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Original Article

Diallyl disulfide administration increases the number of B-lymphocytes in the rat spleen

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ABSTRACT — Diallyl disulfide (DADS), the major sulfur compound in garlic, reduces the number of circulating T-lymphocytes, B-lymphocytes, and monocytes via activation of the hypothalamus-pituitary-adrenal axis. However, the translocation of those cells that migrate in response to DADS administration is still unclear. Therefore, in this study, we examined the effects of DADS administration on a number of lymphocyte subsets and monocyte-derived cells including macrophages (monocytes/macrophages) in spleen, the largest secondary lymphoid organ. Ten-wk-old male Sprague-Dawley rats were orally administered with DADS (dose = 20 mg/kg body weight) or equivalent volume of vehicle. The spleen was harvested 4 hr after administration, and then the splenic cells were isolated and the total number of cells was counted. T-lymphocytes, B-lymphocytes, natural killer (NK) cells, and monocytes/macrophages were fractionated by flow-cytometry and the total number of these cells was calculated. The total number of splenic cells was significantly increased by 1.18-fold after DADS administration. Among the lymphocyte subsets in the spleen, the number of B-lymphocytes significantly increased by 1.28-fold after DADS administration. The number of T-lymphocytes also showed a tendency to increase. However, the number of NK cells and monocytes/macrophages did not change after DADS administration. These results suggest that B-lymphocytes migrate from the circulation and translocate to the spleen in response to DADS administration.

Key words: Diallyl disulfide, T-Lymphocytes, B-Lymphocytes, Natural killer cells, Monocytes-derived cells, Spleen

INTRODUCTION

Garlic (*Allium sativum*) has been consumed as a spice and as a medical herb since early times (Block, 1985). Organosulfur compounds account for approximately 3-5% of all constituents of garlic, and are composed of alliin, allicin, dithiins, sulfides, ajoenes, *S*-allyl cysteine, and *S*-allylmercaptocysteine. When garlic is crushed, allicin is converted to oil-soluble organosulfur compounds such as diallyl sulfide, diallyl disulfide (DADS), and diallyl trisulfide (Lawson and Hughes, 1992). Among these compounds, DADS is the major oil-soluble organosulfur compound with a wide variety of biological activities, which include anti-cancer, anti-inflammatory, and immune-modulatory activities, in addition to the enhancement of sympathetic nerve activity (Lawson, 1996; Sundaram and Milner, 1996; Oi *et al.*, 1999; Wu *et al.*, 2002; Chang *et al.*, 2005; Liu *et al.*, 2006; Huang *et al.*, 2010; Yi and Su, 2013). However, the immune-modulatory effects of DADS need to be fully clarified.

The distribution of white blood cells, including lymphocyte subsets (T-lymphocytes, B-lymphocytes, and natural killer [NK] cells) and granulocyte subsets (neutrophils, eosinophils, and basophils), is associated with the immune responses, because continuous migration of the immune cells ensures detection of antigens and neoplasms, and promotes cellular interactions that enable the immune system to execute rapid and effective responses (Engler *et al.*, 2004a). In addition, the cellular distributions have been reported to be regulated by several

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hormones derived from adrenals (Dhabhar *et al.*, 1995; Engler *et al.*, 2004a, 2004b; Shirato *et al.*, 2007). Recently, we reported that the administration of DADS in a single dose reduced the number of circulating T-lymphocytes, B-lymphocytes, and monocytes without altering the number circulating NK cells and granulocytes, and increased the plasma concentration of corticosterone, in rats (Hashizume *et al.*, 2012). These results suggest that DADS administration changes the distribution of these cells by activating of the hypothalamus-pituitary-adrenal axis (Hashizume *et al*., 2012). However, the translocation of those cells that migrate in response to DADS administration is still unclear.

