Research Note

Food Science

Fermented soybean powder with rice mold in the absence of salt stimulates the cellular immune system and suppresses the humoral immune response in mice

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食塩無添加で米麹により発酵した大豆粉末は細胞性免疫を増強し液性免疫を抑制する

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要約　食塩無添加で米麹により発酵した大豆粉末(NSBP)の免疫調節作用を C3H/HeN マウスを用いて調べた。NSBP を添加して培養した脾臓細胞の $CD11b^+$, $CD49b^+$およびインターフェロン(IFN)-γ^+CD4^+細胞数は有意に増加し，インターロイキン(IL)-4^+CD4^+および $CD19^+$細胞数は有意に減少した。同様に，NSBP 添加飼料で飼育したマウスの脾臓とパイエル板の IL-12^+CD11b^+, $CD49b^+$および IFN-γ^+CD4^+細胞数は，NSBP 無添加飼料で飼育した場合より明らかに増加し，IL-4^+CD4^+および $CD19b^+$細胞数は減少した。また，NSBP 添加飼料で飼育したマウスの腹腔マクロファージによる活性酸素の生産は有意に増加した。一方，NSBP 添加飼料で飼育したマウスの腸管総 IgA および血清総 IgG レベルは減少傾向にあった。マイクロアレイ解析の結果，NSBP 添加飼料で飼育したマウスのパイエル板の細胞性免疫に関わる幾つかの遺伝子発現は増加し，免疫グロブリンの産生に関わる幾つかの遺伝子発現は減少した。これらの結果は，NSBP は C3H/HeN マウスの細胞性免疫応答を増強し，獲得液性免疫応答を抑制することを示している。

Summary

The immunomodulatory effect of fermented non-salty soybean powder (NSBP) was investigated in C3H/HeN mice. The number of splenic $CD11b^+$, $CD49b^+$, and interferon (IFN)-γ^+CD4^+ cells increased significantly, while that of interleukin (IL)-4^+CD4^+ and $CD19^+$ cells decreased significantly in cultures containing NSBP. Similarly, in the spleen and Peyer's patches of mice fed a diet containing NSBP, the number of IL-12^+CD11b^+, $CD49b^+$, and IFN-γ^+CD4^+ cells increased noticeably, whereas the number of splenic IL-4^+CD4^+ and $CD19b^+$ cells was lower compared to mice fed an NSBP-free diet. Superoxide production by peritoneal macrophages was significantly higher in mice fed an NSBP-containing diet. Both intestinal total IgA and serum total IgG levels declined in mice fed the NSBP-containing diet. Microarray analysis of mRNAs extracted from Peyer's patch cells of mice fed an NSBP-containing diet indicated an increase in the expression of several genes related to cellular immune responses, while the expression of genes related to immunoglobulin production decreased. These results indicate that NSBP stimulates the cellular immune response, but suppresses the acquired humoral immune response in C3H/HeN mice.

Key Words: fermented soybean, immunomodulation, cellular immune response, antibody production, mouse

Introduction

Fermented soybean paste, also known as “miso”, is a common traditional food or seasoning in Japan. Miso is generally produced by fermentation of soybeans with rice mold (*Aspergillus oryzae*), which is cultivated on steamed rice under solid-state conditions, in the presence of a high amount of salt (5%-13 %) in order to suppress the growth of saprophytes. Miso is high in protein and rich in vitamins, amino acids, organic acids, minerals, and polyphenols, and is therefore considered an important nutrient in Japan (*1*). However, due to its high salt content, only small amounts of miso are generally consumed in the daily diet.

The authors of the present study devised a method for large-scale fermentation of soybeans that does not require the addition of salt. The resulting powder was termed “non-salty fermented soybean powder” (NSBP), and due to the low salt content, NSBP may be an ideal food material. We reported that NSBP might protect against obesity induced by a high-fat diet (*2*). Moreover, we demonstrated that a soybean protein fraction digested with Peptidase R produced by *Rhizopus oryzae* stimulates the immune response in mice, and identified a glutamine-rich peptide responsible for the immunostimulation (*3, 4*). It has also been reported that fermented soybean products may suppress allergic reactions via modulation of type 1 helper T (Th1) and type 2 helper T (Th2) cell responses in mice (*5*). In this study, we demonstrate that NSBP stimulates the cellular immune system and suppresses the humoral immune response in C3H/HeN mice.

