation modified these immune cells, there would be a health outcome associated with this modification. To quantify this modification we assessed the incidence and severity of illness in the cold and flu season during AGE supplementation.

2. Methods and materials

2.1. Subjects

A total of 120 healthy men ($n = 55$) and women ($n = 65$), ranging in age from 21 to 50 years with a body mass index between 18 and 30 kg/m^2, were recruited to participate in a 90-day double-blind, randomized, placebo-controlled parallel intervention. Subjects were recruited from the University of Florida campus and the Gainesville, Florida community. The University of Florida Institutional Review Board approved the study protocol, and informed written consent was obtained from each subject. Screening for the study occurred by telephone and/or personal interviews. Exclusion criteria consisted of the following: Body mass index above 30, pregnancy or lactation, blood pressure above 140/90, chronic illness or infection, antihypertensive medication, immunosuppressive drugs, antibiotics, chronic use of NSAIDS, dietary supplements, or more than 2 alcoholic beverages per day. Participants were in contact with the enrolling research assistant by e-mail and telephone throughout the study to boost compliance with capsule consumption and illness log entries.

Power analysis, with an alpha level of 0.05 and a power of 0.80, based on previous data where average percentage of γδ T cells in peripheral blood of subjects in the placebo group = 4.7% ± 2.7 and those in a previous intervention group averaged 9.9% ± 5.3, indicates that 15 individuals were needed to detect a statistical difference.

2.2. Study design

The study was conducted from February through May of 2010, which is considered cold and flu season by the CDC (http://www.cdc.gov/flu/; accessed August 2010). The study lasted until the end of the semester, which included subjecting students to the academic stress of final exams.[26] Subjects arriving for the initial blood draw at the clinical laboratory housed in the Food Science & Human Nutrition building blindly drew numbers and were given the corresponding capsule bottles containing either Aged Garlic Extract (AGE) powder (4 capsules: 2.56 g per day) or placebo capsules, which had been sequentially numbered and randomized by the capsule manufacturer, Wakunaga of America, Co. Ltd., Mission Viejo, CA. Both subjects and investigators were blinded as to the treatment groups. The primary outcome measurement was *ex vivo* proliferation of γδ-T cell and NK cells. Activation markers and cytokine production are exploratory because there is less knowledge about their behavior under *ex vivo* conditions. Blood pressure measurements were taken and subjects received an illness diary with instructions for completion. The daily illness diary, also a secondary outcome measure, was kept during the entire 90 d of the study. Subjects were instructed to consume two capsules, twice daily with food, for 45 d. Subjects returned to the clinical laboratory at 45 d for a second blood draw and received capsules to consume for a further 45 d. At 90 d, subjects returned any remaining capsules and their personal illness diary, and completed a final questionnaire. The final questionnaire asked about side effects experienced, consumption of other dietary supplements and medications, and which treatment group they thought they had been in. Compliance was determined by capsule counts in returned bottles and expressed as a percentage of what should have been consumed. In addition, participants self-reported if they missed taking any capsules.

2.3. Blood collection and separation of peripheral blood mononuclear cells (PBMC)

Blood was obtained from fasting subjects on day 0 (baseline) and day 45 and collected into one 10 mL sodium heparin tube for PBMC separation, and one 10 mL SST™ tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) to obtain serum. Tubes for PBMC were maintained at room temperature, while tubes for serum were kept at 4 °C. All tubes were processed within 2 h of blood collection. Blood cell separation and culture procedures were carried out under sterile conditions.

The blood for PBMC separation was diluted 1:1 with 0.9% NaCl, layered over 10 mL of Lympholyte H® Cell Separation Media (CEDARLANE Laboratories Ltd., Burlington, NC) and centrifuged (800 *g*, 25 min, 20 °C). The mononuclear cell layer was removed and cells washed twice with RPMI 1640 (Cellgro; Mediatech, Herndon, VA) complete medium (100,000 U/L penicillin; 100 mg/L streptomycin; 0.25 mg/L fungizone; 50 mg/L gentamicin; 2 mmol/L L-glutamine; 25 mmol/L HEPES buffer). Cell pellets were resuspended in 2 mL RPMI 1640, and counted using a Coulter Z1-S Particle Counter (Beckman Coulter, Brea, CA).