It is generally accepted that white blood cells continuously circulate between blood and secondary lymphoid organs such as spleen, lymph nodes, and Peyer's patches. The spleen, which is the largest secondary lymphoid organ, is composed of a branched splenic artery that eventually ends in venous sinuses (Kraal and Mebius, 2006). Although the terminal arterioles from the central arteriole run through the lymphoid compartment known as white pulp, most of the arterial blood ends in the marginal zone of the spleen (Kraal, 1992; Schmidt *et al.*, 1993; Kraal and Mebius, 2006). The marginal zone of the spleen contains marginal zone macrophages and B-lymphocytes (Kraal and Mebius, 2006). Thus, the site is strategically important for clearance of blood-borne antigens and initiation of antibody production against such pathogens (Kraal *et al.*, 1989). Moreover, the white pulp contains tingible body macrophages, follicular B-lymphocytes, and T-lymphocytes, and these cells also coordinately initiate immune responses in the spleen (Cesta, 2006). When B-lymphocyte production in bone marrow was suppressed by conditional knockout of *Rag-2* in mice, the quantity of follicular B-lymphocytes was gradually reduced (Hao and Rajewsky, 2001). These results indicate at least in part that B-lymphocytes are continuously recruited from the circulation and translocated to the spleen.

Therefore, in the present study, we examined the acute effects of DADS administration on the number of T-lymphocytes, B-lymphocytes, and monocyte-derived cells including macrophages (monocytes/macrophages) in the spleen of rats.

MATERIALS AND METHODS

Experimental procedure and animal care

The experimental protocol used in the present study is shown in Fig. 1. Ten-wk-old male Sprague Dawley rats (CLEA Japan, Tokyo, Japan) were pre-fed for 3 d to allow adaptation to a new environment (Hashizume *et al.*, 2012, 2013). All rats were housed in stainless steel cages at a controlled temperature (23-25°C) and relative humidity (50-60%) (Hashizume *et al.*, 2012, 2013). Lighting was automatically provided from 8:00-20:00. Animal chow (CE-2 cubic type, CLEA Japan) and distilled water were given to the rats ad libitum (Hashizume *et al.*, 2012, 2013). After the adaptation period, the rats were divided into the control (CON) and DADS (dose = 20 mg/kg BW) groups (n = 6/group).

The present study (2012-A102) was approved by the Animal Ethics Committee, Waseda University, and conducted according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, the Physiological Society of Japan (The Physiological Society of Japan, 2004). The experiments were per-

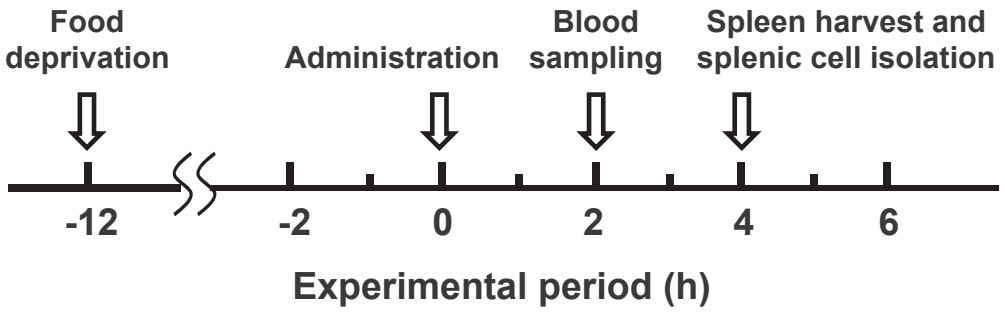

Fig. 1. Experimental protocol. Plasma corticosterone concentration was quantified by enzyme-linked immunosorbent assay 2 hr after the administration of DADS (dose = 20 mg/kg BW). Spleen was harvested and weighed 4 hr after the administration, and then splenic cells were isolated and counted. The fractionation of lymphocyte subsets (T-lymphocytes, B-lymphocytes, and natural killer cells) and monocytes/macrophages from the splenic cells were performed by a direct immunofluorescent staining based on flow-cytometry, and then the number of each cell type was calculated.

formed with the least possible pain or discomfort to rats (Hashizume *et al.*, 2012, 2013).

Oral administration of DADS

DADS (99.5% purity, LKT Lab, St Paul, MN, USA) was dissolved in 2% ethanol, and then supplemented with 0.9% NaCl solution containing 10% Tween 80 as a vehicle to obtain 1.0% DADS solution (Hashizume *et al.*, 2012, 2013). Then, 1.0% DADS solution was administered orally at the dosage of 20 mg/kg body weight (BW) to rats in DADS group (Hashizume *et al.*, 2012, 2013). The dosage of DADS was based on the reports of Munday *et al*. (Munday and Munday, 1999) and our recent studies (Hashizume *et al.*, 2012, 2013). An equivalent volume of vehicle was administered to rats in CON group instead of DADS solution in the same manner (Hashizume *et al.*, 2012, 2013).

