A Dried Tofu-Supplemented Diet Affects mRNA Expression of Inflammatory Cytokines in Human Blood

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Summary In order to develop a new model of diet research, blood was drawn from 12 adult volunteers for 3 wk on regular diets as controls, and for a subsequent 3 wk supplemented with 18.5 g of freeze-dried tofu (Koya tofu) every day. Triplicate aliquots of 0.06 mL each of whole blood were stimulated ex vivo with phytohemagglutinin (PHA)-P, heat aggregated human IgG (HAG), lipopolysaccharide (LPS), zymosan A, and anti-T cell receptor (TCR) monoclonal antibody to activate specific subsets of leukocytes, then the levels of various inflammatory cytokine mRNA were quantified by real time PCR. Koya tofu significantly (p<0.05) augmented the fold increase of PHA-induced tumor necrosis factor superfamily (TNFSF) 15, IL6, and IL8, HAG-induced TNFSF15 and IL8, LPS-induced IL6 and IL8, zymosan-induced TNFSF15, IL6 and IL8, and TCR-induced TNFSF2 in comparison to the regular diet. Such increase was due to the reduction of baseline mRNA expression, not the enhancement of mRNA induction after specific stimulations. Six (TNFSF15), 4 (IL6), and 3 (IL10) subjects showed significant reduction of baseline mRNA during the Koya tofu diet compared to that of the control diet. Despite large individual-to-individual and day-to-day variation of mRNA, the method employed in this study was sensitive enough to identify statistically significant results as a group as well as on an individual basis, which will be a foundation for tailored diet in the future. The results also indicated that Koya tofu had a power to alter mRNA expression in leukocytes, and TNFSF15, IL6, and IL10 would be biomarkers for soy.

Key Words tofu, mRNA, TNFSF15, IL6, IL10

Various epidemiological studies have shown that in Asian countries—where soy products are a major component of diet—the incidences of specific types of cancer are much lower than in the United States (1). Furthermore, Asian immigrants who have adopted a Western diet have higher risks for breast cancer (2). These observations and studies indicate that a high intake of soy-based products may be protective against the development of certain types of cancers. Soy has also been shown to alter the concentrations of various hormones, including those affecting metabolism, and can lower the incidence of cardiovascular diseases, prevent osteoporosis, and attenuate post-menopausal problems (3–4).

The main active ingredients in soy are isoflavones, which include genistein and daidzein, but genistein is present in the highest concentration and is the most active (5, 6). Recent data indicated that isoflavones were capable of inducing immune effects, specifically by acting as anti-inflammatory agents in vitro and modulating the gene expression of various inflammatory markers and cytokines (7, 8). Inhibition of cancer cell growth by genistein was considered the result of the modulation of genes related to homeostatic control of the cell cycle and apoptosis, as well as angiogenesis, invasion and metastasis (9). Genistein has also been attributed with antioxidant properties stemming from its ability to scavenge free radicals, and by virtue of its inhibition of the expression of stress-responsive genes such as nuclear factor kappa B (NF-κB), which effectively reduces their contribution to mutagenesis and the progression of cancer thereof (10). Furthermore, isoflavones have been shown to sensitize cancer cells in vitro to apoptosis as a result of radiotherapy and cytotoxic chemotherapeutic drugs (7, 10).
Dried Tofu Effect on Inflammatory Cytokine mRNAs in Human Blood

Most research regarding soy has been performed in vitro and in vivo using rodents, but for humans the research has been mainly in vitro (11). Furthermore, for human studies, the purified forms of soy’s active ingredients, such as genistein, were used to investigate its effects. While these studies described the potential advantages of these active compounds, it is yet to be determined whether these results can be reproduced successfully in vivo because supraphysiologic concentrations of genistein are often used in such in vitro studies. Standard double blind clinical trials, where control groups are compared to experimental groups, are not a suitable method if soy is beneficial only for a small population of subjects. Thus, a tailored medicine-type approach is required to discriminate responders from non-responders.