2.4. Culture of PBMC for γδ-T cell and NK proliferation and activation

On day 0, 1.0 × 10^6 PBMC in 200 μl of RPMI 1640 from each individual were placed into one well of duplicate 48-well tissue culture plates (Costar, Corning, NY). Another 200 μl of complete medium containing 2 × FBS(20%),2 × β-ME (50 μM) and 2 × PHA-P (10 μg/ml, Sigma, St. Louis, MO), was added to all wells of both plates. The plates were incubated in a humidified 5% CO_2 atmosphere at 37 °C. After 24 h, cells and supernatant fluids from one plate were harvested and centrifuged (500 *g*, 10 min, 4 °C). The supernatant fluids were removed and frozen at −80 °C for cytokine analysis. On days 3 and 7 of culture, wells were fed with 400 μl of RPMI complete, containing recombinant human IL-2 (final concentration of 30 U/ml, BD Biosciences, San Diego, CA). This plate was incubated until day 10, when cells were harvested and processed for flow cytometry.

At both blood draws, cells from original PBMC suspensions, and the same cells after 10 d of culture, were analyzed by flow cytometry using cell surface markers for identification. All antibodies were obtained from eBioscience, San Diego, CA. The combination of PE-α-human CD3 and FITC-α-human γδ-TCR were used to stain PBMC to determine proliferation of γδ-T cells. In addition, FITC-α-human CD56 and PE-α-human CD314 (NKG2D) were used to detect percentage and activation of NK cells. PBMC were stained at 4 °C in the dark for 30 min. Cells were washed, centrifuged, fixed with 1% paraformaldehyde, and analyzed by flow cytometry within 48 h.

The percentages of CD3 positive γδ-T cells and activated NK cells in the PBMC population were determined. Cells of small size and high scatter (20–30%) were gated out of the analysis. The number of live cells per well was not statistically different between the groups, between the draws or due to culturing. A BD Biosciences FACS Sort flow cytometer using CellQuest Pro software (ICBR - Cellomics Division) was used for data acquisition (Becton–Dickinson, San Jose, CA). Data was analyzed using FlowJo (version 7.6.4) Analysis Software (Tree Star, Inc., Ashland, OR).

2.5. Cytokine level determination in supernatant fluids after 24 h cell proliferation

PBMC (1×10^6) from each subject were stimulated with 5 μg/ml PHA-P for 24 h. The undiluted 24 h cell culture supernatants were assayed to determine the levels of three inflammatory cytokines (IFN-γ, IL-12, and TNF-α), using a Human Cytokine Multiplex Immunoassay kit, according to the manufacturer's directions (Millipore Corp. Billerica, MA). The beads were analyzed on a Luminex® 200 instrument (Austin, Texas) with xPONENT 3.1 software. Data is expressed in pg/mL.

2.6. Total glutathione

Individual subject PBMC (2×10^6) from each blood draw were washed once in cold PBS, centrifuged (500 *g*, 4 °C, 5 min), resuspended in 0.5 ml of 5% metaphosphoric acid (MPA) and frozen at −80 °C. PBMC in MPA were subjected to sonication to lyse cells and centrifuged to deproteinate the samples. Resulting supernatant fluids (50 μl) were analyzed, in duplicate, for total glutathione using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, MI) following manufacturer's instructions. After incubation (25 min at room temperature), reactions were read at 405 nm on a SPECTRAmax 340 PC plate reader (Molecular Devices). Total μM glutathione concentration was determined, based on the standard curve supplied with the kit.

2.7. S-allyl cysteine (SAC) analysis

S-allyl cysteine was analyzed by Wakunaga Pharmaceutical Co., Ltd, Osaka, Japan to determine compliance.[27] An extraction of serum collected at 45 d and subsequent HPLC analysis showed good compliance with capsule consumption.