Blood sampling and plasma preparation

As shown in Fig. 1, whole blood was collected with a heparinized microcapillary tube from the tail vein at 0, 2, and 4 hr after the administration. Plasma was obtained by centrifugation of the whole blood at 3,000 × *g* for 5 min at 4°C, frozen at -80°C, and stored until assay (Hashizume *et al.*, 2012, 2013).

Enzyme-linked immunosorbent assay

Plasma corticosterone concentration was determined by using YK240 Corticosterone EIA Kit (Yanaihara Institute, Shizuoka, Japan) (Hashizume *et al.*, 2012).

Harvesting of spleen and isolation of splenic cells

Four hours after the administration of DADS or vehicle, the rats were anesthetized by peritoneal injection of sodium pentobarbital (50 mg/kg BW), and their spleens were immediately harvested and weighed. The spleen tissue was minced using a 22-gauge needle (Terumo, Tokyo, Japan) in phosphate-buffered saline (PBS; pH 7.4), and then filtered with a cell strainer (70 μm; Becton Dickinson and Company [BD], Franklin Lakes, NJ, USA) (Shirato *et al.*, 2013). After centrifuging at 300 × *g* for 3 min at 4°C, the resultant cell pellets were suspended in 15 mL of FACS Lysing Solution (BD), and then incubated for 5 min at room temperature to facilitate hemolysis. After centrifuging at 300 × *g* for 3 min at 4°C, the resultant cell pellets were washed twice with 15 mL of PBS, and then resuspended in 10 mL of PBS and gently mixed. The splenic cells were counted using a disposable one-cell counter (Wakenyaku, Kyoto, Japan) (Shirato *et al.*, 2013).

Flow-cytometry

Lymphocyte subsets (T-lymphocytes, B-lymphocytes, and NK cells) and monocytes/macrophages were fractionated by a direct immunofluorescent staining based on flow-cytometry. For fractionation of the lymphocyte subsets, we used Rat T/B/NK Cell Cocktail (BD) containing allophycocyanin (APC)-conjugated anti-rat CD3 for T-lymphocytes (clone 1F4), fluorescein isothiocyanate (FITC)-conjugated anti-rat CD45RA for B-lymphocytes (clone OX-33), and phycoerythrin (PE)-conjugated anti-rat CD161a for NK cells (clone 10/78) (Akimoto *et al.*, 2009; Someya *et al.*, 2009). Monocytes/macrophages were fractionated by using PE-conjugated Mouse Anti-Rat CD11b (BD). The splenic cells (1×10^6 cells) were collected by centrifugation at 300 × *g* for 3 min at 4°C, and resuspended in 100 μL of PBS supplemented with 2% fetal bovine serum. Five microliter of the cocktail or single antibody was added to the cell suspension, and then incubated for 30 min at room temperature in dark. After the centrifugation at 300 × *g* for 5 min at 4°C, the cell pellets were washed twice with PBS, and then resuspended in 500 μL of PBS. After filtration with a cell strainer (40 μm; BD), the cells were analyzed using a FACSCalibur (BD) with FL1 (FITC), FL2 (PE), and FL3 (APC) detectors. Representative scatter diagrams for lymphocyte subsets and monocytes/macrophages in the spleen are presented in Fig. 2.

Statistical analysis

All data are presented as mean ± standard error of the mean. The effects of DADS administration on plasma coticosterone concentration, the absolute and relative weights of spleen, the total number of splenic cells, and the number of lymphocyte subsets and monocytes/macrophages in the spleen were analyzed by Student's *t*-test. Differences were considered as significant when p was less than 0.05.

RESULTS

Effect of DADS administration on plasma corticosterone concentration

Plasma corticosterone concentration was significantly higher in the DADS group by 1.84 times compared to the CON group (Fig. 3). This result agreed qualitatively with our previous report (Hashizume *et al.*, 2012).