Materials and Methods

Materials. Defined protein-free purified diet (P5765) was obtained from Purina Mills (St. Louis, MO, USA). Ovalbumin (OVA, grade II) was purchased from Kewpie Corporation (Tokyo, Japan). Phycoerythrin (PE)-labeled anti-mouse IL-4 monoclonal antibody (mAb, clone 11B11), PE-labeled anti-mouse IFN-γ mAb (clone XMG1.2), PE-labeled anti-mouse IL-12 p40 mAb (clone C15.6), PE-labeled anti-mouse CD49b mAb (clone DX5), biotin-labeled anti-mouse CD4 mAb (clone RM4-5), biotin-labeled anti-mouse CD11b mAb (clone M1/70), and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin were obtained from BioLegend (San Diego, CA, USA). Brefeldin A (BFA), ionomycin, streptomycin and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Penicillin was obtained from MP Biomedicals (Costa Mesa, CA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium and thioglycolate broth were purchased from Nissui Pharmaceutical (Tokyo, Japan). 2-Methyl-6-p-methoxyphenylethynylimidazopyranizone (MPEC) was obtained from ATTO Corporation (Tokyo, Japan). Latex beads (0.79 μm) were purchased from Polysciences Inc. (Warrington, PA, USA). All chemicals used in this study were of the highest commercially available analytical grade.

Preparation of NSBP. The procedure for preparation of NSBP was reported previously

(*2*).

Feeding procedure. All animal experiments undertaken during this study were conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University, and according to Law no. 105 and Notification no. 6 of the Japanese government. Four-week-old male C3H/HeN mice (*n* = 8) (Japan SLC, Shizuoka, Japan) were fed a commercial mouse powder feed (MF, Oriental Yeast Co., Tokyo, Japan) for 1 week, and were then fed either an NSBP-containing or NSBP-free diet between 5 and 10 weeks of age. The detailed composition of each diet is shown in Table 1. The amount of protein, sugar, oil, and minerals in the NSBP-containing diet conformed to that in the NSBP-free diet. Food and water were supplied *ad libitum* throughout the course of the experiment. Mice were housed at 23 ± 2°C under a standard 12-h light-dark cycle. Serum, intestine, spleen, and Peyer's patch samples were collected immediately following euthanasia via administration of a lethal dose of ether at 10 weeks of age. Samples were analyzed individually to investigate antibody levels, cell function, and gene expression.

Table 1

Cell suspensions and cell cultures. Spleen cell suspensions were prepared as described previously (*6*). Peritoneal macrophage suspensions were prepared according to a previously described procedure (*7*). Macrophages were confirmed to constitute over 95% of the adherent viable cells by treatment with esterase and Giemsa staining. The cell density was adjusted to 1.0×10^6 /ml and cells were placed into wells of a 24-well flat-bottom plate (Sarstedt Inc., Newton, NC, USA). Cells were cultured at 37°C in a humidified incubator

with an atmosphere of 5% CO_2.

Cell identification. Cell surface markers and intracellular cytokines were labeled according to a previously described procedure (*6*). The number of cells was determined using a Guava personal cell functional analyzer (Guava PCA, Guava Technologies, Hayward, CA, USA).

Measurement of macrophage superoxide anion production and phagocytic activity. Peritoneal macrophages were suspended in HEPES-saline solution and incubated for 30 min at 37°C in a humidified incubator with an atmosphere of 5% CO_2, after which 300 μmol/l of MPEC was added to the suspensions. As a negative control, 30,000 U/ml of superoxide dismutase (Biomedicals, Eschwege, Germany) was also added to appropriate suspensions. Chemical luminescence intensity was measured using a Luminesencer-PSN AB-2200 (ATTO). Ingestion of latex beads as an indicator of pagocytosis was measured according to a previously described procedure (*8*).

Determination of immunoglobulin (Ig) levels. Samples of blood and intestinal tract (duodenum to rectum, including contents) were carefully collected from mice at 10 weeks of age. Blood was centrifuged at 450 × *g* for 60 min at 4°C, and the supernatant (serum) was collected and stored at -30°C until use. The intestinal tract (1 g) was ground for 15 min at 2 ± 1°C using a pestle with 1.5 g of sea sand in 2.5 mL of 0.01 M sodium phosphate buffer containing 0.15 M NaCl (PBS, pH 7.2). The ground material was then centrifuged at 1,200 × *g* for 30 min at 4°C, and the supernatant was collected and stored at -30°C until use.

