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Associations of Dietary Patterns with Subpopulations of PBMCs and Their Transcriptomic Profiles in the Korean Elderly

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Abstract

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As human life expectancy has been increased, there has been a growing interest in the aging of immune system, which is associated with a variety of age-related diseases. To date, it has been established that the diet can regulate the immune system by several mechanisms. Recently, in a diet-related epidemiologic research, analyzing habitual dietary patterns based on the food groups has been considered as a comprehensive tool to consider complex combinations of food groups. However, little human re-

search has investigated the associations between dietary patterns and immune function of free-living elderly, and the fundamental mechanisms have not been completely understood. In this study, to elucidate the associations of the dietary patterns with populations and gene expressions of circulating immune cells in the elderly, we conducted a cross-sectional study in which the community-dwelling elderly (≥ 65 years) participated. We recruited 201 volunteers living in Seoul City and Seongnam City and screened the volunteers to select the elderly who had abilities to control basic needs and to perform more complex activities for independent life according to the Activities of Daily Living (ADL) and Instrumental-ADL (I-ADL). We collected one 24-hour recall and two-day food diaries of each subject. Food items were categorized into 15 food groups based on the Korean Nutrition Society database, and the intakes of food group were converted into percentages of daily energy intakes. The 3-day averages of intake of each food group were used to identify dietary patterns by *k*-means clustering. We isolated human peripheral blood mononuclear cells (PBMCs) which include circulating lymphocytes, monocytes and dendritic cells (DCs) from whole blood for fluorescence-activated cell sorting (FACS) analysis and next-generation sequencing (NGS) of mRNA. Serum C-reactive protein (CRP) concentrations were measured by enzyme-linked immunosorbent assay (ELISA). As a result, 92 elderlies completed this cross-sectional study, and cluster analysis identified two dietary patterns of the subjects: Cluster 1 (n = 60) characterized by higher intake of "cereals"; and Cluster 2 (n = 32) characterized by higher intakes of animal foods including "meats and their products", "fishes and shellfishes", "eggs", and "milk and their

products", as well as higher intakes of "potatoes and starches" and "beverage, tea and alcohol". Macronutrients compositions significantly differed in the subjects of two clusters, but the average energy intakes and BMI did not differ. Comparison of PBMCs subpopulation suggested that these two clusters significantly differed in T cells and NK cells subpopulations. Subjects in Cluster 2 had fewer $CD4^+$ T cells (for Cluster 1, 23.83 ± 1.28%; for Cluster 2, 15.77 ± 2.41%; $p < 0.01$) and more $CD8^+$ T cells (For Cluster 1, 29.80 ± 1.08%; for Cluster 2, 35.50 ± 2.03%; $p = 0.02$) and $CD56^{high}$ NK cells (for Cluster 1, 0.71 ± 0.11%; for Cluster 2, 1.37 ± 0.20%; $p < 0.01$). Subgroup analysis suggested that $CD8^+$ T cell frequency could be different according to the BMI. In addition, mRNA-sequencing analysis of PBMCs identified 957 differentially expressed genes (DEGs) between two clusters. These genes are mainly involved in innate immunity, such as binding, uptake and activation of lipopolysaccharide (LPS). Also, we observed higher expressions of genes related to TLR signaling, DC maturation, and antibacterial response in Cluster 2. Linear regression indicated that expressions of the *TLR2*, *TLR4*, *TLR8*, and *CD14* had a negative linear relationship with intakes of cereals (for *TLR2*, $\beta = -0.62$, $p = 0.03$; for *TLR4*, $\beta = -0.61$, $p = 0.04$; for *TLR8*, $\beta = -0.76$, $p < 0.01$; for *CD14*, $\beta = -0.81$, $p < 0.01$) and positive linear relationship with meats and their products intake (for *TLR2*, $\beta = 0.59$, $p = 0.04$; for *TLR4*, $\beta = 0.65$, $p = 0.02$; for *TLR8*, $\beta = 0.69$, $p = 0.01$; for *CD14*, $\beta = 0.86$, $p < 0.01$). In addition, the serum CRP concentration was significantly higher in Cluster 2 than in Cluster 1 (for Cluster 1, 0.59 ± 0.23 mg/L; for Cluster 2, 1.60 ± 0.43 mg/L; $p = 0.04$). It may imply that subjects in Cluster 2 have higher level of inflammation than

subjects in Cluster 1. In conclusion, diet rich in animal food was associated with the lower CD4/CD8 ratio (for Cluster 1, 0.87 ± 0.06; for Cluster 2, 0.45 ± 0.11; $p < 0.01$) and higher frequency of $CD56^{high}$ NK cells with higher expressions of genes involved in infection and inflammation of PBMCs as well as higher concentrations of serum CRP. These results suggest that diet rich in animal food might be associated with the higher susceptibility to infection and chronic inflammation in the community-dwelling elderly. These results provide an insight into the fundamental mechanisms of dietary effects on immune function in the elderly.

Keywords: The community-dwelling elderly, dietary pattern, peripheral blood mononuclear cells (PBMCs), fluorescence-activated cell sorting (FACS) analysis, transcriptomic profile, C-reactive protein (CRP)

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List of Abbreviations

ADL	Activities of daily living
CI	Confidence interval
CRP	C-reactive protein
DC	Dendritic cell
DEG	Differentially expressed gene
FACS	Fluorescence-activated cell sorting
I-ADL	Instrumental activities of daily living
IFN	Interferon
IPA	Ingenuity Pathway Analysis
MFI	Median fluorescence intensity
NGS	Next-generation sequencing
NK cell	Natural killer cell
NSAID	Nonsteroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
RPKM	Exon per million mapped reads
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UN	United Nations.

I. Introduction

Advances in medical and scientific technology have remarkably extended human life expectancy. According to the World Population Prospects: The 2017 Revision of the United Nations (UN), the number of the elderly aged 65 and older is expected to reach approximately 1 billion by 2030, more than double of the number of 2000. Naturally, there has been a growing interest in healthy aging because aging is associated with a functional decline of body systems such as pulmonary, cardiovascular, and immune systems, which increases the mortality in the elderly (Joaquin and Gollapudi 2001). Especially, aging immune system, collectively termed as immunosenescence, including low-grade, chronic inflammation is apparently associated with morbidity and mortality (Nikolich-Zugich 2018, Shaw, Goldstein, and Montgomery 2013). Aged immune system progressively loses the ability to control infection and increases the susceptibility to infections (Aw, Silva, and Palmer 2007, Pera et al. 2015). Therefore, delaying immunosenescence and reducing chronic inflammation are especially important for the elderly.

The role of diet in developing immunity and modulation of immune function has been receiving growing attention (Maijo et al. 2014). It has been well established that several dietary factors such as carotenoids, fiber, vitamin E, zinc, and fish oil or n-3 polyunsaturated fatty acid (PUFA) improve immune function of the elderly (Pae, Meydani, and Wu 2012, Galland 2010). In contrast, intake of saturated fatty acids is considered to be positively associated with inflammation (Santos, Oliveira, and

Lopes 2013).

However, it has been important to consider the whole dietary pattern rather than a single dietary factor, because habitual diet consists of a variety of foods. Dietary pattern derived from factor analysis, dietary indices, or cluster analysis has been considered as a comprehensive tool to reflect complex effects of combined dietary factors (Hu 2002). To date, an increasing body of evidence suggested that dietary patterns is associated with a wide range of physiological conditions such as metabolites (Bouchard-Mercier, Rudkowska, et al. 2013, Gibbons et al. 2017), metabolic syndrome (Xia et al. 2016), and gut microbiome (Claesson et al. 2012, Tindall, Petersen, and Kris-Etherton 2018). Therefore, it has been accepted that the dietary pattern could be related to the health status. For example, Mediterranean dietary pattern is considered as a "healthy dietary pattern", which emphasizes high intake of fruits, vegetables and nuts with olives and extra virgin olive oil. Clinical trials have reported that this dietary pattern is associated with the lower mortality and risk of diseases such as coronary heart disease, stroke, and cancer (Benetou et al. 2008, Fung et al. 2009, Mitrou et al. 2007).

In this regard, many studies have attempted to determine the association of dietary patterns with immune function. A nested case-control study which was a part of Nurses' Health Study (Belanger et al. 1978) reported that the dietary pattern high in sugar-sweetened soft drinks, refined grains, diet soft drinks and processed meat

and low in wine, coffee, cruciferous and yellow vegetables may increase chronic inflammation and risk of type 2 diabetes (Schulze et al. 2005). A case-control study in which Japanese adults aged 50-74 y participated suggested that a healthy dietary pattern which was high in vegetables, fruit, soy products, and fish was associated with low concentration of C-reactive protein (CRP) involved in inflammation (Nanri et al. 2008). This result was consistent with other case-control studies identified the inverse association between healthy dietary pattern and concentrations of inflammatory markers (Nettleton et al. 2006, Centritto et al. 2009, Lopez-Garcia et al. 2004, Esmaillzadeh et al. 2007).

In the immunologic research, human peripheral blood mononuclear cells (PBMCs) has become of importance. PBMCs including lymphocytes, monocytes, and dendritic cells (DCs) which are major regulators of immune defense system continuously differentiate and proliferate (Sen, Kemppainen, and Oresic 2017). Since they systemically circulate in the blood stream and interact with the every cell of human body, PBMCs population and gene expressions can dynamically reflect systemic environment (Liew et al. 2006). So PBMCs analysis is an informative tool to investigate dietary effects on immune system. Previously, several cross-sectional studies reported that PBMCs phenotypes and their mRNA transcripts were associated with the dietary patterns by fluorescence-activated cell sorting (FACS) analysis and microarray (Bouchard-Mercier, Paradis, et al. 2013, Nettleton et al. 2010). Intervention studies of Mediterranean dietary pattern clarified the effect of dietary intake on cell phenotypes and pro-inflammatory gene expressions of PBMCs of the elderly by

FACS analysis and quantitative real time polymerase chain reaction (PCR) analysis (Yubero-Serrano et al. 2012, Camargo et al. 2012, Maijo et al. 2018).

Despite the growing interests and importance, the association of dietary patterns with populations and global gene expressions of PBMCs in the elderly remain not fully understood. It is partly because of the lack of studies investigating dietary patterns among free-living elderly, and absence of systemic screening of global population and whole transcriptomes of PBMCs. To elucidate the association, we conducted a comprehensive analysis of PBMCs across the habitual dietary patterns of the community-dwelling elderly. We aimed to compare broader populations of PBMCs including lymphocytes, monocytes, and DCs and their whole transcriptomic profiles of different dietary patterns. This study may provide a fundamental link of usual dietary intake with PBMCs populations and gene expressions of the elderly.

II. Subjects and Methods

1. Subjects

This study was a two-centered cross-sectional study approved by the Institutional Review Boards of Seoul National University (IRB No. 1801/002-015). Briefly, 201 free-living individuals aged 65 or older were recruited and administered screening tests at each center between May to December 2018 at each center located in Seoul City and Seongnam City, Republic of Korea. Exclusion criteria were as follows: 1) Activities of Daily Living (ADL) score < 7 and/or Instrumental-ADL score < 10; 2) usual supplementation of probiotics and/or n-3 polyunsaturated fatty acids (PUFAs); 3) long-term consumption of antibiotics or anticoagulants within the previous three months; and 4) incomplete dietary records. In total, 92 subjects were included in this cross-sectional study. All subjects provided written informed consent, and visited each study center twice for the dietary assessment and blood collection. With the exception of the blood samples from Seongnam center, only 45 blood samples from Seoul center were included in the further analysis.