Effect of DADS administration on spleen weight and total number of splenic cells

The absolute weight of spleen (Fig. 4A) and its relative weight to the BW (Fig. 4B) were not different between

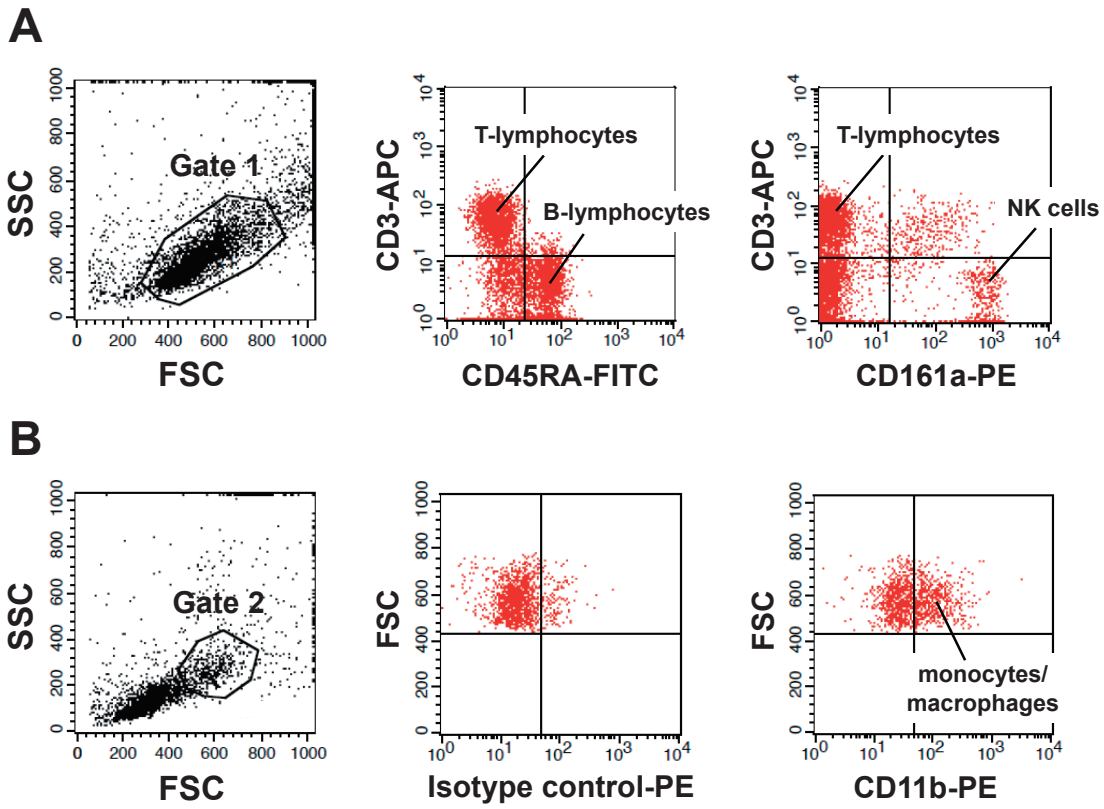

Fig. 2. Representative scatter diagrams for lymphocyte subsets and monocyte-derived cells including macrophages (monocytes/macrophages). Splenic cells were first analyzed based on forward-scattered light (FSC; index for cellular size) and side-scattered light (SSC; index for intracellular complexity). (A) Based on the indexes, the cellular population containing lymphocytes was gated (Gate 1), and the fluorescence intensities for CD3-allophycocyanin (APC), CD45RA-fluorescein isothiocyanate (FITC), and CD161a-phycoerythrin (PE) were analyzed. (B) For the cellular population containing monocytes/macrophages (Gate 2), the fluorescence intensities for CD11b-PE or isotype-control antibody-PE were analyzed, and then CD11b-positive cells were determined as monocytes/macrophages.

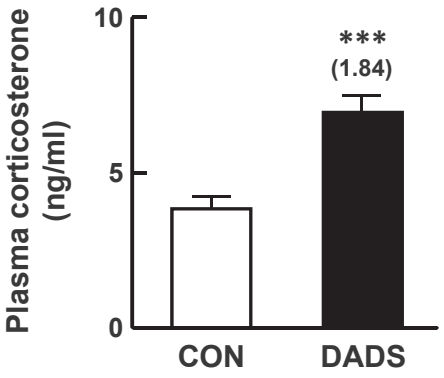

Fig. 3. Effect of DADS administration on plasma corticosterone concentration. Plasma corticosterone concentrations were quantified 2 hr after the administration. The values are shown as mean ± standard error of the mean. Open bar: control (CON) group (n = 6) and closed bar: DADS group (n = 8). Statistics: ***p < 0.001 (vs. CON group, by Student's t-test).

the CON and DADS groups 4 hr after the administration, although the total number of splenic cells was significantly higher in the DADS group by 1.18 times compared to the CON group (Fig. 4C).