Identifying biomarkers for nutrition is challenging due to the requirement to evaluate weak physiological changes that are often very close to the homeostatic state. Disruption of this homeostasis, however, can lend insight into the effectiveness of a compound in restoring homeostasis (8). Leukocytes become fully activated when they migrate to local inflammatory sites or cancer lesions. They respond to various chemotactic factors released from damaged tissues, migrate to the site, then release various effecter molecules to kill the target and establish, augment, and suppress inflammatory reactions. Leukocytes also release additional chemokines to recruit more leukocytes and increase inflammation. In order to simulate such complex functions, we exposed whole blood to various stimuli in vitro. Since messenger RNA (mRNA) expression happens within a couple of hours, we chose specific mRNAs as analytical targets. The 4-h incubation of whole blood used in our study is very physiological and can be considered ex vivo (12). This is an advantage over traditional in vitro culture experiments, where isolation of particular cells is labor-intensive, selection of culture media and calf serum induces variation, and secondary reactions may happen during lengthy culture periods (10).

In the present study, we applied our ex vivo mRNA analysis to determine the effects of a common freeze-dried tofu (Koya tofu), which can be rehydrated to eat or ground into tofu flour (13). This method allowed for the statistical analysis of mRNA levels between the 3-wk normal diet and the 3-wk tofu diet, lending a more specific and significant interpretation of the effects of soy per individual.

**MATERIALS AND METHODS**

**Subjects and study design.** Twelve Japanese adult volunteers, 5 men and 7 women aged 24–58 y (mean±SD = 37.6 ± 10.2) were recruited for the clinical study after obtaining written informed consent. Using a general health assessment and routine blood tests, subjects who showed any diseases of the liver, kidney, heart, blood, endocrine, digestive or respiratory systems were excluded from the study. During the test period of 6 wk, all subjects were asked to not change dietary habits. Additionally, the subjects were prohibited from beginning new weight control programs and/or altering their routine exercise regimen. In order to assess the efficacy of soy diet on an individual basis, blood was drawn 3 times (once/wk) before and during treatment. Four milliliters of fasting venous blood was drawn from each subject in the morning and collected in a heparin container. Blood samples were immediately stored at 4°C. For the first 3 wk, subjects were asked to eat regular meals, and during the subsequent 3 wk, subjects were asked to eat 115 g of cooked tofu (Koya tofu, Asahimatsu Foods Co., Ltd., Iida, Nagano, Japan) daily. This quantity of cooked tofu is equivalent to 18.5 g of dried Koya tofu. The human research protocol was approved by the institutional ethic committee (ICE) at Sekino Clinical Pharmacology Clinic (Japan).

**Ex vivo stimulations.** Phytohemagglutinin (PHA)-P, heat aggregated human IgG (HAG), lipopolysaccharide (LPS), zymosan A, and anti-T cell receptor (TCR) antibody were used to stimulate general T-cell population, IgG (immunoglobulin G) Fc region receptor (FcγR)-bearing leukocytes, innate immunity toll-like receptor (TLR) type 4 and 2, and specifically TCR on T-cells, respectively. PHA, LPS, zymosan A, human serum IgG (Sigma-Aldrich, St. Louis, MO), TCR monoclonal antibody (IgG1 λ), and control mouse IgG1 κ (BioLegend, San Diego, CA) were obtained from the designated suppliers. Anti-TCR monoclonal antibody used in this study was specific to the human α/β chain. HAG was prepared by heating 20 mg/mL human IgG at 63°C for 15 min as described previously (14, 15). To each well of an 8-well microwell strip, 1.2 μL of PHA (2 mg/mL), HAG (10 mg/mL), PBS, LPS (0.5 mg/mL), zymosan (75 mg/mL), TCR (50 μg/mL), or mouse control IgG (50 μg/mL) was added (total 7 wells) and then sealed and stored at −80°C. These strips were prepared in the United States (US), shipped to Japan in a dry ice package, and stored at −80°C until use.

On the same day of the blood draw, 60-μL blood samples were aliquotted into the 8-well microwell strips in triplicate—where PHA, HAG, PBS, LPS, zymosan, anti-TCR, and control IgG had been dispensed previously as described above—incubated at 37°C for 4 h, then stored frozen at −80°C. The number of samples for this study was 1,512 (12 subjects×6 blood draws×7 wells×3 triplicate). Frozen samples were shipped to the US in a dry ice package, then stored again at −80°C until mRNA analysis.