2. Dietary assessment

Dietary intakes were assessed by 24-hour recalls and a two-day dietary records. We tested the 24-hour recall via personal interviews, and instructed the subjects regarding the assessment of portion sizes and procedure to complete the dietary record. The completed dietary records were reviewed to clarify the types and portions of food items by researchers. We input three-day dietary intake data into CAN-Pro 5.0 of Korean Nutrition Society to determine energy and nutrient consumption. Averages of the three-day intake data were analyzed. All researchers were trained to perform the dietary assessment.

3. Dietary pattern analysis

To determine the dietary patterns, we categorized each food item into 15 food groups based on the Korean Nutrition Society database. We converted the intakes of each food group into percentages of daily energy intake. The three-day averages were clustered by using the *k*-means clustering algorithm of SPSS version 25.0 for Windows (IBM cor., Chicago, IL, USA). *K* was based on the results of hierarchical clustering of food intake data.

4. Blood collection

We asked subjects to quit all supplementation for two weeks before drawing the blood. Fasting blood samples were collected from the cephalic vein into BD vacutainer EDTA-containing tubes, serum-separating tubes, and mononuclear cell preparation tubes (BD biosciences, Franklin Lakes, NJ, USA). Serum separated by centrifugation, aliquoted, and frozen at -80°C for subsequent analysis.

5. Assessment of variables

On the day of screening, trained researchers determined education level, medical history and medicine use of all the subjects via personal interview. Education level was considered as a categorical variable (primary, middle or high school, and college or more). Intake of medicine including steroids, non-steroidal anti-inflammatory drugs (NSAIDs) and statins was coded as binary variables (yes or no). Then, all subjects completed anthropometric measurements including height and body weight and a self-administered questionnaire including date of birth, smoking status, drinking quantity, and physical activity level on the day of testing. Subjects reported their smoking status (never, former, or current smoker), drinking quantity (type of alcoholic drink, frequency and quantity), and physical activity level (type of physical activity, frequency and duration). Drinking quantity was converted into a continuous variable of alcohol consumption (grams of consumed alcohol per week) and physical activity level was also converted into a continuous variable (hours of moderate to vigorous physical activity per week).

6. PBMCs population analysis

Frequencies of PBMC subpopulations were analyzed by FACS analysis. We isolated PBMCs from 4 mL of EDTA whole blood obtained from 45 participants via density-gradient centrifugation using Ficoll-Paque PLUS density gradient media. PBMCs were resuspended in FACS buffer (0.1% bovine calf serum and 0.05% sodium azide in phosphate-buffered saline [PBS]) and stained with antibodies for 30 min at 4°C. The following antibodies were used: 1) PE-Cy7-conjugated anti-human CD19 (HIB19, 25-0199-42; Life Technologies, Carlsbad, CA, USA) for B cell frequency analysis; 2) APC-Cy7-conjugated anti-human CD4, Alexa Fluor 700-conjugated anti-human CD8, PE-conjugated anti-human CD197, and FITC-conjugated anti-human CD45RA (RPA-T4, 561839; RPA-T8, 557945; 3D12, 552176; HI100, 555488; BD Pharmingen, San Jose, CA, USA) for T cell subpopulation analysis; 3) BV711-conjugated anti-human CD3 (UCHT1, 563725; BD Pharmingen, San Jose, CA, USA) and Brilliant Violet785-conjugated anti-human CD56 (5.1H11, 362550; BioLegend, San Diego, CA, USA) for NK cell subpopulation analysis; and 4) PE-conjugated anti-human CD14, FITC-conjugated anti-human CD16, APC-conjugated anti-human CD11c and PE-Cy5-conjugated anti-human HLA-DR (M5E2, 555398; NKP15, 347523; B-ly, 559877; HI100, 555488; BD Pharmingen, San Jose, CA, USA) for monocytes and DCs. After staining, we analyzed the frequency of cells using FACS LSRII (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (TreeStar, Ashland, OR, USA).

7. Profiling of PBMCs transcriptomes

To analyze PBMC transcriptomic profile, we conducted next-generation sequencing (NGS). PBMCs were isolated from 8 mL of whole blood collected in BD vacutainer mononuclear cell preparation tubes (BD bioscience, Franklin Lakes, NJ, USA) within 2 h after blood sampling. Total RNA was immediately extracted from PBMCs according to the manufacturer's protocol (RNAqueous-4PCR Kit; Ambion Inc., Austin, TX, USA), aliquoted and frozen at -80°C until the sequencing.

For mRNA sequencing, we randomly selected 12 individuals who did not use medicine including steroids, NSAIDs or statins. We verified the quality of extracted total RNA of 12 subjects using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). None of these samples had RNA integrity number (RIN) values less than eight, and therefore, all the samples were sequenced.

From 1 μg of total RNA, we captured intact mRNA using Dynabeads mRNA DIRECT Micro Kit (Ambion Inc., Austin, TX, USA) and prepared whole transcriptome libraries via reverse transcription using Ion total-RNA Seq Kit v2 (Life Technologies, Carlsbad, CA, USA). These cDNA libraries were purified with the nucleic acid-binding beads and amplified by PCR using Ion Xpress RNA 3′ Barcode Primer (Life Technologies, Carlsbad, CA, USA). We repeated bead purification of cDNA libraries, assessed the yield and size distribution of the libraries by Agilent 2100 Bioanalyzer with High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA), and diluted the libraries with 100 pmol/L. Diluted libraries were amplified

with Ion Sphere Particles (ISPs) using Ion OneTouch 2 system and Ion PI Hi-Q OT2 200 Kit (Life Technologies, Carlsbad, CA, USA). Template-positive ISPs were enriched using the OneTouch Enrichment System Instrument (Life Technologies, Carlsbad, CA, USA). The enriched ISPs were sequenced using the Ion PI Hi-Q Sequencing 200 Kit (Life Technologies, Carlsbad, CA, USA), with sequencing primers annealed. Prepared samples were loaded on the Ion PI Chip v3 (Life Technologies, Carlsbad, CA, USA) with the polymerase, and the chip was placed on the Ion Proton System (Life Technologies, Carlsbad, CA, USA). All procedures were performed following the manufacturers' instructions.

8. Bioinformatics analysis

Using the raw reads, filtered FASTQ files were generated by removing the adapter sequence and low-quality reads, and uploaded to the Torrent Suite software (Life Technologies, Carlsbad, CA, USA). We mapped and aligned these high-quality reads using Bowtie 2 and TopHat to generate aligned BAM files (Trapnell et al. 2010). The transcripts levels were normalized according to reads per kilobase of exon per million mapped reads (RPKM) using a mixed-model approach. Differentially expressed genes (DEGs) were filtered based on the *p* values and fold change ($P < 0.05$; fold change > 1.3 or < -1.3) determined using the *t* test. The bioinformatics analysis was performed via Partek Genomics Suite v 6.6 (Partek Inc., Saint Louis, MI, USA).

For functional annotation of DEGs, we used Ingenuity Pathway Analysis (IPA; QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). *P* values associated with enriched functional categories were determined using the right-tailed Fisher's Exact test.

9. Measurement of serum C-reactive protein concentrations

We measured serum CRP concentrations of 43 participants using enzyme-linked immunosorbent assay (DuoSet ELISA Ancillary Reagent Kit, DY008; DuoSet ELISA Development System, DY1707; R&D Systems, Minneapolis, MN, USA). In a brief, we coated the 96-well plates with capture antibody and incubated overnight at room temperature. After wash process by wash buffer (0.05% Tween 20 in PBS) and blocking process by reagent diluent (1% BSA in PBS), we loaded serum samples and detection antibody according to the protocol. Streptavidin-HRP and substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) were added after repeated wash process and incubation at room temperature. Stop solution (H_2SO_4) was added to terminate the reaction. All samples and standards were duplicated. Optical density of each well was immediately determined by using a microplate reader (Spectramax 190 Microplate Reader, Molecular Devices, San Jose, CA, USA) set to 450 nm. The concentration of each biomarker was directly calculated from the linear regression of log-transformed concentration and optical density.

10. Statistical analysis

Statistical analysis was performed via SPSS 25.0 for Windows (IBM cor., Chicago, IL, USA). Data were tested for normality before the analysis. The *t* test was used to compare the dietary intake, energy consumption, age, and BMI according to different dietary patterns. Chi-square test was used to determine the significance of categorical variables including sex, education and smoking status. PBMC subsets were analyzed using the *t* test or Mann-Whitney *U* test according to their distribution. In order to obtain the odds ratios and 95% confidence intervals (CIs) of diabetes, cardiovascular diseases, hypertension, and dyslipidemia, we conducted logistic regression analysis. To analyze PBMCs subsets and CRP concentration adjusted for potential confounders and to investigate the interaction of dietary clusters with sex and BMI, we used a generalized linear model and obtained estimated marginal means. In order to analyze the correlation and the linear relationship between gene expression and dietary intake, Spearman correlation analysis and linear regression were used. Statistical significance was defined as $p < 0.05$.

III. Results

1. Identification of two distinct patterns of diet in the elderly

In total, the study population consisted of 92 free-living elderly subjects including 53 females and 39 males, whose ages ranged from 65 to 85 years. All participants were initially screened by ADL and I-ADL; therefore, they had abilities to perform basic physical needs such as bathing, dressing, transferring, toileting and eating, and more complex activities such as shopping, financial management and intake of medicine, which are important for independent living in community.

We performed hierarchical clustering analysis of dietary intake data to determine the number of dietary patterns, and the analysis suggested the clear distinction into two groups based on food group intake (Figure 1). So we fixed *k* as two in *k*-means clustering. As a result, the two clusters were clearly distinguished by intakes of food groups (Table 1). Subjects in Cluster 1 consumed higher amounts of "cereals" such as rice, bread, and noodles than those in Cluster 2, while Cluster 2 was characterized by higher intakes of animal foods such as "meats and their products", "eggs", "fishes and shellfishes", and "milk and dairy products", as well as "potatoes and starches" and "beverage, tea and alcohol".

As summarized in Table 2, the macronutrient compositions of two dietary patterns significantly differed, although the energy intakes of two clusters did not significantly differ. Particularly, the intakes of protein and fat from animal foods were

significantly higher in subjects in Cluster 2. In addition, they consumed higher amounts of riboflavin, pantothenic acid, phosphorus, chlorine, coper, fluorine, and selenium than the subjects in Cluster 1.

When general characteristics were compared between two dietary patterns, age, BMI, education level, smoking status, and alcohol consumption were not different, however, the male-to-female sex ratio varied (Table 3). Odds ratios of diabetes, cardiovascular diseases, hypertension and dyslipidemia suggested that the two clusters did not differ in prevalence of these chronic diseases (Table 4).

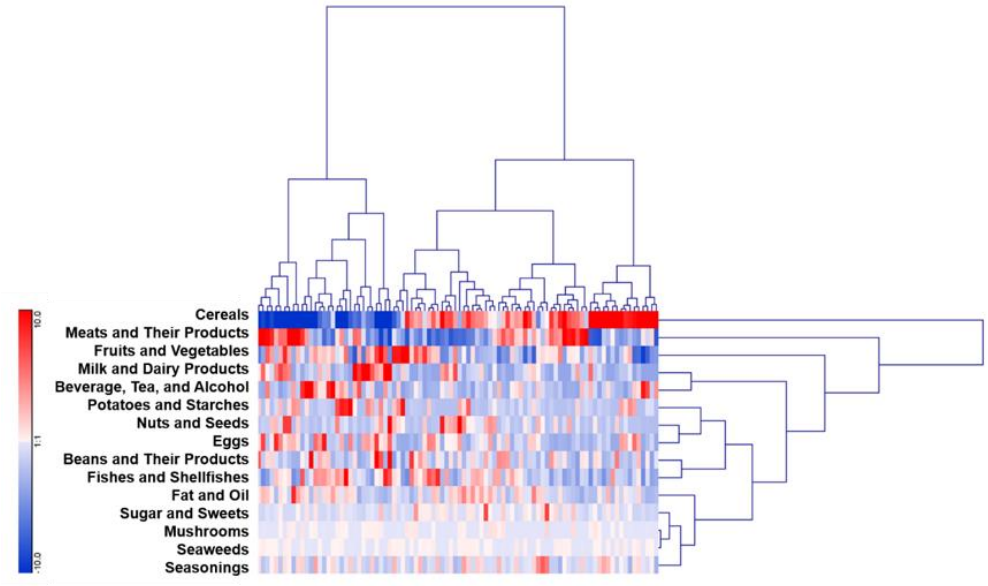

Figure 1. Hierarchical clustering of energy contributions of food groups

A heatmap represents the mean-centered energy contributions of each food group of all participants. Each column represents dietary intake of each subject. Hierarchical clustering analysis based on Ward method displays the subjects could be classified into two clusters based on the dietary intakes.