Effect of DADS administration on the number of lymphocyte subsets and monocytes/macrophages in the spleen

Among the splenic cells, the number of B-lymphocytes in the spleen was significantly higher in the DADS group by 1.28 times compared to the CON group (Fig. 5). In addition, the number of T-lymphocytes in the spleen showed a tendency to increase in the DADS group, as compared to the CON group (Fig. 5). In contrast, the number of NK cells and monocytes/macrophages in the spleen was similar between the CON and DADS groups (Fig. 5).

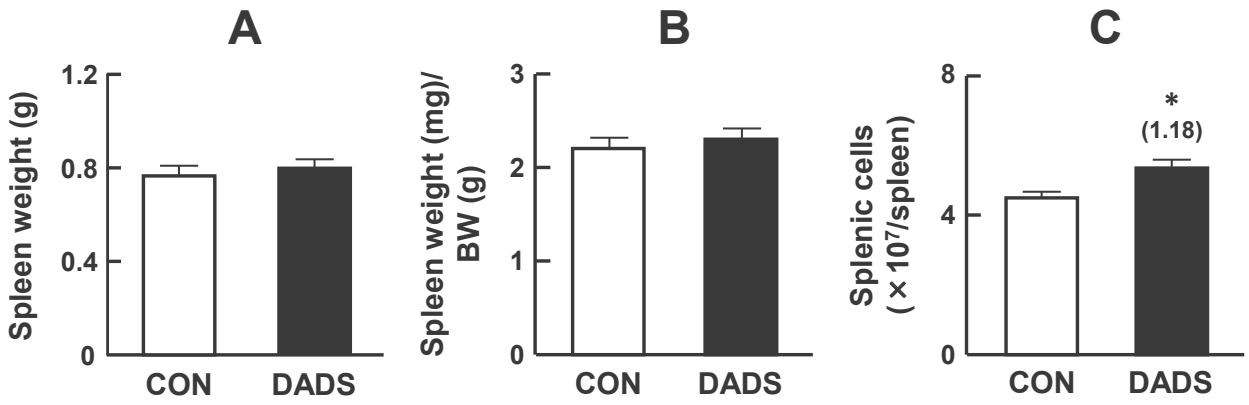

Fig. 4. Effect of DADS administration on spleen weight and total number of splenic cells. A: absolute weight of spleen, B: relative weight per body weight (BW) of spleen, and C: total number of splenic cells. These parameters were analyzed 4 hr after the administration. The values are shown as mean ± standard error of the mean. Open bar: control (CON) group (n = 6), and closed bar: DADS group (n = 6). Statistics: *p < 0.05 (vs. CON group, by Student's t-test).