The levels of serum and intestinal total immunoglobulin were measured using an enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's protocol.

Microarray analysis. Four Peyer's patches were collected from each mouse and the cells were isolated. Peyer's patch cells collected from the mice in each group were pooled. Genome-wide gene expression of pooled Peyer's patch cells was examined using a Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), as described previously (*9*). Data analysis was performed with GeneChip Operating software 1.4 (Affymetrix). Expression data were selected when there was more than a 1.5-fold increase and less than a 0.7-fold decrease in expression compared to the control group.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the Student's *t*-test. Differences were considered significant when the *P*-value was less than 0.05.

Results

Effect of NSBP on the number of immunocompetent spleen cells in culture

Fig. 1 shows the number of immunocompetent cells among C3H/HeN mouse spleen cells cultured in the presence and absence of NSBP. The number of $CD11b^+$, $CD49b^+$, and IFN-γ^+CD4^+ cells increased significantly in the presence of NSBP. In contrast, the number of IL-4^+CD4^+ and $CD19b^+$ cells decreased significantly when cultured with NSBP.

Fig. 1

Effect of NSBP consumption on the number of immunocompetent cells in the spleen and Peyer's patches

Five-week-old C3H/HeN mice were fed an NSBP-containing or NSBP-free diet for 5 weeks. No significant difference in body weight was observed between the mice in each group (data not shown). Fig. 2 presents the number of immunocompetent cells in spleen and Peyer's patches of the10-week-old mice. The number of IL-12$^+$CD11b$^+$ cells in the spleen and Peyer's patches of mice fed an NSBP-containing diet was noticeably higher than in mice fed an NSBP-free diet. The number of CD49b$^+$ cells in the spleen and Peyer's patches tended to be higher in mice fed an NSBP-containing diet than in mice fed an NSBP-free diet. Moreover, the number of IFN-γ^+CD4$^+$ cells was significantly higher in the spleen and noticeably higher in the Peyer's patches of mice fed an NSBP-containing diet, while the number of IL-4$^+$CD4$^+$ cells in both the spleen and Peyer's patches of mice fed an NSBP-containing diet were noticeably lower than in mice fed an NSBP-free diet. The number of CD19b$^+$ cells in the spleen was also noticeably lower in mice fed an NSBP-containing diet.

Fig. 2

Effect of NSBP consumption on ingestion of latex beads and production of superoxide by peritoneal macrophages

Fig. 3 shows the ingestion of latex beads and the production of superoxide by peritoneal macrophages of 10-week-old mice. The number of ingested latex beads taken up by macrophages of mice fed an NSBP-containing diet was noticeably higher than that of

Fig. 3

macrophages of mice fed an NSBP-free diet. The level of superoxide production was significantly higher in macrophages of mice fed an NSBP-containing diet.

Effect of NSBP consumption on total Ig levels

Fig. 4 presents the levels of intestinal total IgA and serum total IgG of 10-week-old mice. The levels of both intestinal total IgA and serum total IgG were noticeably lower in mice fed an NSBP-containing diet than in mice fed an NSBP-free diet.

Fig. 4

Effect of NSBP consumption on gene expression in Peyer's patch cells

Table 2 shows the expression of immunity related genes increased more than 1.5-fold or decreased less than 0.7-fold in Peyer's patch cells of 10-week-old mice. The expression of transcripts representing the genes *Cd4*, *Jun*, *Fos*, *B2m*, and *Ctla4* increased by more than 1.5-fold in mice fed an NSBP-containing diet compared to mice fed an NSBP-free diet. In contrast, the expression of transcripts representing the genes *Igk-V1*, *Igkv12-46* and *Pigr* decreased by less than 0.7-fold in mice fed an NSBP-containing diet.