Table 1. Average energy contribution of food groups across different dietary patterns[1]

	Cluster 1 (n = 60)	Cluster 2 (n = 32)	P[2]
Cereals	52.87 ± 6.91	33.14 ± 6.61	<0.01
Meats and their products	7.76 ± 5.56	11.55 ± 7.81	0.02
Eggs	2.37 ± 2.36	5.08 ± 3.50	<0.01
Fishes and shellfishes	4.38 ± 2.97	6.04 ± 4.10	0.03
Milk and dairy products	2.13 ± 2.48	6.34 ± 5.76	<0.01
Potatoes and starches	1.45 ± 2.33	3.37 ± 4.08	0.02
Beverage, tea and alcohol	3.05 ± 3.03	5.49 ± 5.52	0.03
Sugar and sweets	1.47 ± 1.58	0.99 ± 0.68	0.10
Beans and their products	3.45 ± 2.39	4.84 ± 4.62	0.12
Nuts and seeds	2.08 ± 2.66	2.53 ± 3.27	0.48
Fruits and vegetables	11.56 ± 4.85	13.35 ± 5.32	0.11
Mushrooms	0.17 ± 0.31	0.17 ± 0.19	0.97
Seaweeds	0.17 ± 0.15	0.14 ± 0.16	0.37
Fat and oil	4.00 ± 1.97	4.15 ± 2.22	0.74
Seasoning	3.09 ± 1.79	2.80 ± 1.54	0.44

[1] Dietary patterns were derived by k-means clustering. Data are expressed as means ± SDs.

[2] P values were based on t test.

Table 2. Average nutrients intakes of each dietary pattern[1]

	Cluster 1	Cluster 2	*P*[2]
Energy intake (kcal)	1584.31 ± 327.70	1555.84 ± 418.70	0.72
Carbohydrate (% of total energy)	64.75 ± 6.12	54.12 ± 7.08	< 0.01
Fiber (g)	24.76 ± 7.29	25.21 ± 9.16	0.60
Protein (% of total energy)	15.05 ± 2.63	18.11 ± 2.73	< 0.01
Vegetable protein (% of total energy)	8.57 ± 1.33	7.64 ± 2.01	0.02
Animal protein (% of total energy)	6.48 ± 2.51	10.43 ± 2.97	< 0.01
Fat (% of total energy)	19.80 ± 4.57	26.29 ± 6.81	< 0.01
Vegetable fat (% of total energy)	10.97 ± 2.67	11.37 ± 3.81	0.60
Animal fat (% of total energy)	8.37 ± 3.72	14.44 ± 5.80	< 0.01
n-3 PUFAs (g)	0.83 ± 0.88	0.83 ± 0.61	0.97
Saturated fatty acids (g)	8.32 ± 5.21	10.73 ± 5.12	0.04
Cholesterol (mg)	203.73 ± 137.37	353.57 ± 149.29	< 0.01
Riboflavin (mg)	1.18 ± 0.45	1.53 ± 0.55	< 0.01
Pantothenic acid (mg)	4.00 ± 1.00	4.91 ± 2.19	0.03
Phosphorus (mg)	974.73 ± 37.24	1110.17 ± 57.19	0.04
Chlorine (mg)	94.94 ± 9.55	196.99 ± 29.62	< 0.01
Copper (μg)	547.53 ± 204.83	940.55 ± 929.03	0.02
Fluorine (mg)	0.01 ± 0.01	0.02 ± 0.02	0.01
Selenium (μg)	51.33 ± 22.52	71.02 ± 32.01	< 0.01

[1] Dietary patterns were derived by *k*-means clustering based on the energy contributions of each food group. Data are expressed as means ± SDs. PUFA, polyunsaturated fatty acid.

[2] *P* values were based on the *t* test.

Table 3. General characteristics of participants[1]

	Total population	Cluster 1	Cluster 2	*P*
Age (year)	71.09 ± 4.59[2]	71.47 ± 5.13	70.38 ± 3.29	0.22[3]
BMI (kg/m²)	23.72 ± 2.83	24.13 ± 2.83	22.95 ± 2.73	0.06
Sex (*n*)	53 F / 39 M	30 F / 30 M	23 F / 9 M	0.04[4]
Education (*n*)				
Primary	25	19	6	
Middle or high school	43	28	15	
College or more	24	13	11	0.28
Smoking status (*n*)				
Never	80	49	31	
Former	7	6	1	
Current	5	5	0	0.10
Alcohol consumption (g/week)	18.96 ± 42.36	16.95 ± 34.08	22.74 ± 55.09	0.54
Physical activity (h/week)	5.42 ± 4.28	5.50 ± 4.53	5.27 ± 3.84	0.81

[1] General characteristics of total populations and each cluster are suggested.

[2] Data are expressed as means ± SDs (all such values).

[3] *P* values of age, BMI, alcohol consumption and physical activity were obtained by the *t* test.

[4] *P* values of sex, education and smoking status were based on the Chi-square test.

Table 4. Odds ratios and 95% confidence intervals of chronic diseases[1]

	Cluster 1	Cluster 2	*P*
Diabetes			
Model 1[3]	Ref	2.677 (0.394, 18.185)[2]	0.31
Model 2[4]	Ref	2.871 (0.387, 21.288)	0.30
Cardiovascular Diseases			
Model 1	Ref	5.390 (0.617, 47.092)	0.13
Model 2	Ref	4.584 (0.408, 51.548)	0.22
Hypertension			
Model 1	Ref	0.794 (0.260, 2.423)	0.69
Model 2	Ref	0.824 (0.252, 2.694)	0.75
Dyslipidemia			
Model 1	Ref	0.566 (0.097, 3.306)	0.53
Model 2	Ref	0.805 (0.115, 5.631)	0.83

[1] Odds ratios and *p* values were obtained from logistic regression.

[2] All values are expressed as odds ratios (95% CIs).

[3] Adjusted for age (y), sex (F/M), and BMI (< 18.5, ≥ 18.5 and < 23, ≥ 23 and < 25, ≥ 25 and < 30, ≥ 30 kg/m^2).

[4] Adjusted as model 1 + alcohol consumption (g/week), smoking status (never, former, or current smoker), and physical activity (h/week).

2. Populations of PBMCs

To investigate the association between dietary patterns and immune cell population, we examined the frequencies of subpopulations in PBMCs via FACS analysis using antibodies to the cell surface makers. In lymphoid cells, we analyzed B cells ($CD19^+$ cells/total live cells), $CD4^+$ T cells ($CD4^+$ $CD8^-$ cells/lymphocytes), $CD8^+$ T cells ($CD8^+$ $CD4^-$ cells/lymphocytes), $CD56^{low}$ NK cells ($CD3^-$ $CD56^{low}$ cells/lymphocytes), and $CD56^{high}$ NK cells ($CD3^-$ $CD56^{high}$ cells/lymphocytes). In myeloid cells, we analyzed the frequencies of classical monocytes ($CD14^+$ $CD16^-$ /monocytes), nonclassical monocytes ($CD14^-$ $CD16^+$/monocytes), and DCs ($CD11c^+$ $HLA\text{-}DR^+$ cells/$CD14^-$ $CD16^-$ cells).

Among lymphoid cells, B cells did not differ in the two dietary patterns, but we found differences in subpopulations of T cells and NK cells, even after all the potential confounders were adjusted (Table 5; Figure 3). Compared to subjects in Cluster 1, subjects in Cluster 2 showed fewer $CD4^+$ T cells (for Cluster 1, 23.83 ± 1.28%; for Cluster 2, 15.77 ± 2.41%; $p < 0.01$) and more $CD8^+$ T cells (for Cluster 1, 29.80 ± 1.08%; for Cluster 2, 35.50 ± 2.03%; $p = 0.02$), resulting a significantly lower CD4/CD8 ratio (for Cluster 1, 0.87 ± 0.06; for Cluster 2, 0.45 ± 0.11; $p < 0.01$). In addition, the proportion of $CD56^{high}$ NK cells was significantly higher in Cluster 2 than in Cluster 1 (for Cluster 1, 0.71 ± 0.11%; for Cluster 2, 1.37 ± 0.20%; $p < 0.01$). However, there was no differences in myeloid cells including classical and nonclassical monocytes and DCs. (Table 5; Figure 4).

Additionally, to examine the T cell aging status, we analyzed $CD4^+$ and $CD8^-$ T cell subpopulations including naïve T cells ($CD45RA^+$ $CD197^+$), central memory T cells (CM T cells; $CD45RA^-$ $CD197^+$), effector memory T cells (EM T cells; $CD45RA^-$ $CD197^-$), and effector memory T cells RA (EMRA T cells; $CD45RA^+$ $CD197^-$). However, we found no statistical significance (Table 6; Figure 5).

Next, we categorized the participants according to the sex (F/M) and BMI (< 25 or ≥ 25 kg/m^2) to perform subgroup analysis of $CD4^+$ and $CD8^+$ T cells, CD4/CD8, and $CD56^{high}$ NK cells in each cluster and to investigate the interaction between dietary clusters and sex or BMI. As suggested in Table 7 and Figure 6, sex was not a significant factor in these markers. However, subgroups categorized by BMI showed different trends in $CD8^+$ T cell frequency (Table 8; Figure 7). In Cluster 2, $CD8^+$ T cell frequency was significantly different according to the BMI subgroups ($p = 0.048$). Underweight or normal subjects (BMI < 25 kg/m^2) showed lower frequency than the overweight or obese subjects (BMI ≥ 25 kg/m^2). Also, when we compared the $CD8^+$ T cell frequencies of overweight or obese subjects in Cluster 1 and Cluster 2, there was no statistical significance (for Cluster 1, 29.80 ± 2.07; for Cluster 2, 27.45 ± 4.40; $p = 0.53$), which was a different trend compared to BMI < 25kg/m^2 subgroups (for Cluster 1, 30.01 ± 1.41; for Cluster 2, 37.90 ± 2.58; $p = 0.02$).