**mRNA Quantification.** Various inflammatory cytokine mRNAs were quantified by quantitative polymerase chain reaction (qPCR). We selected tumor necrosis factor superfamily (TNFSF) 2 (=TNFα) and 15 (=TL1A) as an inducer of apoptosis, interleukin (IL) 2, IL4, interferon (IFN)γ, and granulocyte-macrophage colony stimulating factor (GMCSF) to augment immune reaction. IL10 for the suppression of immune reaction, and IL6, 8, CXCL10 (chemokine (C-X-C motif) ligand 10, =IP10) as a recruiter of leukocytes. Vascular endothelial growth factor (VEGF) and an opioid peptide β-endorphin precursor, proopiomelanocortin (POMC) mRNA, were also selected as markers of local angiogen-
time PCR (1 min, mixed vigorously, then 50°C) was conducted in a thermal cycler (PRISM 7900, Applied Biosystems) as described previously. The cycle threshold (Ct), which was the cycle of PCR to generate a certain amount of PCR products (fluorescence), was determined using analytical software (SDS, Applied Biosystems). For SYBR green PCR, the melting curve was analyzed in each case to confirm that PCR signals were derived from a single PCR product.

Data analysis. To determine the fold increase of each stimulation, Ct values of PHA-, HAG-, LPS- or zymosan-treated samples were subtracted individually from the mean Ct values of the PBS-treated sample to calculate ΔCt, and the fold increase was calculated as 2ΔCt according to our previous publications (11, 14–16, 22). TCR-treated samples were subtracted from control IgG-treated ones. The Ct values of the mRNA targets were normalized to the reference gene ACTB by subtracting the Ct value of the mRNA target from the Ct value of the reference gene and obtaining a ΔCt. The normalized values are given as percentage of ACTB (%ACTB) and calculated as 2ΔCt×100. The number of PCR reactions in this clinical study was 10,152 (47 PCR×12 subjects×6 blood draws×3 triplicate).

For statistical analyses, Ct values (average of 3 measurements) from PBS or control IgG-treated triplicate whole blood samples were compared with Ct values (average of 3 measurements) from triplicate whole blood samples stimulated with either PHA, HAG, LPS, zymosan, or TCR to calculate the probability (p) values by t-test. Furthermore, fold increase and %ACTB of each mRNA were compared by t-test between weekly blood samples obtained during 3 wk of normal diet and weekly blood samples obtained during 3 wk of Koya tofu diet for each subject as well as a group of 12 subjects. Since the values for fold increase and %ACTB had wide distributions, a t-test was employed for both linear and log scale.

A fold increase greater than 2 was considered a positive result according to our previous studies (23), although less than a 1.5 fold increase often resulted in p<0.05. Then, the incidence of positive responses among 12 subjects was compared between normal diet and Koya tofu diet for each mRNA. Because the study population became 2 groups (fold increase greater than 2 and less than 2), and a t-test is only applicable to a
The results for Ct values of TNFSF15 (H17005/H17009) and reference mRNAs in whole blood were compared between the 2 diet groups.

Comparison of ex vivo stimulation versus un-stimulated mRNAs in subjects on regular and Koya tofu diet regimens was minimal (Ct difference <3). We also observed that the levels of TNFSF15 mRNA showed larger subject-to-subject variation than that of ACTB (Fig. 1). Interestingly in one subject (#3), HAG failed to induce TNFSF15 during the regular diet, whereas it induced TNFSF15 during the Koya tofu diet (Fig. 1). The repeatability of the mRNA analysis is indicated by the standard deviation bars and clear differences are observed.

### RESULTS

**Characteristics of qPCR measurements of leukocyte target and reference mRNAs in whole blood**

Using the method of mRNA analysis described above, the results for Ct values of TNFSF1 (Δ●) and ACTB (○) mRNA in both HAG (△●)- and PBS (△○)-treated samples are shown in Fig. 1. The levels of ACTB were consistent within the same individual (Ct difference <1, with the exception of the second blood draw for subjects #5 and 11), and subject-to-subject variation was minimal (Ct difference <3). We also observed that the levels of TNFSF15 mRNA showed larger subject-to-subject variation than that of ACTB (Fig. 1). Interestingly in one subject (#3), HAG failed to induce TNFSF15 during the regular diet, whereas it induced TNFSF15 during the Koya tofu diet (Fig. 1). The repeatability of the mRNA analysis is indicated by the standard deviation bars and clear differences are observed for TNFSF15 between PBS (△) and HAG (△●) treatments for all subjects.