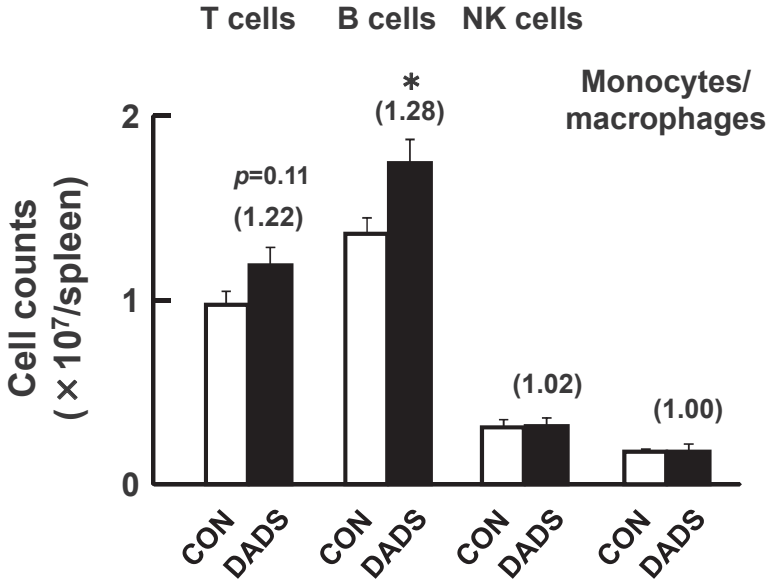

Fig. 5. Effect of DADS administration on the number of splenic lymphocyte subsets and monocytes/macrophages in the spleen. The fractionation and count analysis of T-lymphocytes (T cells), B-lymphocytes (B cells), natural killer (NK) cells, and monocytes-derived cells including macrophages (monocytes/macrophages) were performed 4 hr after the administration. The values are shown as mean ± standard error of the mean. Open bar: control (CON) group (n = 6), and closed bar: DADS group (n = 6). Statistics: *p < 0.05 (vs. CON group, by Student's t-test).

DISCUSSION

In the present study, we verified that the plasma corticosterone concentration increased 2 hr after the administration of DADS (Fig. 3). We have previously demonstrated that the elevation of plasma corticosterone occurs 2 hr after DADS administration (Hashizume *et al.*, 2012); the present results indicated the reproducibility of this phenomenon. Moreover, DADS can bind to and activate transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1) (Bautista *et al.*, 2005; Koizumi *et al.*, 2009). Activation of these ionic

channels in sensory neurofibers leads to noradrenalin and adrenalin secretion (Iwasaki *et al.*, 2006, 2008), and such sympathetic nervous activation stimulates the hypothalamus-pituitary-adrenal axis. Therefore, the production of corticosterone in response to oral administration of DADS was probably induced at an earlier stage.

The major finding of this study is that the number of B-lymphocytes in spleen increased after the administration of DADS (Fig. 5). In addition, the increase in the number of B-lymphocytes was accompanied by an increase in the total number of splenic cells (Fig. 4C) but not by an increase in spleen weight (Figs. 4A and B). Kuttan (2000) demonstrated that the daily administration of DADS (dose = 20 mg/kg BW) for 5 days increased the count of total white blood cells including antibody producing cells in spleen, which was accompanied by an increase in the weights of spleen and thymus. This indicates that continuous administration of DADS promotes the delivery of immune cells to lymphoid organs, and this in turn leads to the hypertrophy of the organs. We have previously reported that a single administration of DADS (20 mg/kg BW) reduced the number of circulating lymphocytes, including B-lymphocytes (Hashizume *et al*., 2012). Furthermore, our results indicated that the administration of similar doses of DADS increases the number of B-lymphocytes in spleen without affecting the organ weight. These findings indicate that the administration of DADS can promote the accumulation of lymphocytes, particularly antibody-producing B-lymphocytes, in the spleen via the intermittent action of glucocorticoids stimulated by a single injection of DADS.

Zamani *et al.* (2009) reported that oral consumption of garlic increased interleukin-4 (IL-4) production, and also decreased the interferon-γ production of splenic lymphocytes in rats, indicating that garlic intake can change the balance between Th1/Th2 cytokine productions and promote a Th2 type or hormonal immune responses. IL-4 has been shown to promote the migration of B-lymphocytes from the circulation to the spleen (Mori *et al.*, 2000). The constituents of garlic that mediate the immune-modulatory effects are yet unknown. However, it is possible that the increase in the number of B-lymphocytes after a single administration of DADS is mediated at least in part by the modulation of the balance between Th1/Th2 cytokine production.

On the other hand, the number of monocytes/macrophages in spleen was not changed after DADS administration (Fig. 5). An *in vitro* study demonstrated that treatment of human monocyte-like cell line U937 or murine peritoneal macrophages with DADS resulted in an increased expression of leukocyte function-associated antigen-1 (LFA-1) on the cell surfaces and enhanced the cellular adhesion mediated by LFA-1 without affecting cellular migration (Huang *et al.*, 2010). We have previously reported that a single administration of DADS reduced the number of circulating monocytes in rats (Hashizume *et al.*, 2012). Thus, it is conceivable that DADS administration caused the increase in the adhesion of monocytes in target sites such as vascular endothelial cells.