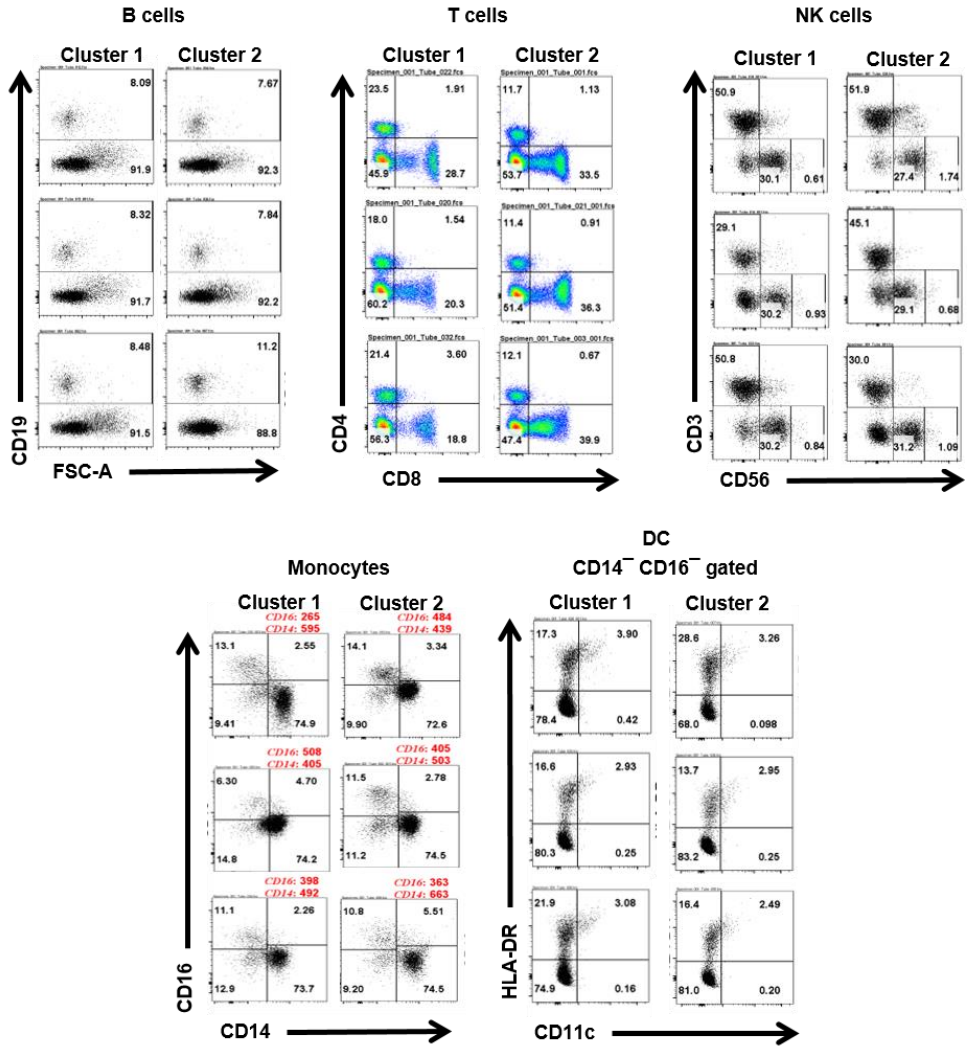

Figure 2. Representative plots of B cells, T cells, NK cells, monocytes and DCs frequency analysis

Table 5. Estimated marginal means of PBMCs populations across different dietary patterns[1]

	Cluster 1 (n = 34)	Cluster 2 (n = 11)	*P*			
			Crude[2]	Model 1[3]	Model 2[4]	Model 3[5]
B cell						
$CD19^+$ (%)	8.22 ± 0.54	9.71 ± 1.02	0.26	0.23	0.20	0.22
T cell						
$CD4^+$ (%)	23.83 ± 1.28	15.77 ± 2.41	0.02	0.02	< 0.01	< 0.01
$CD8^+$ (%)	29.80 ± 1.08	35.50 ± 2.03	0.05	0.05	0.05	0.02
CD4/CD8	0.87 ± 0.06	0.45 ± 0.11	0.04	< 0.01	< 0.01	< 0.01
NK cell						
$CD56^{high}$ (%)	0.71 ± 0.11	1.37 ± 0.20	0.33	0.04	< 0.01	< 0.01
$CD56^{low}$ (%)	32.00 ± 1.80	34.03 ± 3.39	0.67	0.85	0.73	0.61
Monocyte						
$CD14^- CD16^+$ (%)	11.70 ± 0.69	11.75 ± 1.30	0.87	0.74	0.80	0.97
$CD14^+ CD16^-$ (%)	74.65 ± 1.14	73.05 ± 2.14	0.67	0.37	0.78	0.53
Dendritic cell						
$CD11c^+$ $HLA\text{-}DR^+$ (%)	3.01 ± 0.20	2.78 ± 0.37	0.59	0.77	0.47	0.60

[1] Data are expressed as estimated marginal means ± SEs of model 3. Frequencies of each cell were obtained by FACS analysis using antibodies of cell surface marker. Gating strategies were as follows: B cells, $CD19^+$ cells/total live cells; T cells, $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells/lymphocytes; NK cells, $CD3^-$ $CD56^+$/lymphocytes; Monocytes, $CD14^-$ $CD16^+$ or $CD14^+$ $CD16^-$/total monocytes; dendritic cells, $CD11c^+$ $HLA\text{-}DR^+$ cells/$CD14^-$ $CD16^-$ cells. CD4/CD8 represents the ratio of frequencies of $CD4^+$ T cells and $CD8^+$ T cells.

[2] Unadjusted p values were obtained from t test or Mann-Whitney U test depending on the distributions.

[3,4,5] Multivariable-adjusted p values were based on the generalized linear model.

[3] Adjusted for age (y), BMI (kg/m^2), and sex (F/M).

[4] Adjusted as model 1 + energy intake (kcal/d), smoking status (never, former, or current smoker), alcohol consumption (g/week), and physical activity level (h/week).

[5] Adjusted as model 2 + medicine including steroid, NSAID, and statin use (y/n).

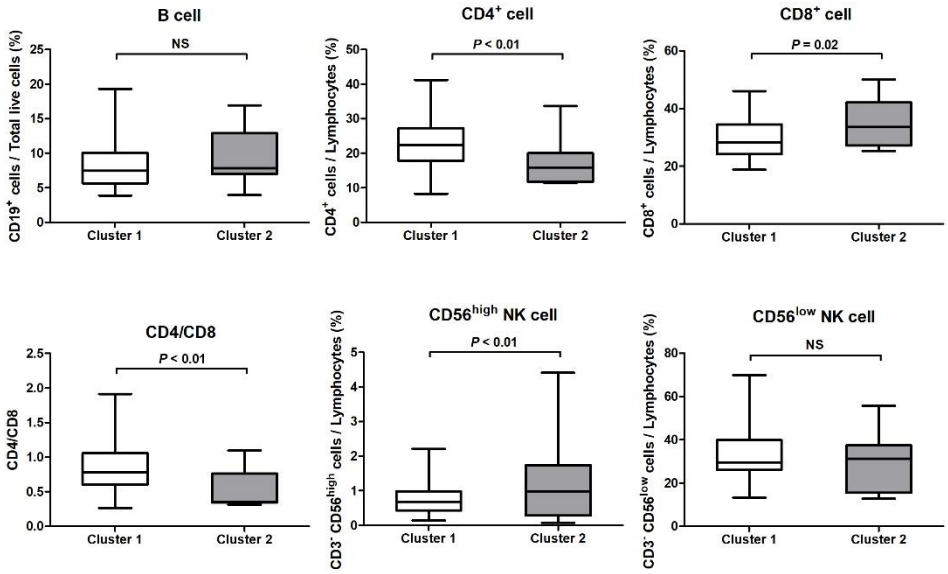

Figure 3. Lymphocytes subpopulations

Box and whisker plots display the distributions of the lymphocytes subpopulations. *P* values were based on the generalized linear models with the adjustments of potential confounders including age (y), BMI (kg/m^2), sex (F/M), energy intake (kcal/d), smoking status (never, former, or current smoker), alcohol consumption (g/week), physical activity (h/week), and medicine including steroids, NSAID, and statin use (y/n). NS, not significant.

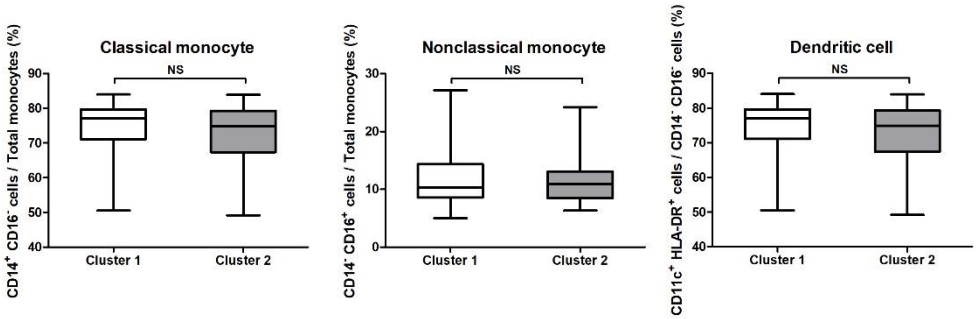

Figure 4. Frequencies of myeloid cells

Box and whisker plots display the distributions of the myeloid cell subpopulations. Significances were based on the generalized linear models with the adjustments of potential confounders including age (y), BMI (kg/m^2), sex (F/M), energy intake (kcal/d), smoking status (never, former, or current smoker), alcohol consumption (g/week), physical activity (h/week), and medicine including steroids, NSAID, and statin use (y/n). NS, not significant.

Table 6. Estimated marginal means of subpopulations of $CD4^+$ and $CD8^+$ T cells in peripheral blood across different dietary patterns[1]

	Cluster 1 (n = 34)	Cluster 2 (n = 11)	*P*			
			Crude[2]	Model 1[3]	Model 2[4]	Model 3[5]
Gated on $CD4^+$						
Naïve ($CD45RA^+$ $CD197^+$)	42.30 ± 2.36	38.63 ± 4.44	0.37	0.44	0.52	0.49
CM ($CD45RA^-$ $CD197^+$)	24.55 ± 0.96	25.92 ± 1.80	0.37	0.25	0.63	0.52
EM ($CD45RA^-$ $CD197^-$)	27.75 ± 1.87	29.66 ± 3.51	0.36	0.71	0.64	0.65
EMRA ($CD45RA^+$ $CD197^-$)	5.39 ± 0.47	5.78 ± 0.89	0.91	0.78	0.72	0.71
Gated on $CD8^+$						
Naïve ($CD45RA^+$ $CD197^+$)	4.15 ± 0.68	5.15 ± 1.28	0.65	0.46	0.55	0.51
CM ($CD45RA^-$ $CD197^+$)	0.52 ± 0.05	0.53 ± 0.10	0.46	0.58	0.94	0.97
EM ($CD45RA^-$ $CD197^-$)	25.11 ± 1.17	21.37 ± 2.20	0.89	0.19	0.16	0.15
EMRA ($CD45RA^+$ $CD197^-$)	70.23 ± 1.41	72.95 ± 2.65	0.80	0.41	0.36	0.39

[1] Frequencies of naïve, CM, EM and EMRA cells/$CD4^+$ or $CD8^+$ T cells were measured by FACS analysis using antibodies to cell surface markers CD45RA and CD197. All frequency values are expressed as estimated marginal means ± SEs of model 3. CM, central memory; EM, effector memory; EMRA, effector memory RA.

[2] Unadjusted *p* values were obtained from *t* test or Mann-Whitney *U* test depending on the distributions.

[3,4,5] Multivariable-adjusted *p* values were based on the generalized linear model.

[3] Adjusted for age (y), BMI (kg/m^2), and sex (F/M).

[4] Adjusted as model 1 + energy intake (kcal/d), smoking status (never, former, or current smoker), alcohol consumption (g/week), and physical activity level (h/week).

[5] Adjusted as model 2 + medicine including steroid, NSAID, and statin use (y/n).