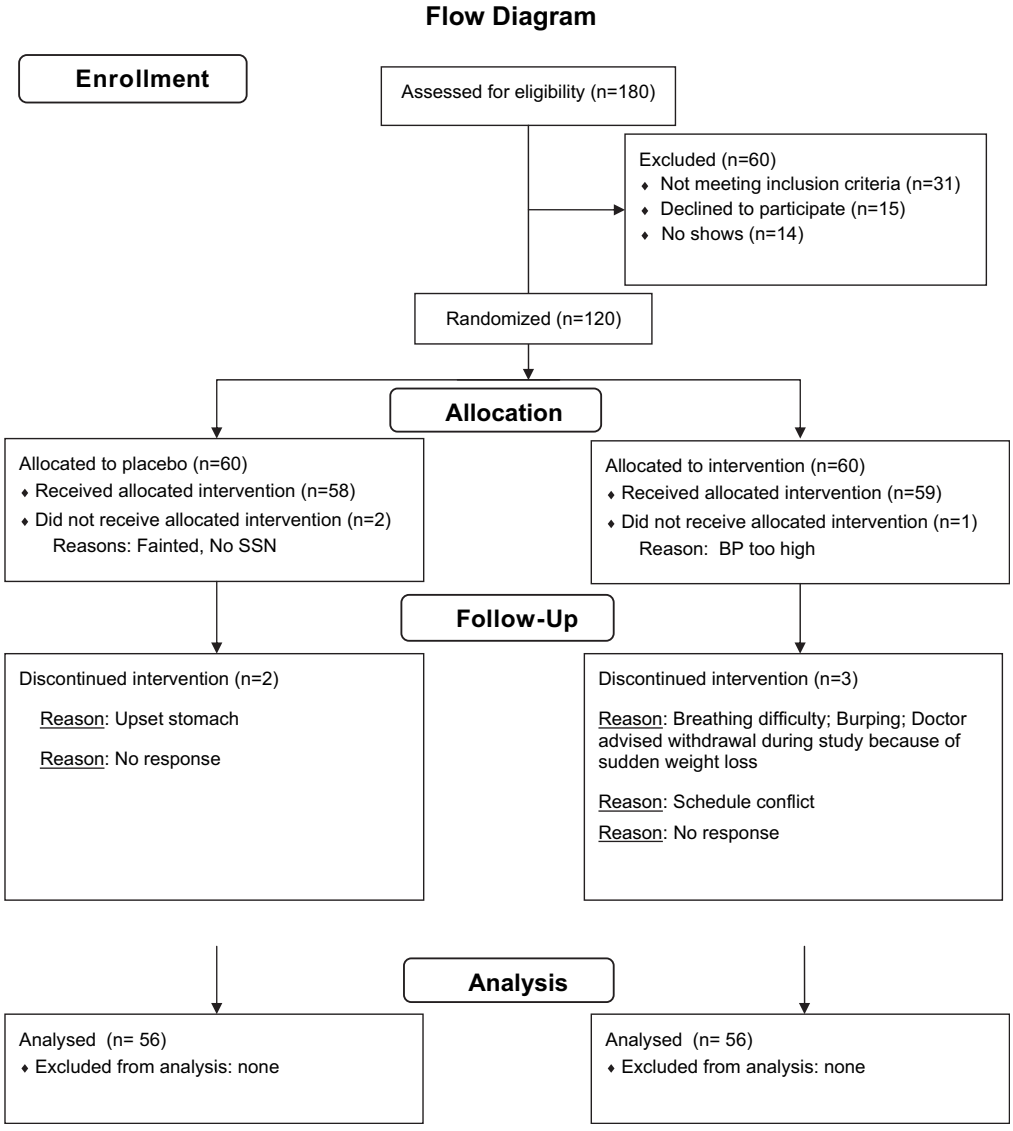

Fig. 1. Flow diagram of persons assessed for eligibility, enrolled in the study, allocated to the AGE or placebo and statistically analyzed.

2.8. Cold and flu symptomology

Illness logs were kept for each day through the 90 days of the supplementation. Incidence was determined by counting the number of illnesses per group. Illness was defined as more than 1 symptom for more than 1 day. When subjects felt sick, they checked what symptoms they had for that day and for each day thereafter. Severity was defined as: average duration of illnesses (days); total number of symptoms; the incidence of a report of a decrease in normal activities; number of missed days of school or work; and number of doctor visits. Eight symptoms were listed in the illness log: runny/congested nose; cough; sneezing; fever/chills; sore throat; headache; body aches/pains; and other (to be specified by the individual). They recorded if they missed school or work; if they felt too ill to function optimally (which is referred to as DIA or Decrease in Activity) and both the incidence and the number of days were recorded. They recorded if medical attention was sought; and which medications were taken, both over the counter and prescribed. Subjects were instructed as to the differences between cold, flu and allergy symptoms. Medications taken for symptoms also helped to distinguish between illness and allergy. Allergy symptoms were not included in the data analysis.

2.9. Statistical analysis

Statistics were performed using SigmaStat version 3.11, SystatSoftware or JMP version 8 (Cary, NC). A 2-way repeated measure ANOVA was used to examine differences in time (baseline to 45 d) and in treatment (AGE vs. placebo capsules). Both time and treatment differences were considered important measures of immune enhancement. To judge the evidence of an interaction when comparing treatment and time, the criterion was relaxed to $p = 0.15$, based on the explanation of Bancroft.[28] The proliferation index of NK and γδ-T cells was calculated by determining the ratio (fold increase) between cultured and uncultured cells. A 2-way RM ANOVA was performed for time and treatment. When cells were not normally distributed or of unequal variance, the data was transformed using the natural log. The change from baseline was calculated by subtracting baseline ratio (day 0) values from the 45 d ratio values and testing the differences by the *t*-test. A *z*-test of proportions was used to determine the significance differences in the proportion of cold and influenza characteristics among the groups.