In conclusion, a single administration of DADS results in the recruitment of lymphocytes, particularly B-lymphocytes, from the circulation to the spleen. Moreover, the immune-modulatory effects may be mediated at least in part by the activation of the hypothalamus-pituitary-adrenal hormonal cascade.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Akimoto, S., Tanihata, J., Kawano, F., Sato, S., Takei, Y., Shirato, K., Someya, Y., Nomura, S., Tachiyashiki, K. and Imaizumi, K. (2009): Acute effects of dihydrocapsaicin and capsaicin on the distribution of white blood cells in rats. J. Nutr. Sci. Vitaminol., **55**, 282-287.

Bautista, D.M., Movahed, P., Hinman, A., Axelsson, H.E., Sterner, O., Hogestatt, E.D., Julius, D., Jordt, S.E. and Zygmunt, O.M. (2005): Pungent products from garlic activate the sensory ion channel TRPA1. Proc. Natl. Acad. Sci. USA, **102**, 12248-12252.

Block, E. (1985): The chemistry of garlic and onions. Sci. Am., **252**, 114-119.

Cesta, M.F. (2006): Normal structure, function, and histology of the spleen. Toxicol. Pathol., **34**, 455-465.

Chang, H.P., Huang, S.Y. and Chen, Y.H. (2005): Modulation of cytokine secretion by garlic oil derivatives is associated with suppressed nitric oxide production in stimulated macrophages. J. Agric. Food Chem., **53**, 2530-2534.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. and Spencer, R.L. (1995): Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. J. Immunol., **154**, 5511-5527.

Engler, H., Dawils, L., Hoves, S., Kurth, S., Stevenson, J.R., Schauenstein, K. and Stefanski, V. (2004a): Effects of social stress on blood leukocyte distribution: the role of α- and

β-adrenergic mechanisms. J. Neuroimmunol., **156**, 153-162.

Engler, H., Bailey, M.T., Engler, A. and Sheridan, J.F. (2004b): Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. J. Neuroimmunol., **148**, 106-115.

Hao, Z. and Rajewsky, K. (2001): Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J. Exp. Med., **194**, 1151-1164.

Hashizume, Y., Shirato, K., Abe, I., Kobayashi, A., Mitsuhashi, R., Shiono, C., Sato, S., Tachiyashiki, K. and Imaizumi, K. (2012): Diallyl disulfide reduced dose-dependently the number of lymphocyte subsets and monocytes in rats. J. Nutr. Sci. Vitaminol., **58**, 292-296.

Hashizume, Y., Shirato, K., Sato, S., Matsumoto, A., Tachiyashiki, K. and Imaizumi, K. (2013): Dose-dependent effects of diallyl disulfide on plasma glucose and free fatty acid levels in rats. J. Toxicol. Sci., **38**, 879-884.

Huang, T.C., Teng, K.C., Liou, Y.Y., Ho, C.T., Liu, H.J. and Chuang, K.P. (2010): Diallyl disulphide, but not diallyl sulphide, increased leukocyte function-associated antigen-1 expression and cellular adhesion in monocytes. Food Chem., **120**, 113-120.

Iwasaki, Y., Morita, A., Iwasawa, T., Kobata, K., Sekiwa, Y., Morimitsu, Y., Kubota, K. and Watanabe, T. (2006): A nonpungent component of steamed ginger--[10]-shogaol--increases adrenaline secretion via the activation of TRPV1. Nutr. Neurosci., **9**, 169-178.

Iwasaki, Y., Tanabe, M., Kubota, K. and Watanabe, T. (2008): TRPA1 agonists-allyl isothiocyanate and cinnamaldehyde-induce adrenalin secretion. Biosci. Biotechnol. Biochem., **72**, 2608-2614.

Koizumi, K., Iwasaki, Y., Narukawa, M., Iitsuka, Y., Fukao, T., Seki, T., Ariga, T. and Watanabe, T. (2009): Diallyl sulfides in garlic activate both TRPA1 and TRPV1. Biochem. Biophys. Res. Commun., **382**, 545-548.

Kraal, G., Ter Hart, H., Meelhuizen, C., Venneker, G. and Claassen, E. (1989): Marginal zone macrophages and their role in the immune response against T-independent type 2 antigens: modulation of the cells with specific antibody. Eur. J. Immunol., **19**, 675-680.