### Table 1. Summary of mRNA fold increase after stimulations.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>% Responders</th>
<th>Fold Increase</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regular +Koya tofu</td>
<td>Regular diet + Koya tofu</td>
<td>t-test (linear)</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>0 0</td>
<td>1.00 ± 0.00</td>
<td>0.8012</td>
</tr>
<tr>
<td>TNFSF2</td>
<td>75 75</td>
<td>4.77 ± 2.57</td>
<td>0.0014</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>100 100</td>
<td>19.2 ± 13.5</td>
<td>0.0011</td>
</tr>
<tr>
<td>IL6</td>
<td>100 100</td>
<td>26.0 ± 17.2</td>
<td>0.5497</td>
</tr>
<tr>
<td>IL8</td>
<td>100 100</td>
<td>82.7 ± 12.7</td>
<td>0.4487</td>
</tr>
<tr>
<td>HAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>6 0</td>
<td>2.21 ± 0.17</td>
<td>0.1717</td>
</tr>
<tr>
<td>TNFSF2</td>
<td>25 25</td>
<td>2.99 ± 1.3</td>
<td>0.0015</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>92 100</td>
<td>7.57 ± 3.9</td>
<td>0.0042</td>
</tr>
<tr>
<td>IL6</td>
<td>36 36</td>
<td>3.77 ± 1.44</td>
<td>0.5918</td>
</tr>
<tr>
<td>IL8</td>
<td>100 100</td>
<td>18.1 ± 20.9</td>
<td>0.1757</td>
</tr>
<tr>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTB</td>
<td>0 0</td>
<td>4.47 ± 18.7</td>
<td>0.0081</td>
</tr>
<tr>
<td>TNFSF2</td>
<td>83 89</td>
<td>5.05 ± 3.76</td>
<td>0.0016</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>100 100</td>
<td>156 ± 327</td>
<td>0.0057</td>
</tr>
<tr>
<td>IL6</td>
<td>100 100</td>
<td>51 ± 38.1</td>
<td>0.0036</td>
</tr>
<tr>
<td>IL10</td>
<td>97 97</td>
<td>8.47 ± 7.7</td>
<td>0.0073</td>
</tr>
<tr>
<td>CXCL10</td>
<td>100 100</td>
<td>52.6 ± 70.7</td>
<td>0.4587</td>
</tr>
<tr>
<td>INFγ</td>
<td>100 100</td>
<td>287 ± 221</td>
<td>0.4490</td>
</tr>
</tbody>
</table>

1 The fold increase of IL2, IL4, and GMCSF was omitted because the Ct of PBS-treated samples was very high.

2 Responders were considered to be samples with more than 2-fold increase. n=72 (12 subjects, 6 blood draws each).

3 Values are means ±SD.

4 Up arrows indicate significant increase of ratios in comparison to control diet.
induced by PHA, LPS, or TCR, whereas HAG and zymosan occasionally induced ACTB. Stimulation by PHA induced TNFSF2, IL6, 8, 10, CXCL10, and IFNγ; HAG induced IL8; LPS induced TNFSF2, IL6, 8, CXCL10, and IFNγ; and zymosan induced TNFSF2, 15, IL6, and 8 in all samples for both regular and Koya tofu diet groups. For the other mRNAs, two populations of responders and non/unclear responders were observed and the percentage of responders to non/unclear responders was the same regardless of the diet regimen (Table 1). As shown in Table 1, Koya tofu significantly augmented the fold increase of PHA-induced TNFSF15, IL6, and IL8, HAG-induced TNFSF15 and IL8, LPS-induced IL6 and IL8, zymosan-induced TNFSF15, IL6 and IL8, and TCR-induced TNFSF2 in comparison to the regular diet.

**TNFSF15 and IL6 correlation**

In Fig. 2, the summary of the fold increase of TNFSF15 (x-axis) and IL6 mRNAs (y-axis) for PHA (○), HAG (▲), LPS (●), and zymosan (×)-treated samples is given. Interestingly, the distribution of each symbol is clearly distinguished from the others, suggesting that each stimulation might activate different subsets of leukocytes (PHA: T cells, HAG: FcyR-bearing leukocytes, LPS: TLR4-bearing leukocytes, and zymosan: TLR2-bearing leukocytes, respectively). The $r^2$ value between two mRNAs is summarized in Supplemental Table 2. Although TNFSF15, IL6, and IL8 mRNAs induced by one stimulation were correlated to those of other stimulations ($r^2>0.3$), the induction of other target mRNAs was specific to each stimulation. Moreover, the induction of TNFSF15 and IL6 mRNA was correlated in all four stimulations (PHA, HAG, LPS, and zymosan).