3. Results

3.1. Demographics

A total of 180 individuals were assessed for eligibility (Fig. 1). Of this group, 31 did not meet the inclusion criteria, 15 declined to participate, and 14 did not return for the first blood draw. Of the remaining 120 subjects, 60 were randomized into each of two groups and asked to consume four capsules daily, of either Aged Garlic Extract powder (2.56 g per day) or a placebo that was identical in appearance to the treatment. Five subjects dropped out due to scheduling conflicts at the second blood draw and three dropped out for minor gastrointestinal discomfort during the study, four in each group.

Subjects in the two groups were similar with regard to age and gender distribution (Table 1) and also in their vaccination status and personal hygiene assessment. The individuals in this study represent a healthy adult population up to age 50. Compliance, as determined from capsule counts, did not differ between groups. On average, subjects took 85% of their capsules over the 90 d of the study.

Table 1
Demographics and other characteristics of the study population.

	All subjects	Placebo	Age	*p* value
Demographics				
n	112	56	56	
Age (y)	26.0 ± 6.5	26.9 ± 7.1	25.4 ± 5.7	
Gender (M)	49	26	23	
Gender (F)	63	30	33	
Compliance (%consumed)	85.8 ± 0.8	85.6 ± 1.2	85.9 ± 1.2	
Blinding (% guessing correctly)		69.2%	68.1%	
Flu shot				
Yes	38	17	21	0.355
No	71	38	33	0.578
Hygiene rating				
Excellent	44	24	20	0.439
Very good	47	21	26	0.349
Good	15	8	7	0.605
Fair	3	2	1	

Values are the means ± SD. Differences between groups were determined by a *z*-test of proportions.

3.2. Functional immune and cytokine assays

The NK cell proliferation index is the ratio of the percentage of cells that were positive for CD56 and CD314 cell surface markers on day 10 of culture, relative to the uncultured cells. In the placebo group, there was a difference of about 20% in the proliferation index of NK cells between the pre-consumption baseline and 45 d after dietary intervention (Fig. 2A). The NK cells from subjects in the AGE group proliferated two times more after the supplement was taken compared to those in the placebo group, which was significant ($p = 0.043$). The activation state of NK cells was determined by the median fluorescence of CD314 (NKG2D) per cell. There was a significant increase ($p = 0.031$) in the activation state of the NK population in the AGE group (Fig. 2B).

γδ-T cell proliferation, calculated in the same way as the NK cell data, was found to be of unequal variance, and was subsequently transformed. Results showed a significant 8-fold increase in γδ-T cell proliferation after AGE capsule consumption compared to the placebo ($p = 0.039$) (Fig. 3). γδ-T cell activation as indicated by increased expression of the γδ-TCR after AGE supplementation tended to be improved (5% greater), but did not achieve significance compared to the placebo (1% greater) ($p = 0.094$) (data not shown). The change in median fluorescence was minimal comparing uncultured to 10 d cultured cells.

TNF-α and IFN-γ mean values are reported in Table 2. No IL-12 was detected in the culture supernatants from either group (results not shown). There was a significant effect of time on TNF-α secretion: the placebo group secreted higher levels of TNF-α at 45 d than they did at baseline, while levels of those ingesting AGE did not change from baseline. Similarly, IFN-γ levels were significantly greater at 45 d in the placebo group compared to baseline, while levels from those consuming AGE did not change.

3.3. Thiol determination

Glutathione was measured in isolated PBMC from day 0 and 45 d samples. Glutathione levels increased significantly from baseline to 45 d in the group that took the AGE capsules, while the placebo group, on average, showed a slight reduction over time (Fig. 4). Compliance was determined by measuring S-allyl cysteine in serum, and it was confirmed it is significant greater in the AGE group compared to the placebo (Fig. 5; $p = 0.002$).