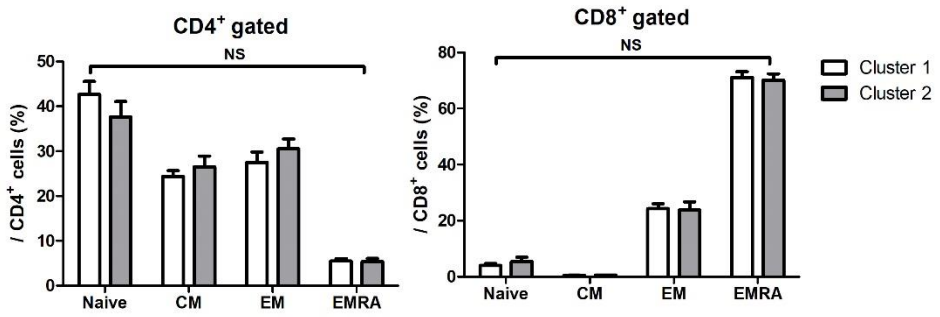

Figure 5. Subpopulations of $CD4^+$ and $CD8^+$ T cells

Bar plots indicate means with SEMs. CM, central memory; EM, effector memory; EMRA, effector memory RA. NS, not significant.

Table 7. $CD4^+$ and $CD8^+$ T cells, CD4/CD8, and $CD56^{high}$ NK cell by sex (F/M) in each dietary pattern[1]

	Cluster 1 (n = 34)			Cluster 2 (n = 11)			
	Female (n = 16)	Male (n = 18)	P_{sex}[2]	Female (n = 5)	Male (n = 6)	P_{sex}[2]	$P_{Interaction}$[3]
$CD4^+$ T cell (%)	23.95 ± 1.82	22.32 ± 1.93	0.42	18.12 ± 3.08	17.33 ± 3.41	0.87	0.82
$CD8^+$ T cell (%)	31.07 ± 1.70	28.94 ± 1.60	0.55	38.09 ± 2.59	32.51 ± 2.34	0.13	0.80
CD4/CD8 (ratio)	0.91 ± 0.09	0.76 ± 0.10	0.29	0.60 ± 0.12	0.48 ± 0.13	0.52	0.64
$CD56^{high}$ NK cell (%)	0.82 ± 0.10	0.69 ± 0.11	0.40	1.55 ± 0.45	0.84 ± 0.50	0.32	0.25

[1] Data are expressed as estimated marginal means ± SEs obtained from a generalized linear model adjusting for the age (y) and BMI (kg/m^2). Frequencies of each cell were obtained by FACS analysis using antibodies of cell surface marker. Gating strategies were as follows: $CD4^+$ or $CD8^+$ T cells, $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells/lymphocytes; $CD56^{high}$ NK cells, $CD3^-$ $CD56^{high}$/lymphocytes. CD4/CD8 represents the ratio of frequencies of $CD4^+$ T cells and $CD8^+$ T cells.

[2,3] P values were based on the generalized linear model adjusting for age (y) and BMI (kg/m^2).

[3] $P_{Interaction}$ indicates p values for interaction between dietary cluster and sex (F/M).

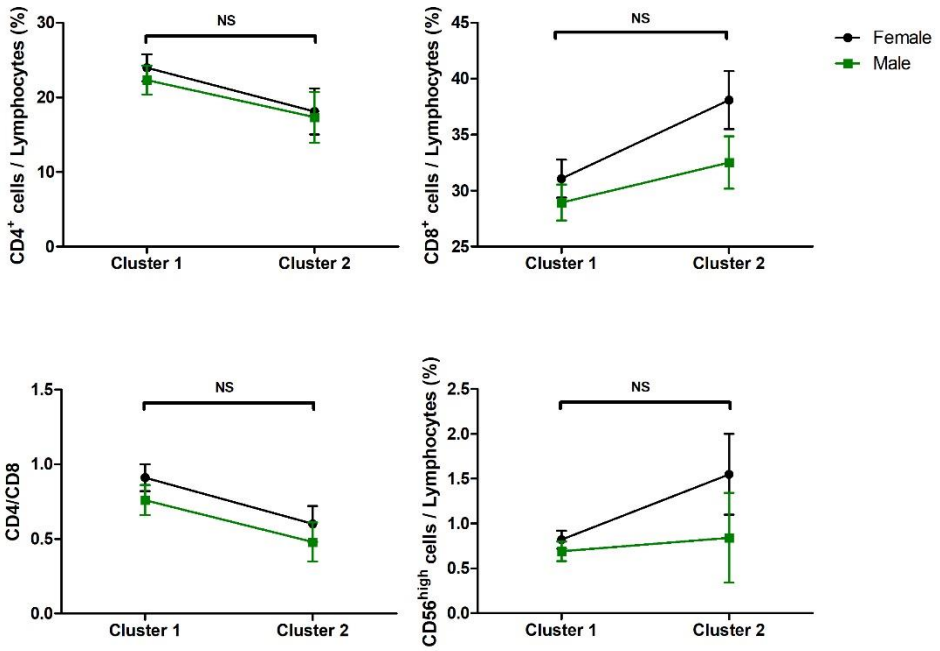

Figure 6. $CD4^+$ and $CD8^+$ T cells, CD4/CD8, and $CD56^{high}$ NK cell by sex (F/M) in each dietary pattern

Dots and error bars represent estimated marginal means ± SEs obtained from a generalized linear model adjusting for the age (y) and BMI (kg/m^2). NS, not significant.

Table 8. $CD4^+$ and $CD8^+$ T cells, CD4/CD8, and $CD56^{high}$ NK cell by BMI (< 25 and ≥ 25 kg/m²) in each dietary pattern[1]

	Cluster 1 (n = 34)			Cluster 2 (n = 11)			
	BMI < 25 kg/m² (n = 23)	BMI ≥ 25 kg/m² (n = 11)	P_{BMI}[2]	BMI < 25 kg/m² (n = 8)	BMI ≥ 25 kg/m² (n = 3)	P_{BMI}[2]	$P_{Interaction}$[3]
$CD4^+$ T cell (%)	23.17 ± 1.60	23.21 ± 2.35	0.99	18.45 ± 2.54	15.92 ± 4.34	0.63	0.76
$CD8^+$ T cell (%)	30.01 ± 1.41	29.80 ± 2.07	0.94	37.90 ± 2.58	27.45 ± 4.40	0.05	0.13
CD4/CD8 (ratio)	0.87 ± 0.08	0.79 ± 0.12	0.58	0.53 ± 0.10	0.60 ± 0.18	0.74	0.59
$CD56^{high}$ NK cell (%)	0.83 ± 0.09	0.62 ± 0.13	0.21	1.36 ± 0.37	0.85 ± 0.63	0.50	0.88

[1] Data are expressed as estimated marginal means ± SEs obtained from a generalized linear model adjusting for the age (y) and sex (F/M). Frequencies of each cell were obtained by FACS analysis using antibodies of cell surface marker. Gating strategies were as follows: $CD4^+$ or $CD8^+$ T cells, $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ cells/lymphocytes; $CD56^{high}$ NK cells, $CD3^-$ $CD56^{high}$/lymphocytes. CD4/CD8 represents the ratio of frequencies of $CD4^+$ T cells and $CD8^+$ T cells.

[2,3] P values were based on the generalized linear model adjusting for age (y) and sex (F/M).

[3] $P_{Interaction}$ indicates p values for interaction between dietary cluster and BMI (< 25 and ≥ 25 kg/m²).

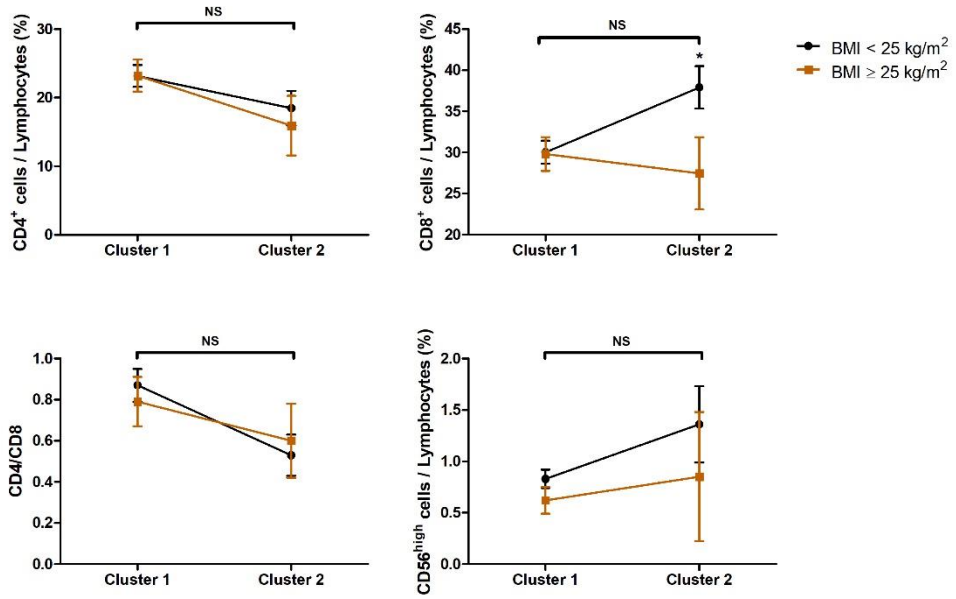

Figure 7. $CD4^+$ and $CD8^+$ T cells, CD4/CD8, and $CD56^{high}$ NK cell by BMI (< 25 and ≥ 25 kg/m^2) in each dietary pattern

Dots and error bars represent estimated marginal means ± SEs obtained from a generalized linear model adjusting for the age (y) and sex (F/M). NS, not significant; **P* < 0.05.

3. PBMCs transcriptomic profiles

FACS analysis suggested that each cluster has different population of PBMCs. Then. we screened gene expression profile of PBMCs among 12 randomly selected participants from two clusters. Six mRNA samples were loaded on ISP beads to be stacked to one Ion PI^{TM} chip. In each chip, three samples per each dietary cluster were loaded to reduce batch effects. Average loading density of Ion beads were 93.5%, and the loading density plots are presented in Figure 8. As summarized in Table 9, all samples were successfully sequenced and aligned.

According to the statistical criteria of $p < 0.05$ and fold change > 1.3 or < -1.3, we found that 957 genes were differentially expressed in the two dietary patterns. Principal component analysis (PCA) and hierarchical clustering of these DEGs indicated that PBMCs gene expression profiles were clearly distinguished by the two dietary patterns (Figure 9; Figure 10). As a result of functional categorization of this DEGs set by IPA, we observed that genes belonging to the functional categories of nutrients metabolism, cell interaction, and cell movement were differentially regulated (Figure 11A). Among them, carbohydrate metabolism was the most enriched function in this set of DEGs, and therefore, we analyzed the subfunction of carbohydrate metabolism. As shown in Figure 11B, top 5 enriched subfunctions of carbohydrate metabolism did not include nutrients metabolic pathway, such as glycolysis, gluconeogenesis or glycogenolysis. The most significant pathways included binding, uptake and activation of bacterial recognition components such as lipopolysaccharide

(LPS), carbohydrate and polysaccharide.

So we compared expressions of genes related to detection and killing of bacteria and activation of innate immunity, and we found differences in Toll-like receptor (TLR) signaling, DC maturation, and antibacterial responses (Figure 12). All genes of TLR signaling including the most downstream gene, *IL1R1*, were highly expressed in Cluster 2. We also found that 9 out of 11 genes involved in activation of myeloid cells were highly expressed in Cluster 2 (Figure 13). In addition, as shown in Figure 14, we observed that subjects of Cluster 2 also showed higher expressions of *IFNGR2*, *TNFAIP2*, *TNFRSF10C*, and *TNFSF13* which are related to interferon (IFN) and tumor necrosis factor (TNF), the pro-inflammatory cytokine.