*Comparison of %ACTB from ex vivo stimulation and control samples in response to diet treatments*

The fold increase is derived from two values (stimulation and PBS control); thus the increase shown in Table 1 may be due to increased gene expression resulting from stimulation or from lower gene expression in the unstimulated PBS control. To compare mRNAs from stimulated versus control in blood samples from the two diet regimens, normalization was performed using a reference, ACTB. Although PHA and TCR stimulations did not induce ACTB, it was occasionally induced in other ex vivo stimulations (Table 1). The target mRNAs in samples from the two diet regimens treated as controls (not stimulated, PBS) were analyzed as a percentage of ACTB expression (Table 2). The entire data set normalized as a percentage of ACTB expression (PHA-, HAG-, LPS, zymosan-, and TCR-treatments with TNFSF2, 15, IL2, 4, 6, 8, 10, CXCL10, GMCSF, IFNγ, POMC, and VEGF mRNA for 6 blood draws) is summarized in Supplemental Table 3. As shown in Table 2, values of TNFSF15, IL6, IL8, and IL10 were significantly decreased during the Koya tofu diet compared to control/regular diet values.

Since we have 3 data points each for regular and tofu diet, we then calculated the statistical differences between the 2 diet treatments for each individual. We found that 6 (subject #3 (●), 6 (○), 7 (▲), 10 (▲), 11 (■), 12 (□)) out of 12 subjects (50%) showed significant

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**Table 2. %ACTB for control samples (unstimulated, PBS-treated).**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Control1</th>
<th>Koya tofu1</th>
<th>p-value2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>t-test (linear)</td>
</tr>
<tr>
<td>TNFSF2</td>
<td>2.243±0.9612</td>
<td>1.950±0.5674</td>
<td>0.1207</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>0.9679±0.8464</td>
<td>0.6391±0.5118</td>
<td>0.0499 ↓</td>
</tr>
<tr>
<td>IL6</td>
<td>0.4306±0.247</td>
<td>0.2714±0.2771</td>
<td>0.0672</td>
</tr>
<tr>
<td>IL8</td>
<td>10.184±8.6204</td>
<td>6.0978±3.6731</td>
<td>0.0108 ↓</td>
</tr>
<tr>
<td>IL10</td>
<td>0.1671±0.0806</td>
<td>0.1170±0.0564</td>
<td>0.0032 ↓</td>
</tr>
<tr>
<td>CXCL10</td>
<td>6.1902±9.9885</td>
<td>3.9421±4.8911</td>
<td>0.2259</td>
</tr>
<tr>
<td>INFγ</td>
<td>0.0841±0.0855</td>
<td>0.0694±0.0655</td>
<td>0.4149</td>
</tr>
<tr>
<td>POMC</td>
<td>0.0421±0.0217</td>
<td>0.0415±0.022</td>
<td>0.9085</td>
</tr>
<tr>
<td>VEGF</td>
<td>1.1274±0.5703</td>
<td>1.0702±0.4666</td>
<td>0.6427</td>
</tr>
</tbody>
</table>

1 Values are means±SD.
2 Down arrows indicate significant decrease of ratios in comparison to control diet.
Reduction of control levels of TNFSF15 mRNA after the tofu diet (Fig. 3A, solid lines). When all 12 subjects were combined, it was still significant \( (p=0.007) \) (Fig. 1A, Table 1 (log)). Moreover, 1 subject (#4 (●)) showed a significant increase of HAG-induced TNFSF15 after the tofu diet (Fig. 3B) without reduction of baseline expression of TNFSF15 mRNA. Subject #7 (▲) showed a significant increase of zymosan-induced TNFSF15 during the tofu diet with a significant reduction of baseline TNFSF15 mRNA (Fig. 3A, C).