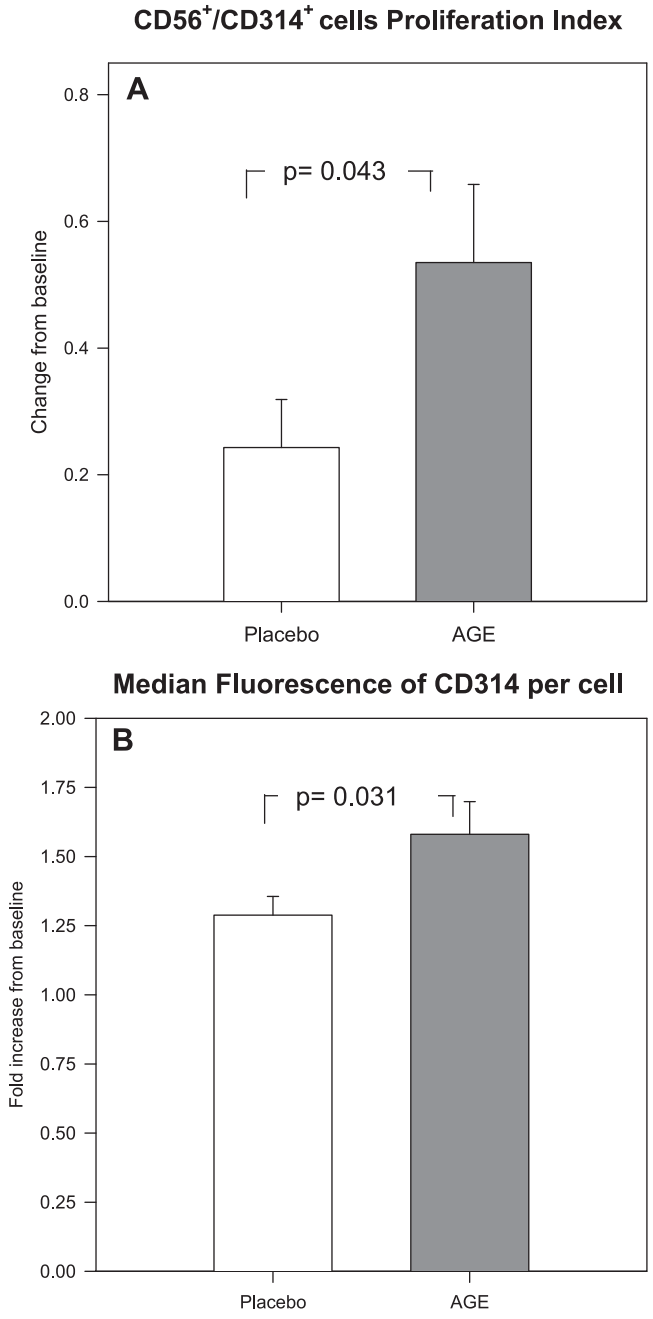

Fig. 2. NK cell proliferation and activation PBMC were isolated after supplementation with AGE or placebo for 45 d. PBMCs were cultured *ex vivo* for 10 days. After culture, the cells were examined by flow cytometry as the percent events by quadrants and the median fluorescence. The means were calculated as a ratio of values at 10 d of culture to uncultured cells, and then baseline was subtracted from 45 d. Panel A was the percent of cells in the $CD56^{+}/CD314^{+}$ quadrant and Panel B was the median fluorescence of the activation marker, CD314. Bars represent the mean ± SEM and were statistically compared by *t*-test.

3.4. Illness characteristics

The incidence of illness was not different between the AGE and placebo groups (Table 3). Total days of illness in both groups were also not significantly different. The sum of symptoms during the entire 90 d period was significantly greater in the placebo group. Subjects were asked to report a decrease in activity (DIA) each day they were too sick to perform their

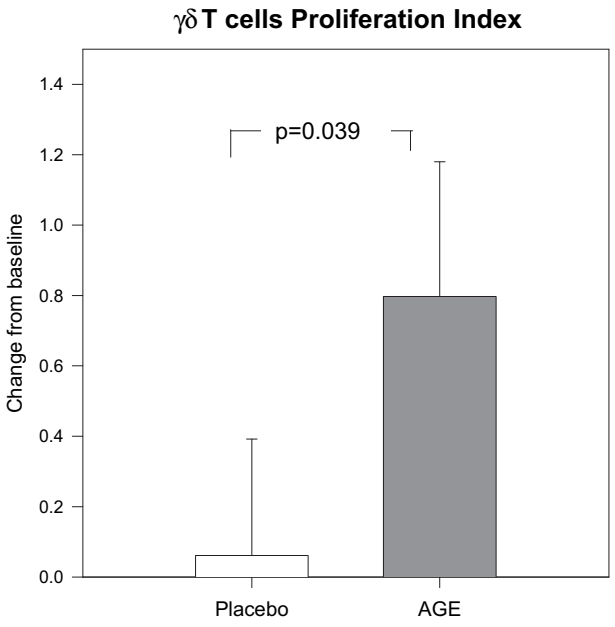

Fig. 3. γδ-T cell proliferation. PBMC were isolated after supplementation of AGE or placebo for 45 d. PBMCs were cultured *ex vivo* for 10 days. After culture, the cells were examined by flow cytometry to enumerate the percent events of $CD3^{+}/\gamma\delta\text{-}TCR^{+}$ from quadrant analysis. The means were calculated as a ratio of values at 10 d of culture to uncultured cells, and then baseline was subtracted from 45 d. Bars represent the mean ± SEM and were statistically compared by *t*-test.