Kraal, G. (1992): Cells in the marginal zone of the spleen. Int. Rev. Cytol., **132**, 31-74.

Kraal, G. and Mebius, R. (2006): New insights into the cell biology of the marginal zone of the spleen. Int. Rev. Cytol., **250**, 175-215.

Kuttan, G. (2000): Immunomodulatory effect of some naturally occurring sulphur-containing compounds. J. Ethnopharmacol., **72**, 93-99.

Lawson, L.D. and Hughes, B.G. (1992): Characterization of the formation of allicin and other thiosulfinates from garlic. Planta Med., **58**, 345-350.

Lawson, L.D. (1996): The composition and chemistry of garlic cloves and processed garlic. In: Garlic: the Science and Therapeutic Application of Allium Sativum L and related Species (Koch, L.P. and Lawson, L.D., ed.), pp.37-107, Williams & Wilkins, Baltimore

Liu, K.L., Chen, H.W., Wang, R.Y., Lei, Y.P., Sheen, L.Y. and Lii, C.K. (2006): DATS reduces LPS-induced iNOS expression, NO production, Oxidative stress, and NF-κB activation in RAW 264.7 macrophages. J. Agric. Food Chem., **54**, 3472-3478.

Mori, M., Morris, S.C., Orekhova, T., Marinaro, M., Giannini, E. and Finkelman, F.D. (2000) IL-4 promotes the migration of circulating B cells to the spleen and increases splenic B cell survival. J. Immunol., **164**, 5704-5712.

Munday, R. and Munday, C.M. (1999): Low doses of diallyl disulfide, a compound derived from garlic, increase tissue activities of quinone reductase and glutathione transferase in the gastrointestinal tract of the rat. Nutr. Cancer, **34**, 42-48.

Oi, Y., Kawada, T., Shishido, C., Wada, K., Kominato, Y., Nishimura, S., Ariga, T. and Iwai, K. (1999): Allyl-containing sulfides in garlic increase uncoupling protein content in brown adipose tissue, and noradrenaline and adrenalin secretion in rats. J. Nutr., **129**, 336-342.

Schmidt, E.E., MacDonald, I.C. and Groom, A.C. (1993): Comparative aspects of splenic microcirculatory pathways in mammals: the region bordering the white pulp. Scanning Microsc., **7**, 613-628.

Shirato, K., Tanihata, J., Motohashi, N., Tachiyashiki, K., Tomoda, A. and Imaizumi, K. (2007): β_2-Agonist clenbuterol induced changes in the distribution of white blood cells in rats. J. Pharmacol. Sci., **104**, 146-152.

Shirato, K., Sato, S., Sato, M., Hashizume, Y., Tachiyashiki, K. and Imaizumi, K. (2013): β_2-Agonist clenbuterol suppresses bacterial phagocytosis of splenic macrophages expressing high levels of macrophage receptor with collagenous structure. Biol. Pharm. Bull., **36**, 475-480.

Someya, Y., Tanihata, J., Sato, S., Kawano, F., Shirato, K., Sugiyama, M., Kawashima, Y., Nomura, S., Tachiyashiki, K. and Imaizumi, K. (2009): Zinc-deficiency induced changes in the distribution of rat white blood cells. J. Nutr. Sci. Vitaminol., **55**, 162-169.

Sundaram, S.G. and Milner, J.A. (1996): Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis, **17**, 669-673.

The Physiological Society of Japan (2004): Guiding principle for the care and use of animals in the field of physiological sciences. Jpn. J. Physiol., **54**, 98.

Wu, D.D., Sheen, L.Y., Chen, H.W., Kuo, W.W., Tsai, S.J. and Lii, C.K. (2002): Differential effects of garlic oil and its three major organosulfur components on the hepatic detoxification system in rats. J. Agric. Food Chem., **50**, 373-383.

Yi, L. and Su, Q. (2013): Molecular mechanisms for anti-cancer effects of dially disulfide. Food Chem. Toxicol., **57**, 362-370.

Zamani, A., Vahidinia, A. and Ghannad, M.S. (2009): The effect of garlic consumption on Th1/Th2 cytokines in phytohemagglutinin (PHA) activated rat spleen lymphocytes. Phytother. Res., **23**, 579-581.