We then identified the associations of specific food groups with gene expressions. Since TLR signaling is the most upstream responses of DC maturation and antibacterial response, we determined the correlation between the expressions of genes involved in TLR signaling and food-group intake via Spearman correlation analysis. We found that *TLR2*, *TLR4*, *TLR8* and *CD14* expressions had significant correlations with intakes of cereals and meats and their products (Figure 15). Therefore, we investigated the linear relationships between the expressions of these genes and the intakes of cereals and meats and their products. As can be seen in Figure 16, these genes showed a linear relationship with the intake of cereals (for *TLR2*, β = -0.62, p = 0.03; for *TLR4*, β = -0.61, p = 0.04; for *TLR8*, β = -0.76, $p < 0.01$; for *CD14*,

β = -0.81, $p < 0.01$) and meats and their products (for *TLR2*, $\beta = 0.59$, $p = 0.04$; for *TLR4*, $\beta = 0.65$, $p = 0.02$; for *TLR8*, $\beta = 0.69$, $p = 0.01$; for *CD14*, $\beta = 0.86$, $p < 0.01$).

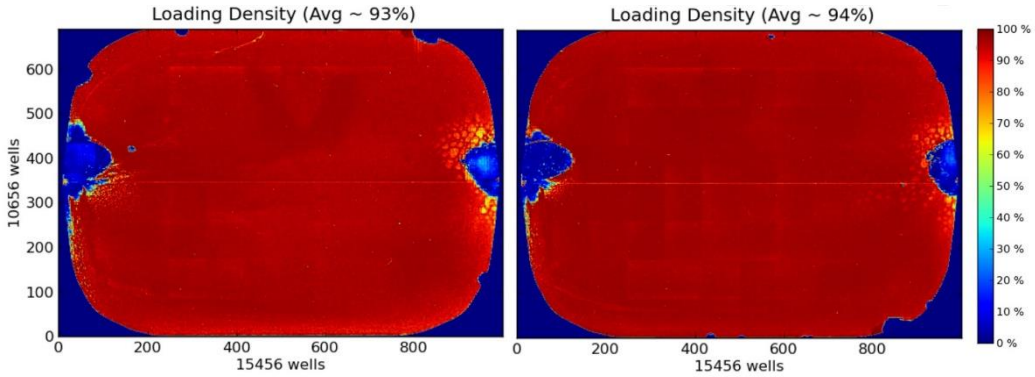

Figure 8. Loading density of ISP beads on the Ion PI™ chips

Table 9. Summary of results from RNA sequencing[1]

Cluster	Aligned reads	Total reads	Percent aligned	Mean read length
Cluster 1	13,311,203	13,555,778	98.20%	147 bp
	10,072,085	10,209,723	98.65%	172 bp
	12,200,032	12,343,832	98.84%	159 bp
	14,788,257	15,002,185	98.57%	148 bp
	14,432,327	14,732,834	97.96%	115 bp
	15,824,032	16,101,879	98.27%	141 bp
Cluster 2	12,903,929	13,026,273	99.06%	161 bp
	12,916,728	13,133,578	98.35%	157 bp
	16,565,178	16,908,874	97.97%	129 bp
	17,031,849	17,361,885	98.10%	132 bp
	16,951,283	17,246,671	98.29%	141 bp
	15,223,843	15,447,321	98.55%	139 bp

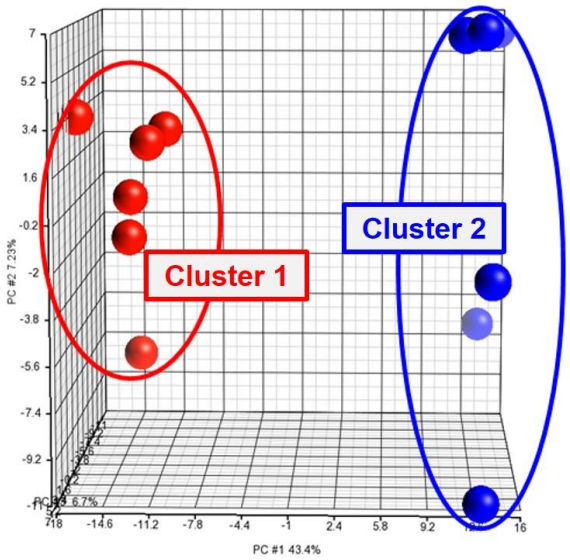

Figure 9. Principal components analysis (PCA) plots of PBMCs mRNA transcriptomic profiles

Each sphere represents each subject. Circles on the plot indicate that the transcriptomic profiles of two clusters were classified by PCA.

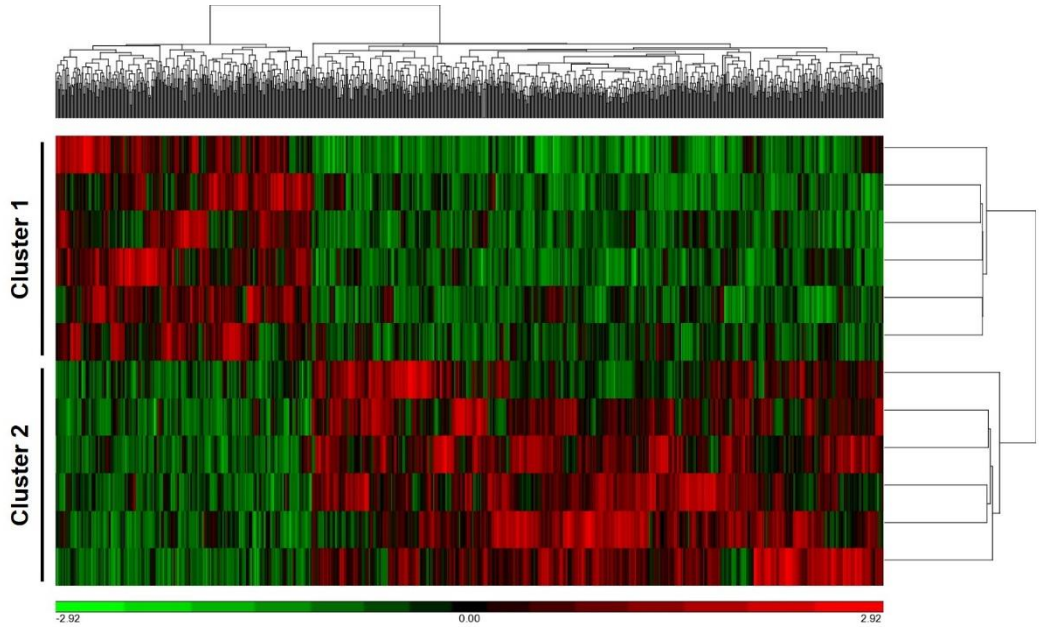

Figure 10. Hierarchical clustering of 957 differentially expressed genes (DEGs)

The heatmap indicates the normalized expression levels of DEGs. Each row represents gene expressions of an individual subject.

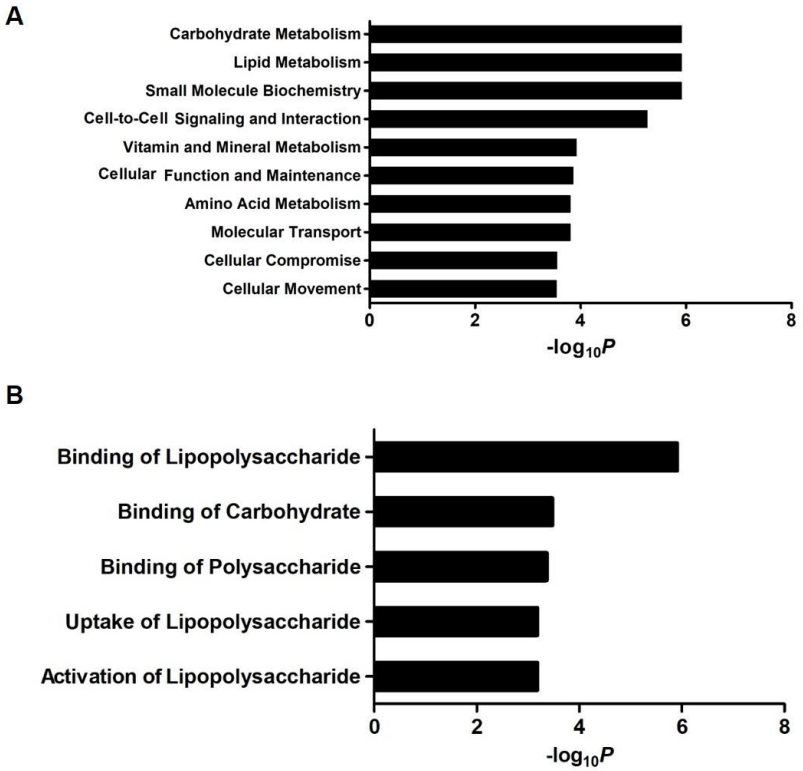

Figure 11. The most enriched functional categories of 957 DEGs identified by Ingenuity Pathway Analysis (IPA)

IPA identified (A) top 10 enriched functions of 957 DEGs and (B) top 5 enriched subfunctions of carbohydrate metabolism. Significances were based on Fisher Exact test and data are presented by $-\log_{10}P$.

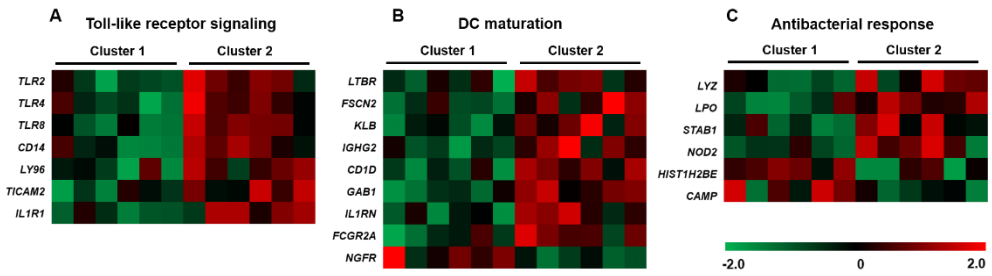

Figure 12. Expression levels of genes related to Toll-like receptor signaling, DC maturation and antibacterial response.

Heatmap represents z-scored expression levels of genes involved in (A) TLR signaling, (B) DC maturation, and (C) antibacterial response. Each column represents gene expression of each participant. DC, dendritic cell.

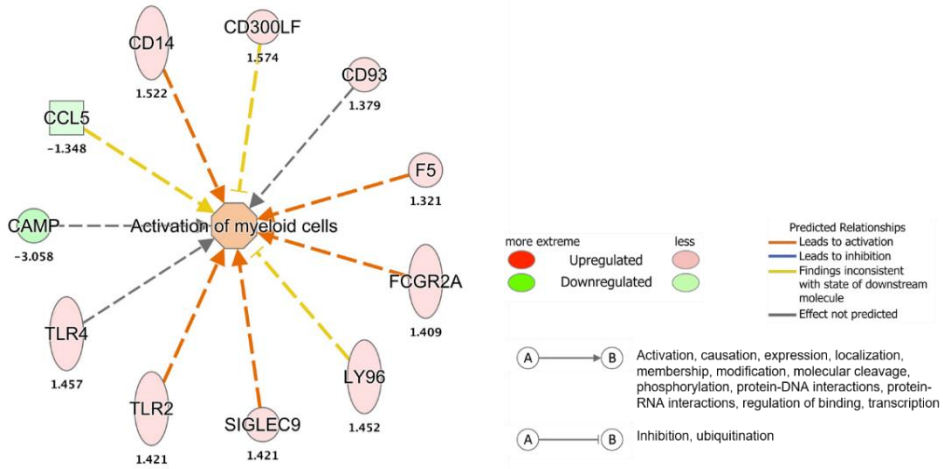

Figure 13. Genes involved in activation of myeloid cells

Among DEGs, genes related to the activation of myeloid cells are suggested. The values under the gene symbol indicates fold change based on the contrast of Cluster 2 vs. Cluster 1. Red color represents higher expression in Cluster 2 and green color means higher expression in Cluster 1.