Similarly, 4 (#3 (●), 6 (○), 11 (■), 12 (□)) out of 11 subjects (baseline IL6 mRNA was undetectably low for subject #9), and 3 (#3 (●), 11 (■), 12 (□)) out of 12 subjects showed significant reduction of the control values of IL6 (Fig. 4A) and IL10 (Fig. 4B) mRNA after the tofu diet, respectively, and 4 (#2 (◇), 4 (○), 5 (●), 11 (■)) subjects showed significant reduction of PHA-induced IL10 mRNA after the tofu diet (Fig. 4C). The data in Figs. 3A, 4A, and 4B were further transformed into plots to visualize the correlations of these %ACTB among the three mRNAs. The \( r^2 \)-value for (A) is 0.60 \((n=11, \ p<0.01)\).
expression was correlated with that of IL6 mRNA with $r^2=0.60$ ($n=11$, $p<0.01$) (Fig. 5A), whereas no correlation was found between TNFSF15 and IL10 (Fig. 5B), or between IL6 and IL10 (Fig. 5C). Supplemental Table 2 also demonstrated that PHA-induced IL10 in the regular diet was not correlated with other stimulations or mRNAs. Thus the effect of the tofu diet is specific in influencing the expression of TNFSF15, IL6, and IL10 mRNA, and the former 2 mRNAs are correlated to each other.

**DISCUSSION**

In this study, whole blood was intentionally used without isolating specific cell populations because it is physiological, allowing cell-to-cell contact and cell-to-plasma interactions. Traditional cell culture experiments are not suitable for the characterization of individual variation, due to the large technical variation introduced during labor-intensive and technique-dependent cell isolation procedures. Instead, we stimulated specific cells in crude whole blood using specific agents, and function-related mRNA was quantified. PHA is a typical stimulator of T cells, and HAG is a classical model of immune complex to stimulate FcyR-bearing leukocytes including B cells, macrophages, natural killer (NK), and polymorphonuclear leukocytes. LPS is a gram-negative bacteria toxin and zymosan is gram-positive bacteria toxin to stimulate toll-like receptor (TLR) 4 and 2, respectively, as a model of innate immunity. The α/β chains of TCR complex are membrane proteins which recognize specific structures of foreign body to initiate (on CD4 positive cells) or induce (CD8 positive cells) cytotoxic function. This is a unique system to characterize a wide variety of leukocyte functions from a small volume of blood (1.26 mL=7 stimulations× 0.06 mL/well×3 (triplicate)).

The effects of diets or dietary supplements are generally weak, and it is difficult to identify clear effects. We initially thought the effect of Koya tofu might be too mild for detection of significant changes. Thus, the study protocol was designed to identify the differences on an individual basis by taking three blood samples during both the regular and the Koya tofu diet. Although the levels of mRNA fluctuated during the 6-wk period, the present study demonstrated that addition of 18.5 g of dried Koya tofu to a regular diet significantly reduced control values of TNFSF15, IL6, IL8, and IL10 mRNA, leading to an enhanced fold increase of PHA-, HAG-, LPS-, and zymosan-induced gene expression. Significant results were obtained as a group as well as on an individual basis. The present study clearly indicates that our mRNA assay is sensitive enough to identify small changes, and is applicable as a unique research model for a wide variety of diets and dietary supplements in the future.

The main bioactive components in soy are the isoflavones genistein and daidzein. Intake of soy isoflavone-enriched foods has been found to be associated with lower concentrations of serum C-reactive protein (CRP), which is a chronic inflammation biomarker (24, 25). Genistein has been shown to inhibit nitric oxide (NO) and prostaglandin E2 (PGE2) production as pro-inflammatory responses in LPS-induced macrophages in vitro at concentrations found naturally in soy (8, 11). It is also known to ameliorate clinical signs of experimental autoimmune encephalomyelitis purportedly through up-regulation of IL10 and down-regulation of pro-inflammatory cytokines IFNγ, TNFa, and IL12 (26). Rodent studies have shown that genistein produces a dose-dependent suppression of both humoral and cell-mediated immune response, and induces thymic atrophy in mice (27, 28). Other non-phytochemical, bioactive ingredients present in soy are its protein and n-3 fatty acids, soyasaporins, sterols, lignans, and phytate (29). These components have also been shown to have beneficial bioactivity, mostly anticarcinogenic and anti-oxidant. Sterols, soy proteins, and n-3 fatty acids in particular have been shown to have immune modulatory effects (29, 30). Sterols can balance Th1- and Th2-type immunity, specifically by increasing the cytokines of Th1-type cells (IL2, IFNγ) while decreasing those of Th2-type cells (IL4). Additionally, sterol anti-inflammatory effects arise from inhibition of IL6 and TNFa (30). N-3 fatty acids and soy protein have also been shown to have anti-inflammatory effects, as well as to down regulate pro-inflammatory cytokines (31). Although numerous reports indicated the effects of soy against immune-inflammatory functions, these were not conclusive since previous results were derived from in vitro or animal experiments.