Table 2

Discussion

The number of splenic IL-12^{+}CD11b^{+}, CD49b^{+}, and IFN-γ^{+}CD4^{+} cells increased significantly when cultured in the presence of NSBP, and the number of these cells increased significantly or noticeably in the spleen and Peyer's patches of mice fed an NSBP-containing diet compared to mice fed an NSBP-free diet (Figs. 1 and 2). CD11b is a typical surface antigen of macrophages and dendritic cells (*10*), while IL-12 is one of the

major cytokines produced by these cells (*11*). Similarly, CD49b is a typical surface antigen of natural killer (NK) cells, and IL-12 induces the proliferation and activation of NK cells (*11*). The observed increase in the number of NK cells may therefore be due to an increase in the number of macrophages and/or dendritic cells. On the other hand, CD4 is a typical surface antigen of helper T cells, and Th1 cells express both Cd4 and IFN-γ (*12*). It is known that production of IFN-γ by helper T cells is stimulated by IL-12 produced by macrophages and dendritic cells, i.e., IL-12 produced by these cells enhances the differentiation of naïve T cells into Th1 cells (*11, 12*). Hence, the observed increase in the number of Th1 cells could have been due to an increase in the number of macrophages and/or dendritic cells. These data indicate that NSBP might enhance the innate cellular immune response and stimulate Th1 cell function in mice.

It is known that macrophages ingest microorganisms and then kill them via oxidative burst reactions that include production of superoxide anion (*13, 14*). In this study, the number of latex beads ingested by peritoneal macrophages increased noticeably in mice fed an NSBP-containing diet, while the level of superoxide production was significantly higher in peritoneal macrophages of mice fed an NSBP-containing diet compared to mice fed an NSBP-free diet (Fig. 4). These results indicate that NSBP may enhance the phagocytic and antimicrobial functions of macrophages, as well as the production of cytokines.

It has been well established that Th2 cells express both CD4 and IL-4 (*12*). IL-4 produced by Th2 cells induces the differentiation of B cells into plasma cells and the

subsequent production of immunoglobulin (*15, 16*). CD19 is a typical surface antigen of B cells, but not of plasma cells (*17*). As shown in Fig. 2, the number of IL-4^+CD4^+ cells in the spleen and Peyer's patches of mice fed an NSBP-containing diet were noticeably lower than in mice fed an NSBP-free diet. The number of $CD19^+$ cells was also noticeably lower in mice fed an NSBP-containing diet. Moreover, the levels of intestinal total IgA and serum total IgG were noticeably lower in mice fed an NSBP-containing diet (Fig. 5). These data suggest that decreased levels of intestinal total IgA and serum total IgG result from an NSBP-induced decrease in the number of Th2 cells.

Microarray analyses demonstrated that the expression of several genes (*Cd4*, *Jun*, *Fos*, *B2m*, and *Ctla4*) encoding proteins related to immune functions increased more than 1.5-fold as a result of NSBP consumption, while the expression of other immune system-related genes (*Igk-V1*, *Igkv12-46*, and *Pigr*) decreased by less than 0.7-fold. The genes *Cd4*, *Jun*, *Fos*, *B2m*, and *Ctla4* encode CD4 antigen, c Jun oncogene, FBJ osteosarcoma oncogene, beta-2 microglobulin (b2m), and cytotoxic T-lymphocyte-associated protein 4 (Ctla4), respectively. Cd4, Jun, and Fos are known to enhance Th1 cell differentiation (*18*). As shown in Fig. 2, the number of IFN-γ^+CD4^+ (Th1) cells was significantly higher in the spleen and elevated in the Peyer's patches of mice fed an NSBP-containing diet compared to mice fed an NSBP-free diet. The observed increases in gene expression are thus consistent with an increase in the number of Th1 cells. B2m induces the differentiation of $CD8^+$ T cells and NKT cells, both of which are involved in the

clearance of microorganisms (*19*). Expression of Ctla4 is correlated with the extent of pro-inflammatory responses and host resistance to protozoan infection-induced acute immunity (*20*). These facts suggest that NSBP enhances host defenses against microorganisms.

The genes *Igk-V1*, *Igkv12-46*, and *Pigr* encode immunoglobulin kappa chain variable 1 (Igk-V1), immunoglobulin kappa variable (Igkv) 12-46, and polymeric immunoglobulin receptor region (Pigr), respectively. Both Igk-V1 and Igkv12-46 are components of immunoglobulin kappa variable chains (*21, 22*), while Pigr is essential for transepithelial transport of IgA in mucosal tissues (*23*). As shown in Fig. 4, the levels of intestinal total IgA and serum total IgG were noticeably lower in mice fed an NSBP-containing diet compared to mice fed an NSBP-free diet. The observed decrease in the expression of these genes is consistent with the observed decreases in intestinal total IgA and serum total IgG levels.