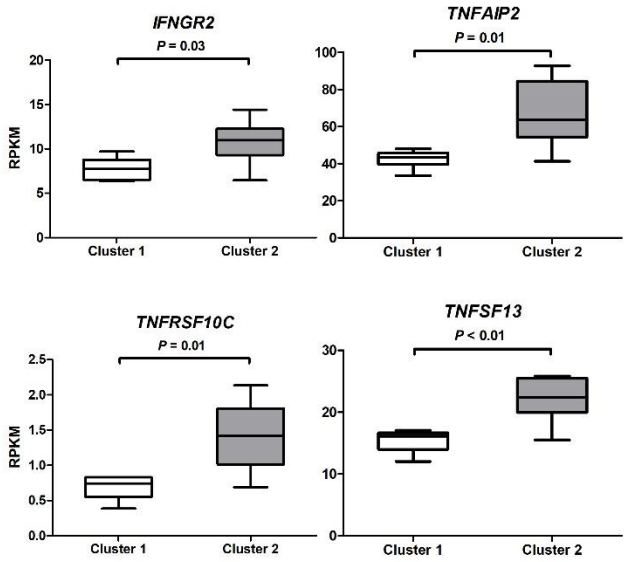

Figure 14. Expressions of genes related to interferon and tumor necrosis factor

Box and whisker plots display the distributions of expression levels.

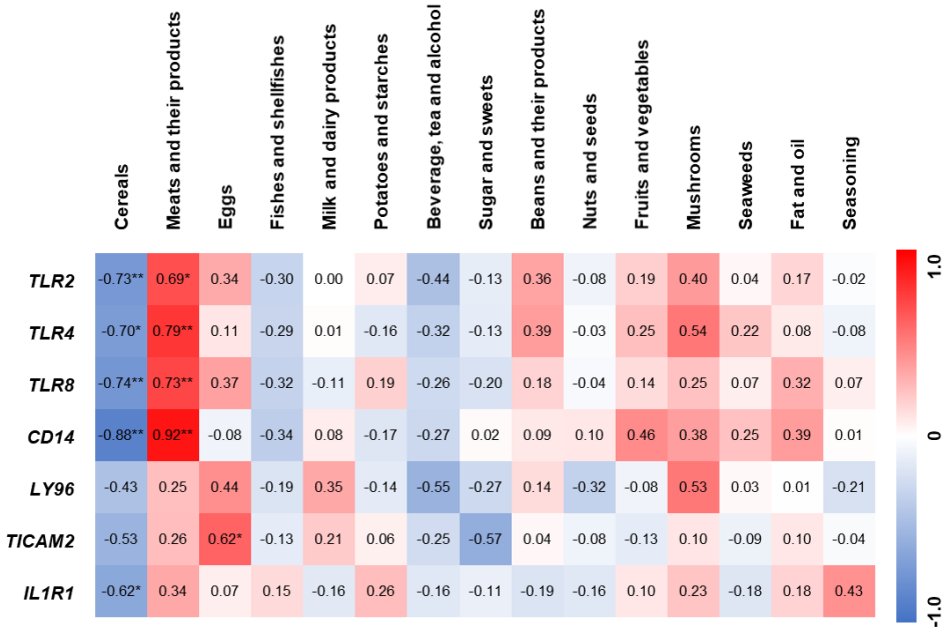

Figure 15. Correlations between expressions of genes involved in Toll-like receptor signaling and food-group intake

Heatmap represents the Spearman correlation coefficients. *$P < 0.05$, **$P < 0.01$

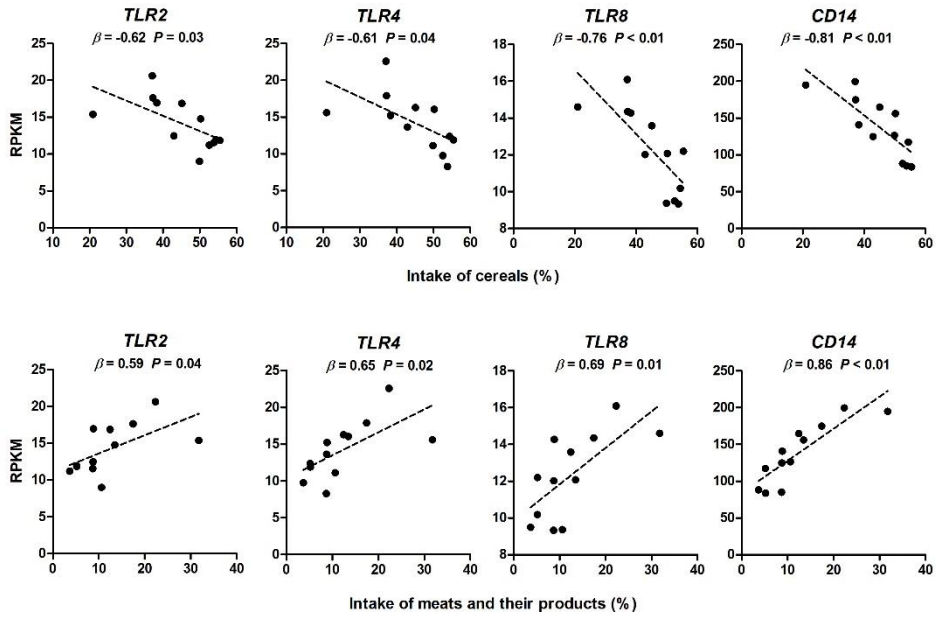

Figure 16. Linear relationships between the expressions of *TLR2*, *TLR4*, *TLR8*, and *CD14* and intake of cereals and meats and their products

Linear regression was used to determine the linear relationship. Expressions of *TLR2*, *TLR4*, *TLR8*, and *CD14* of PBMCs showed negative linear relationship with intake of cereals and positive linear relationship with intake of meats and their products. Each dot represents the each subject.

4. Serum C-reactive protein concentrations

To determine whether these differences in PBMC populations and their transcriptomic profiles were actually reflected in systemic inflammation, we measured the serum CRP concentrations of 43 participants by ELISA. The unadjusted mean (± SD) concentration of all the available data was 0.81 (± 1.47) mg/L. When potential confounders were adjusted, Cluster 2 showed a significantly higher CRP concentration than Cluster 1 (Figure 17).

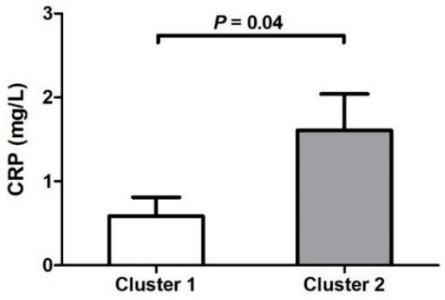

Figure 17. Serum C-reactive protein concentrations of two dietary patterns

Serum CRP concentrations were measured by ELISA in 43 participants. Bar plot represents estimated marginal means with SEs and p value obtained from the generalized linear model. Potential confounders including age (y), BMI (kg/m²), sex (F/M), smoking status (never, former, or current smoker), alcohol consumption (g/week), and physical activity (h/week) were used as covariates. CRP, C-reactive protein.

IV. Discussion

The growing interest in the association between dietary patterns and immune function promoted this study to identify the habitual dietary patterns and to correlate them with profiles of PBMC population and transcriptome signatures in the elderly. In this study, we found that dietary patterns were significantly associated with T cells and NK cells and correlated with 957 DEGs of PBMCs mRNA.

We used *k*-means clustering to analyze dietary patterns of this study population. The cluster analysis among the method of dietary pattern analysis can classify the individuals into distinct clusters based on the food intake (Hu 2002). In this study, we conducted hierarchical clustering of dietary intake data before *k*-means clustering to fix *k*. As a result, we derived two main dietary patterns of 92 community-dwelling elderlies. Subjects in Cluster 1 were characterized by higher intake of cereals than those in Cluster 2, but their mean intake is adequate when compared with recommendations for Korean. In contrast, Cluster 2 was characterized by higher intakes of animal foods than Cluster 1. Also, subjects in Cluster 2 showed higher intake of animal protein and animal fat than in Cluster 1, although daily energy intake and BMI were not different in two dietary patterns.

Compositions of PBMCs are dynamically changed by the microenvironment of immune system (Gaber, Strehl, and Buttgereit 2017). In this regard, we broadly assessed the frequencies of lymphocytes, monocytes, and DCs by FACS analysis and

observed the significant differences of T cell and NK cell population in the two different dietary clusters. Fewer $CD4^+$ T cells and more $CD8^+$ T cells resulted in a lower CD4/CD8 ratio in Cluster 2 than in Cluster 1. Inverted CD4/CD8 ratio (CD4/CD8 < 1) represents an immune risk phenotype (IRP), which is a set of bioparameters related to poor immune function (Boren and Gershwin 2004). The lower CD4/CD8 of Cluster 2 could be related to the increased morbidity and mortality in the elderly (Serrano-Villar et al. 2014).

We also examined the T cell aging status by using antibodies of surface marker CD45RA and CD197 (Fulop, Larbi, and Pawelec 2013). As T cells age, naïve T cells decrease, while memory T cells increase to reduce the adaptive immune response (Moro-Garcia, Alonso-Arias, and Lopez-Larrea 2013, Fagnoni et al. 2000, Kovaiou and Grubeck-Loebenstein 2006). However, the subpopulations of $CD4^+$ and $CD8^+$ T cells did not differ in the two dietary patterns. These results indicate that dietary pattern may not be related to the T cell aging in our older population.

Human NK cells are divided into two subclasses based on CD56 expressions: $CD56^{high}$ NK cells express low levels of perforin, but are specialized for cytokine production; $CD56^{low}$ NK cells are characterized by high expression of perforin and high level of cytotoxicity (Stabile et al. 2017). In our study, only the frequency of $CD56^{high}$ NK cells in lymphocytes was significantly different. Frequency of Cluster 2 was significantly higher than in Cluster 1. Although $CD56^{high}$ NK cell is a minor subset of cells in peripheral blood, these cells efficiently produce pro-inflammatory

cytokines such as IFN-γ and TNF-α, mediating a wide range of innate and adaptive immune responses (Zhang and Tian 2017, Stabile et al. 2017). This finding implies that Cluster 2 might display increased inflammatory response mediated by pro-inflammatory cytokines.

We then performed subgroup analysis of all the significant markers to control for residual confounders and to investigate interaction effects of dietary clusters with sex and BMI. We could not find the significance in interaction effects, but in Cluster 2 characterized by higher intake of animal food, obese or overweight subjects had significantly fewer $CD8^+$ T cells than the normal or underweight subjects. In addition, it was observed that BMI $\geq$ 25 kg/m^2 subgroup showed a reversed trend in the $CD8^+$ T cell frequency; subjects in Cluster 2 had fewer $CD8^+$ T cells than subjects in Cluster 1, although it was not significant. Previously, it has been reported that high scores of Diet Quality Index (DQI) and Healthy Eating Pattern (HEP) with lower intake of saturated fat and meats were associated with higher frequency of $CD8^+$ T cell and lower CD4/CD8 ratio in obese or overweight postmenopausal women whose BMI was 25-45 kg/m^2 (Boynton et al. 2007). These results are consistent with our results of BMI $\geq$ 25 kg/m^2 subgroups.

Next, we profiled whole transcriptomes of PBMCs to investigate the genetic mechanisms of signatures of higher inflammation and poor immunity in Cluster 2 which was suggested by FACS analysis. In human transcriptome analysis, analyzing six samples per each group is considered adequate to detect DEGs in clinical study

(Fu et al. 2010), and thus, we found that 957 genes were differentially expressed. Functional categorization indicated that genes related with binding, uptake and activation of LPS were significantly enriched in this DEGs set.