normal routine. Both the incidence of a DIA and the total number of days subjects experienced a DIA were significantly higher in the placebo group, indicating more severe illnesses compared to the AGE group. In addition, subjects taking the placebo missed more days of work or school than those taking AGE capsules. The number of visits to a doctor was not significantly different between the two groups.

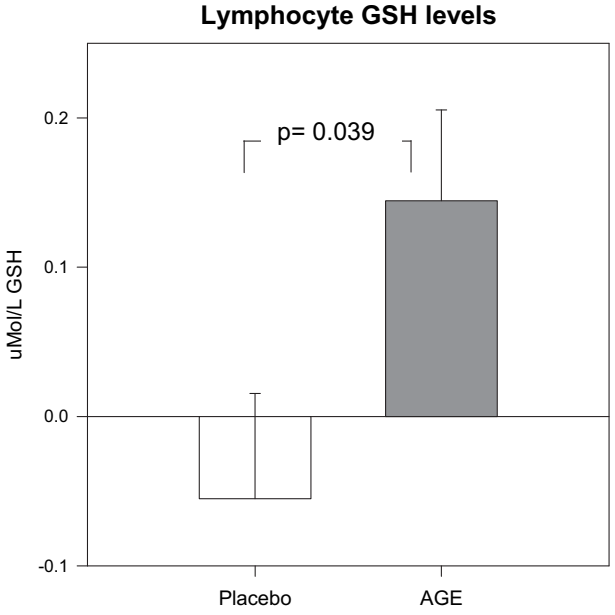

Fig. 4. Glutathione levels in peripheral blood mononuclear cells. Glutathione was determined colorimetrically from PBMC homogenates. Means ± SEM is calculated by subtracting baseline values from 45 d values.

Table 2
Cytokine secretion by PBMC cultured for 24 h with PHA-P.

	Placebo		Age	
	Baseline	45 d	Baseline	45 d
TNF-α	96.8 ± 11.0^{A}	129.6 ± 11.0^{B}	89.2 ± 11.2^{AB}	109.6 ± 11.2^{AB}
Time		$p = 0.018$		
Treatment		$p = 0.216$		
Interaction		$p = 0.579$		
t-test 10 d-Baseline	32.7 ± 18.7		20.4 ± 8.9	
$p = 0.555$				
Interferon-γ	14.7 ± 5.8^{A}	32.8 ± 5.8^{B}	15.8 ± 5.9^{A}	14.4 ± 5.9^{A}
Time		$p = 0.161$		
Treatment		$p = 0.164$		
Interaction		$p = 0.101$		
t-test 10 d-Baseline	17.1 ± 8.7		-1.4 ± 3.0	
$p = 0.049$				

Values are the means ± SEM. $n = 55$ placebo and $n = 54$ AGE. Means in a row with different superscripts indicate a significant difference ($p < 0.05$).

4. Discussion

4.1. Changes in biological function of immunity

Ex vivo proliferation of immune cells is an index of the cell's potential *in vivo*. If proliferation *ex vivo* is improved after consuming a bioactive compound then that cell type most likely has a greater pathogen fighting ability when called upon *in vivo*. Both NK cells and γδ-T cells showed greater proliferation in culture after AGE supplementation. NK cells expressed more NKG2D suggesting that consumption of the supplement improved the capability of this cell type to become activated. NKG2D plays an important role in the NK cell's cytotoxicity arsenal. The improvement in proliferation of both of these cells might result from an interaction of the AGE bioactive compounds with PRR on the cell surface, but as the NK cell does not require sensitization, the mechanism may be different from what has been hypothesized for bioactive compounds and the γδ-T cell.[29,30] Other studies have shown that consumption of AGE improves NK cell cytotoxicity,[24,25] but mechanistic studies are rare. To our knowledge this is the first time that γδ-T cells have been investigated in this type of intervention.

In general, the consumption of AGE resulted in lower inflammatory cytokine secretion by the cells. The cytokine status of an individual is influenced by their history of illness, as well as their environment and genetics. In this study, with no dietary change (placebo) and no illness, levels of cytokine secretion by PBMC were low. After AGE was consumed, cells secreted significantly less IFN-γ relative to baseline (day 0) secretion levels, suggesting that the immune system in these subjects could eradicate pathogens using a lower level of this inflammatory cytokine.