In this study, we demonstrated that NSBP may stimulate the cellular immune response and suppress the acquired humoral immune response, although we did not identify the responsible NSBP component(s). Using a high performance liquid chromatography, we previously estimated that the levels of the glycoside types of genistein and daidzein in NSBP are 20.7 and 35.4 ppm, while the levels of the aglycone types are 118.0 and 188.0 ppm, respectively. Zhang *et al*. reported that daidzein enhances the phagocytic activity in mice (*24*), while Guo *et al*. reported that genistein increases the activity of cytotoxic T cells

and NK cells in mice (*25*). Moreover, our group demonstrated that a glutamine-rich peptide from a soybean protein fraction digested with Peptidase R stimulates the cellular immune response in mice (*4*). These facts suggest that NSBP-mediated enhancement of cellular immune responses is associated with polyphenols such as genistein and daidzein, and/or peptides. We are currently characterizing the immunomodulatory component(s) of NSBP.

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Figure Legends

Fig. 1. Number of immunocompetent cells among mouse spleen cells cultured with and without NSBP. Spleens were obtained from 6-week-old C3H/HeN mice bred with MF. Spleen cells were cultured for 48 h with NSBP at a final concentration of 10, 50 or 100 μg/ml of in the absence of NSBP. Data are presented as the mean ± SD (n = 5). *P<0.05.

Fig. 2. Number of immunocompetent cells in the spleen and Peyer's patches (PP) of mice fed an NSBP-containing (solid columns) or NSBP-free (open columns) diet. Data are presented as the mean ± SD (n = 8). *P<0.05.

Fig. 3. Ingestion of latex beads and production of superoxide by peritoneal macrophages of mice fed an NSBP-containing (solid columns) or NSBP-free (open columns) diet. Data are presented as the mean ± SD (n = 8). *P<0.05.

Fig. 4. Intestinal total IgA and serum total IgG levels in mice fed an NSBP-containing (solid columns) or NSBP-free (open columns) diet. Data are presented as the mean ± SD (n = 8).

Table 1. Composition of diets

Component	NSBP-free diet	NSBP-containing diet
	%	
NSBP powder	-	23.72
Protein-free purified diet	74.26	53.32
Ovalbumin	24.75	17.84
Dextrin	-	4.55
Soy oil	0.99	-
Calcium monohydrogen phosphate	-	0.34
Potassium dihydrogen phosphate	-	0.23
Total	100.00	100.00

Table 2. Expression of immunity related genes in Peyer's patch cells of C3H/HeN mice.

Gene symbol	Gene description	Relative mRNA expression (NSBP-containing diet/NSBP-free diet)
Increased genes		
Cd4	CD4 antigen	1.9
Jun	c Jun oncogene	1.9
Fos	FBJ osteosarcoma oncogene	2.9
B2m	beta-2 microglobulin	1.7
Ctla4	cytotoxic T-lymphocyte-associated protein 4	1.7
Decreased genes		
Igk-V1	immunoglobulin kappa chain variable 1	0.6
Igkv12-46	immunoglobulin kappa variable 12-46	0.7
Pigr	polymeric immunoglobulin receptor	0.5