Although no significant difference was observed in monocyte composition, we found that subjects in Cluster 2 showed higher expressions of *CD14* gene coding the LPS receptor CD14 than in Cluster 1. Previous cross-sectional studies were also reported the associations of whole diet with monocyte subpopulations (Dias et al. 2015) and median fluorescence intensity (MFI) of CD14 (Nettleton et al. 2010) in middle-aged and older adults. The studies reported that healthy diets have inverse associations with frequency of $CD14^+$ monocytes and CD14 MFI. However, in our study, high *CD14* expressions did not induce the difference in monocytes composition.

In addition, Cluster 2 showed higher of TLR signaling and antibacterial response, suggesting increased reactivity to the infection. TLR promotes immune response to infection by sensing the pathogen-associated molecular patterns (PAMPs) and maturating DCs (Medzhitov 2001). DCs activated by TLR pathway can efficiently present antigens derived from phagocytosis of bacteria or dead cells to $CD8^+$ T cells to initiate cytotoxic immune response (Alloatti et al. 2015). These results may be associated with immune tolerance (Agrawal, Agrawal, and Gupta 2017). High expression of DC maturation signaling might contribute to loss of tolerance and increased reactivity against self, which can play an important role in age-related diseases (Audiger et al. 2017). Given that regulatory $CD4^+$ T cells are also important to

maintain immune tolerance (Sakaguchi et al. 2008), a diet rich in animal food might be decrease the tolerance, as well as enhance cytotoxic activity.

Genes related to the pro-inflammatory cytokine IFN and TNF, *IFNGR2*, *TNFAIP2*, *TNFRSF10C*, and *TNFSF13* were also highly expressed in subjects of Cluster 2. They are dynamically involved in inflammatory response. *IFNGR2* expression is tightly regulated to generally limit the responsiveness of IFN-γ. Previous research reported that IFN-γ-exposed $CD4^+$ T_H2 cells display high levels of *IFNGR2* and are related to apoptotic program (Schroder et al. 2004). TNF-α inducible protein 2 coded by *TNFAIP2* gene primarily responds to TNF-α. This protein mediates inflammation and regulates cell proliferation and migration (Jia et al. 2018). IFN-γ and TNF-α are produced by $CD56^{high}$ NK cells, and thereby these genes could be highly expressed by higher frequency of $CD56^{high}$ NK cells of subjects in Cluster 2. These results also suggest that subjects of Cluster 2 may show high level of basal inflammatory response.

Correlation analysis and linear regression suggested that these different TLR signaling may be caused by intakes of cereals and meats and their products. Since these two food groups are the major sources of energy, higher intake of cereal is related to reduced intake of meats and their products. So we speculated that the differences in intake of meats and their products mainly contributes to differences in TLR signaling, resulting in an extensive immune reaction. This result is consistent with previous studies on dietary intake and TLRs expressions (Kopp et al. 2018, Nettleton

et al. 2010). Meat products contain well-known TLRs activators, saturated fatty acids (Snodgrass et al. 2013). In addition, high intake of meats and their products containing organic sulfur may break the gut mucus network, so the bacteria could penetrate the mucus layer and activate TLR signaling (Kopp et al. 2018).

We then evaluated the actual inflammatory status by measuring the serum CRP concentration, which was positively correlated with increased mortality and morbidity in the elderly (Velissaris et al. 2017). As a result, Cluster 2 showed higher concentration than Cluster 1, confirming the association. CRP is a marker of acute-phase inflammation, so higher level of CRP could be associated with the inflammatory response. Previous research reported that CRP induces the inflammation via TLR4 signaling in vascular smooth muscle cells, causing cardiovascular diseases (Liu et al. 2010). Our transcriptomic profiles and serum CRP concentration also suggested the association between TLR signaling and CRP concentration. A systematic review reported that meat-based dietary pattern is associated with higher CRP concentration (Barbaresko et al. 2013).

In conclusion, our study highlights the association of dietary patterns based on food groups with global PBMCs populations and transcriptomic profiles, as well as the inflammatory marker in the community-dwelling elderly. Dietary pattern rich in animal food displayed activated immune response and increased susceptibility to infection, which could be characteristics of aging, in addition to the elevated serum CRP concentration. To the best of our knowledge, this is the first research which

comprehensively investigated the PBMCs population and their transcriptomic profile with inflammatory biomarkers across the dietary intake pattern derived from *k*-means clustering in the free-living elderly.

However, several limitations should be considered to interpret our data. First, this study was cross sectional, so we cannot conclude the causality. Second, we did not consider the differences in intakes of whole grains and refined grains, which have different effects on the immune function (Vanegas et al. 2017). Finally, despite our attempt to control for potential confounders, there may be substantial residual confounders. So further large cohort is necessary to ascertain the associations.

Notwithstanding these limitations, this study demonstrated that subpopulations and transcriptomic profiles of PBMCs differed depending on the patterns of dietary intake based on different food groups. These results represent valuable data based on PBMCs compositions and gene expressions in the community-dwelling elderly population of Korea. This study may contribute to the understanding the mechanisms of how the dietary patterns are associated with the immune cell population and gene expressions in the elderly.

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국문초록

한국 노인의 식사 섭취 패턴과 말초혈액단핵세포의 조성 및 전사체 프로파일의 연관성 연구

서울대학교 대학원 식품영양학과

차 리 나

전세계적으로 노인 인구가 증가하면서, 다양한 노인성 질환과 연관되는 면역계의 노화에 대한 관심이 늘고 있다. 이에 따라 면역 기능과 식사와의 관련성에 대해 많은 연구가 이루어졌으며, 특히 최근에는 단일적인 식이 요인이 아닌 전반적인 식품군 섭취량을 반영한 일상적인 식사 패턴 연구의 필요성이 대두되고 있다. 그러나 현재까지 노인을 대상으로, 특히 지역 사회에서 독립적인 생활을 하고 있는 노인들을 대상으로 식사 섭취 패턴과 면역 기능의 연관성을 규명한 연구는 부족한 실정이며, 관련된 기전이 아직 밝혀지지 않고 있다. 따라서 본 단면 연구를 통해 노인의 전반적인 식사 패턴을 분류하고, 식사 패턴과 면역 세포의 분포와 유전자 발현과의 연관성을 살펴보고자 하였다.

서울특별시와 경기도 성남시에서 모집 공고를 통해 65세 이상의 노인 총 201명을 모집하였고, 한국형 일상생활활동 측정도구(ADL) 및 도구적 일상생활활동 측정도구(I-ADL)를 통해 독립적인 일상생활이 가능한 노인을 선별하였다. 총 92명의 노인이 1일의 24시간 회상법과 2일의 식사기록지 작성을 수행하여 3일의 식사 섭취 기록을 제출하였다. 수집된 식사 섭취 기록에 나타난 식품들을 한국영양학회의 데이터베이스에 따라 15개의 식품군으로 분류하였고, 각 식품군 별 섭취량을 하루 총 에너지 섭취량 대비 백분율로 환산하였다. 3일간의 식품군 섭취량 백분율의 평균을 계산하여, *k*-means clustering 분석을 통해 식사 섭취 패턴을 분류하였다. 또한, 혈액에서 순환하고 있는 림프구와 단핵구 및 수지상세포를 포함하는 말초혈액단핵세포를 분리하여, fluorescence-activated cell sorting (FACS) 분석과 RNA-sequencing 분석을 진행하였다. Enzyme-linked immunosorbent assay (ELISA)를 통해 혈청의 C-reactive protein 농도를 측정하였다. 본 연구 결과, 노인기 식사 섭취 패턴은 크게 두 개의 cluster로 구분되었다. Cluster 1 (60명)은 곡류 및 그 제품군의 섭취량이 많았으며, Cluster 2 (32명)는 감자 및 그 제품군과 음료, 차류, 및 주류를 비롯해, 동물성 식품인 육류 및 그 제품군, 난류군, 어패류 및 그 제품군과 우유류 및 그 제품군의 섭취량이 많았다. 두 그룹에서 다량 영양소의 구성도 유의한 차이를 보였으며, 특히 Cluster 2에서 더 많은

동물성 단백질과 동물성 지방을 섭취하고 있었다. 그러나 평균 에너지 섭취량과 체질량지수는 다르지 않았다. 말초혈액단핵세포의 분포를 일반화선형모형으로 비교한 결과, 두 그룹은 T 세포와 NK 세포의 조성에서 유의한 차이를 보였다. Cluster 1의 피험자들과 비교했을 때, Cluster 2의 피험자들에게서 $CD4^+$ T 세포는 더 적게 발견되었으며 (Cluster 1: 23.83 ± 1.28%; Cluster 2: 15.77 ± 2.41%; $p < 0.01$), $CD8^+$ T 세포 (Cluster 1: 29.80 ± 1.08%; Cluster 2: 35.50 ± 2.03%; $p = 0.02$)와 $CD56^{high}$ NK 세포 (Cluster 1: 0.71 ± 0.11%; Cluster 2: 1.37 ± 0.20%; $p < 0.01$)는 더 많이 발견되었다. 하위 그룹 분석 결과, 체질량지수에 따라 $CD8^+$ T 세포 분포가 다르게 나타났다. 말초혈액단핵세포의 전사체 분석 결과, 총 957개의 유전자의 발현이 두 그룹에서 유의하게 달랐다. 이 유전자들은 주로 lipopolysaccharide (LPS)의 결합 및 활성화 등에 관여하는 선천성 면역 반응과 관련된 유전자들이었으며, 특히 Toll-like receptor (TLR) signaling, 수지상세포의 성숙 및 항박테리아 반응과 관련한 유전자들이 Cluster 2에서 높게 발현됨을 확인할 수 있었다. 이때 선형회귀분석을 통해 *TLR2*, *TLR4*, *TLR8* 및 *CD14*의 발현이 곡류 및 그 제품군 섭취와 음의 선형 관계를 (*TLR2*: β = -0.62, $p = 0.03$; *TLR4*: β = -0.61, $p = 0.04$; *TLR8*: β = -0.76, $p < 0.01$; *CD14*: β = -0.81, $p < 0.01$), 그리고 육류 및 그 제품군 섭취와 양의 선형 관계를 가진다는 것을 확인할 수 있었다 (*TLR2*: β = 0.59, $p = 0.04$; *TLR4*: β =

0.65, $p = 0.02$; *TLR8*: $\beta = 0.69$, $p = 0.01$; *CD14*: $\beta = 0.86$, $p < 0.01$). 여기에, 혈청 C-reactive protein의 농도가 Cluster 2에서 더 높게 관찰되었다 (Cluster 1: 0.59 ± 0.23 mg/L; Cluster 2: 1.60 ± 0.43 mg/L; $p = 0.04$). 결론적으로, 동물성 식품을 많이 섭취하는 식사 패턴은 더 낮은 CD4/CD8 비율 (Cluster 1: 0.84 ± 0.40; Cluster 2: 0.55 ± 0.30; $p < 0.01$)과 더 많은 $CD56^{high}$ NK 세포와 연관되어 있었고, 선천적 면역 반응 및 염증 반응에 관여하는 유전자가 더 높게 발현되고 있었으며, 높은 혈청 C-reactive protein 농도를 보였다. 이는 동물성 식품이 풍부한 식사 섭취 패턴이 노인의 높은 감염 감수성 및 지속적인 염증 반응과 연관될 수 있음을 나타낸다. 본 연구 결과는 식사 섭취 패턴이 노인의 면역 기능에 어떻게 영향을 미치는지에 대한 근본적인 메커니즘을 구축하는데 기여할 수 있다.

주요어: 노인기, 식사 섭취 패턴, 말초혈액단핵세포, 유세포 분석, 전사체, C-reactive protein

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