4.2. Changes in illness

The common cold is usually benign and annoying, whereas influenza can be deadly. Turner estimated that there are 23 million days people are absent from work and $2 billion dollars spent for over-the-counter medications in response to a cold,[31] while Keech has estimated a total economic burden of annual influenza epidemics at $87 billion.[32] Thus, preventing or reducing the duration and/or severity of colds and flu would have a significant economic impact. Very limited information exists regarding the impact of garlic on the health outcome of colds or flu. A systematic review[33] was published in 2009 based on only one study.[34] We found that the incidence of cold and flu illnesses was not different between those consuming AGE compared to a placebo. However, the severity of illness was significantly lower in the group that consumed AGE, as demonstrated by fewer symptom-days and less loss of productivity during an illness.

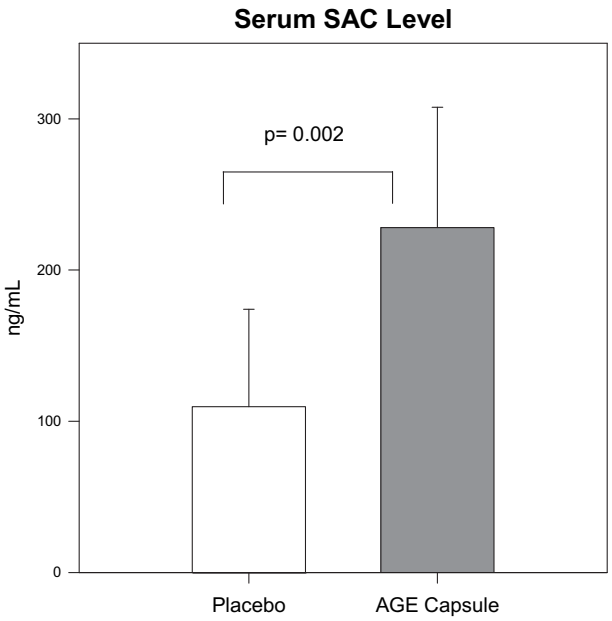

Fig. 5. Serum levels of S-allyl cysteine. Analysis was performed by Wakunaga Pharmaceutical Co., Ltd., Osaka, Japan. Serum samples that were collected at 45 d were extracted and analyzed by HPLC.[27]

4.3. Thiol-redox status

Much of our previous work has focused on the action of bioactive compounds priming γδ-T cells, suggested by others[5] to be a weak interaction with the PRR of these cells. AGE has several

Table 3
Characteristics of illnesses (cold and flu symptoms) over 90 days.[a]

	Placebo ($n = 56$)	Age ($n = 56$)	*p* value
Number of people that got ill/not ill	28/28	26/30	0.848
Reported incidence of illnesses (percent)	58 (55%)	48 (45%)	0.442
Number of days having symptoms in the group that got sick	358	317	0.132
Total number of symptoms reported during the study	737	584	<0.001
Average number of symptoms per illness incident	14.0 ± 17.6[b]	11.9 ± 17.0	0.536
Reported incidence of DIA[c]	38	15	<0.001
Total days of DIA during the study	126	53	<0.001
Number of work days missed	19	8	0.035
Total number of visits to doctor	4	8	0.221

Illness logs were kept through the 90 days of the study. When subjects felt sick, they checked what symptoms they had for that day and for each day thereafter. Illness was defined as more than 1 symptom for more than 1 day. The number of days of an illness as well as the number of total symptoms experienced during the illness was determined. For each day that the subject was ill, they recorded whether or not they had a decrease in their desire or ability to carry out their normal routine (DIA). Incidence is the number of times subjects reported "yes, they had a decrease in their daily activities". Out of 58 (PL) or 48 (AGE) reported illnesses, there was 38 or 15 reports of a decrease in activity sometime during that illness, respectively. The number of days that they felt this decrease in activity was also recorded.

[a] All values in the table were tested by the *z*-test of proportions except for Average Symptoms per group which was tested by the *t*-test.

[b] Mean ± SEM.