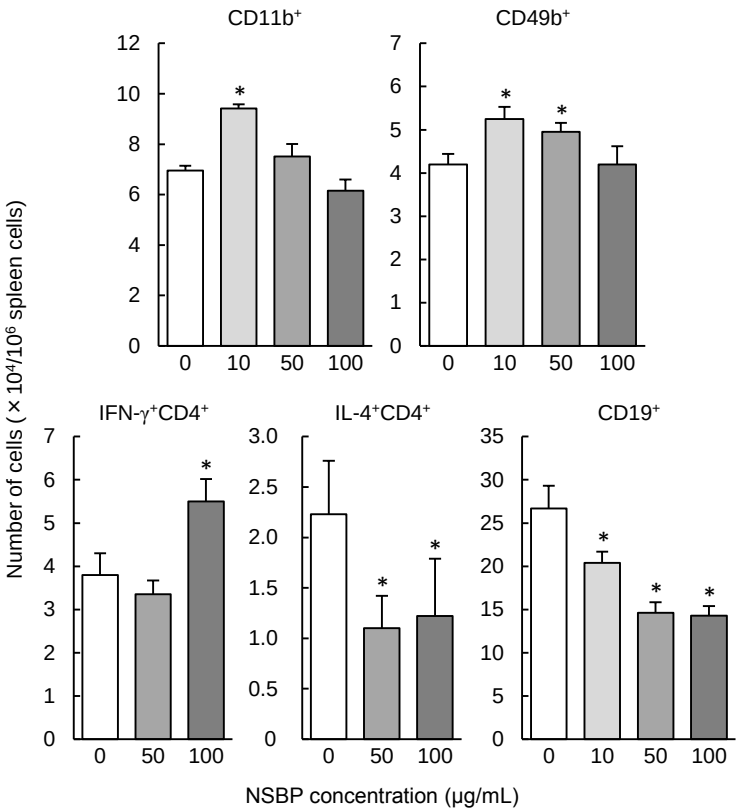

Fig. 1. Karasawa *et al.*

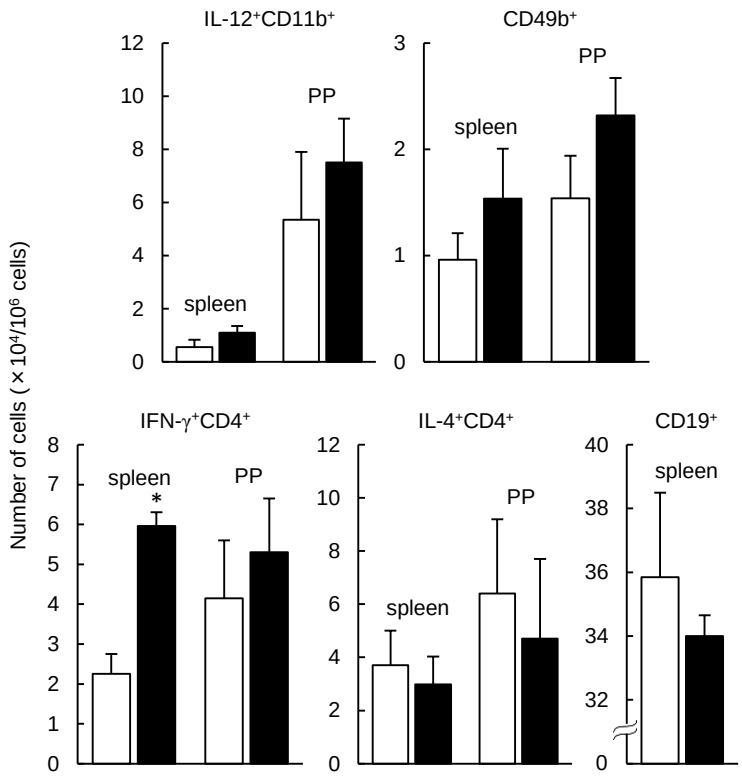

Fig. 2. Karasawa *et al.*

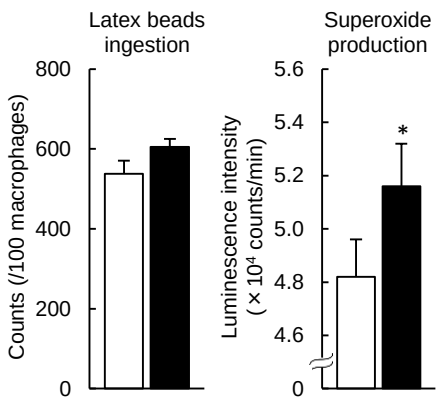

Fig. 3. Karasawa *et al.*

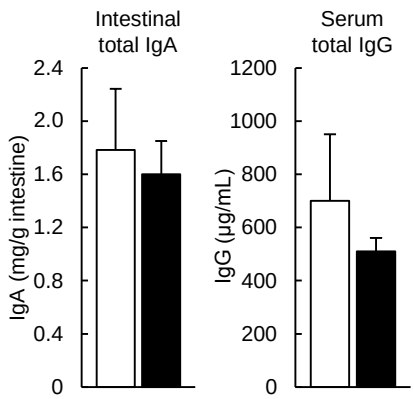

Fig. 4. Karasawa *et al.*