Our study showed a significant reduction in baseline mRNA expression of TNFSF15 after Koya tofu consumption. This indicates that tofu may possibly stabilize leukocytes’ pro-inflammatory status in terms of TNFSF15 mRNA levels; however, such suppression is not harmful since ligand-specific activation is not inhibited. TNFSF15 has been identified as TNF-like factor (TL1A) and is a ligand for DR3, a death domain-containing receptor which is preferentially expressed by lymphocytes and up-regulated in activated T cells, and can activate NF-κB (32–34). Although the present study does not provide concrete answers, it suggests that TNFSF15 may be an important research topic for future studies of soy and inflammation. Koya tofu was also able to lower the baseline expression of IL6 in 4 out of 11 subjects. Interestingly, we also found that there is a correlation between IL6 and TNFSF15 after tofu consumption, which may be attributed to the effects of soy isoflavones on NF-κB activation, which is required for IL6 expression, or the antioxidant effects of other soy bioactive ingredients. Specifically, down-regulation of NF-κB is mediated through soy isoflavones directly; as well as indirectly through lowered TL1A-DR3 interaction, leading to lowered IL6 expression (35). The ability of soy to act on lowering TNFSF15 levels suggests that soy and its bioactive ingredients could potentially be used as targeted anti-inflammatory agents, suppressing IL6-induced inflammatory responses where lymphocytes are recruited by TNFSF15. The genistein found in soy has been shown to decrease IL10 secretion (36).
Similarly, the Koya tofu diet was able to lower the baseline expression of IL10 in 3 out of 12 healthy subjects. In an immune response simulation, we were able to show that Koya tofu prevents the induction of IL10 mRNA expression in two thirds of the subjects tested. In the other 4 subjects, IL10 mRNA levels were actually decreased, possibly improving the immune reaction to T cell activation by allowing further uninterrupted cytokine synthesis. Furthermore, it is important to note that PHA-induced IL10 was not correlated with any other stimulations or mRNAs, indicating that the effect of Koya tofu is specific to the baseline reductions seen in TNFSF15, IL6, and IL10 mRNA. In fact, the maintenance and even reduction of IL10 indicate that the reductions of IL6 and TNFSF15 were not influenced by IL10 cytokine inhibitory action.

Our study has shown that the physiologic soy bioactive ingredient concentration needed to produce significantly lowered levels of TNFSF15, IL6 and IL10 is attainable with a soy diet, and these cytokines are not completely blocked as they are still inducible with stimulations. We also show that a common tofu diet can be used on healthy subjects in a longer time frame (3 wk) than can be done in tissue culture experiments to attain the physiologically relevant concentrations of soy bioactive ingredients in vivo that elicit the proven effects of soy. Furthermore, we were able to utilize our quantitative mRNA technology to reproduce significant and reliable results in accordance with previously reported findings, proving our diet research method is reliable. Data shown in Figs. 1, 3, 4, Table 1, and Supplemental Table 3 may be used as reference values for future mRNA analysis. Although PHA-induced TNFSF2, 15, and IL2; HAG-induced TNFSF2, 15, IL8, and CXCL10; LPS-induced TNFSF2 and 15; and TCR-induced TNFSF2 and CXCL10 mRNA were reported previously (13, 15–18), the induction of other mRNAs in whole blood was unique to this study, including zymosan-induced POMC and VEGF.

Authors’ contributions

MY, SH, and MM designed the research; VT, MO, AK, SK, KS, MK, and MM conducted the research; VT, MO, AK, SK, KS, MK, MY, SH, and MM analyzed the data; VT and MM wrote the paper; VT, MO, AK, SK, KS, MK, MY, SH, and MM reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We would like to thank Dr. Melanie Oakes (Hitachi Chemical Research Center) for her critical review of the manuscript.

All authors declared no conflict of interest.

REFERENCES


### Supplemental Table 1. Summary of sequences of primers and probes used in the study.

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Supplemental Table 2. mRNA-to-mRNA fold increase correlations¹.

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¹Values in bold text indicate $r^2$ values between 0.3 and 0.4; values in bold italics indicate $r^2$ values of more than 0.4.
### Supplemental Table 3. Summary of data (average %ACTB).

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### Supplemental Table 3. (Continued.)

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### Supplemental Table 3. (Continued.)

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1. Values are means, n = 3.