[c] DIA = Decrease in Activity is defined as an inability to carry out one's normal routine.

constituents that could potentially prime innate cells by a weak interaction with PRR, such as lectins, fructooligosaccharide,[1,2] polyphenols (e.g. apigenin), N (alpha) fructosyl arginine, and tetrahydro-carbolines. To more fully investigate this theory in the future, in vitro studies using individual compounds to determine interactions with NK and γδ-T cells are necessary. There is a second hypothesis that may also partially explain the changes to these immune cells. The thiol status of individuals was improved by consuming AGE, as shown by an increase of both intracellular glutathione and serum SAC levels. SAC is not found in fresh garlic, but is a water soluble, milder constituent formed in AGE during the aging process. Increased serum SAC levels in subjects from the AGE group indicate its bioavailability. The benefits of SAC have been shown in rodents [35–37] and in cell culture,[38] however no studies, thus far, have examined the immune modulating capabilities of SAC.

Greater lymphocyte GSH concentration also demonstrated the bioavailability of sulfhydryl compounds from AGE. Changes in GSH/GSSG have been associated with changes in proliferation, apoptosis, and post-translational protein modification for regulating signaling and metabolic pathways.[39–41] During inflammation and infection, GSH levels are reduced in neutrophils[42] and primary bronchial respiratory epithelial cells.[43] According to Wu et al.,[44] increased cellular GSH improved human PBMC proliferation by mitogenic stimulation, increased some cytokine secretion and decreased prostaglandin E2 and leukotriene B4. Improved thiol status may be responsible for improvements in cellular proliferation and activation, resulting in reduced illness; however, more work is needed to show cause and effect.

4.4. Strengths & limitations

One limitation of this study was the self-reported illnesses, without confirmation of pathogen presence. We did not distinguish between colds and the flu; however, we did not include allergy symptoms in the data analysis. Functional immune tests in humans are variable, not only because of genetic and environmental differences, but also due to variability in individual past illness profiles and vaccination history. We were not able to determine whether two illnesses ran back-to-back, which might underestimate incidence, although it is unlikely that back-to-back illnesses occurred in one group and not the other. We did not ask subjects to record their perception of symptom severity, but rather totaled the number of reported symptoms and the number of days that a particular symptom occurred. The perception of symptom intensity has been shown to be modified by diet.[26]

Proliferation and activation of two immune cell types, NK and γδ-T cells, were examined. Changes in these cells may or may not be responsible for the reduced number of symptoms. That being said, supplementation of AGE improved the ability of both NK and γδ-T cells to proliferate *ex vivo*. Each of these cell types kills virally infected host cells, and an enhancement of their functions by AGE may be responsible, in part, for reduced severity of illness.

Some of the compounds in AGE are unique due to the processing while some compounds may be found in both AGE and other garlic preparations. Since we do not know the biologically active compounds, we cannot extend these results of this study to other forms of garlic.

The strength of this experimental design is that changes in immune function are measured after an individual has consumed the product for a specified time period, rather than adding compounds to the cells while in vitro. Systemic cells do not interact with compounds found in the food itself due to the metabolism of compounds in the intestine and liver, thus adding food compounds in vitro is rarely physiological. Critics of in vitro work also mention that some compounds are not bioavailable; however, the absorption of compounds is not necessarily a prerequisite for modifying immunity. The intestine is one of the largest immune organs in the body. Immune cells that sample the contents of the lumen are not static, rather they continuously migrate though the blood and lymph system. Modification of intestinal immune cells that occurs during transit of microorganisms and bioactive compounds though the gut can ultimately be measured from systemic blood.

In summary, AGE supplementation reduced the severity of cold and flu symptoms and this was associated with changes in NK and γδ-T cell function, a reduction in inflammatory cytokine secretion and improved thiol status. Future work will be directed at examining which compounds in AGE contribute to these immune changes and exploring the ability of the compounds to interact with cells via the PRR.

Conflict of interest statement

The funding was provided by Wakunaga of America, Inc. and the Agricultural Experiment Station, IFAS, UF. The study sponsors had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication. SSP receives no other compensation outside of the funding for this research. No authors stand to benefit from the interpretation of the data or publication of this manuscript.

Statement of authorship

SSP is solely responsible for the content of the manuscript, interpretation of the results and design of the study. MPN, CAR, CM, RAC and JMS were responsible for data acquisition and writing of methodology.

Funding

ClinicalTrials.gov Identifier: NCT01390116.

Funding was provided by Wakunaga of America, Inc. and the Agriculture Experiment Station, IFAS, UF.

Acknowledgments

We thank the expertise of Neal Benson and staff at the Flow Cytometry Core Facility, ICBR, University of Florida. We acknowledge with gratitude the help of Cory Pollard and Denise Driscoll for their involvement in subject recruitment and retention. We also thank Linda Sweet for phlebotomy and James Colee for statistical assistance. Intervention protocol can be accessed at http://clinicaltrials.gov/ct2/show/NCT01390116.

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