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RESEARCH ARTICLE | *Nutrient Sensing, Nutrition, and Metabolism*

Water intake increases mesenteric lymph flow and the total flux of albumin, long-chain fatty acids, and IL-22 in rats: new concept of absorption in jejunum

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Nagashio S, Ajima K, Maejima D, Sanjo H, Kajihara R, Hayashi M, Watanabe-Asaka T, Kaidoh M, Yokoyama Y, Taki S, Kawai Y, Ohhashi T. Water intake increases mesenteric lymph flow and the total flux of albumin, long-chain fatty acids, and IL-22 in rats: new concept of absorption in jejunum. *Am J Physiol Gastrointest Liver Physiol* 316: G155–G165, 2019. First published November 15, 2018; doi:10.1152/ajpgi.00325.2018.—The traditional Japanese health care custom recommends that a suitable volume of water is consumed. However, physiological and immunological mechanisms in support of this practice are unknown. Therefore, we conducted rat and rabbit in vivo experiments to investigate the effects of intragastric administration of distilled water on the jejunal-originated lymph flow and the concentrations and total flux of cells, albumin, long-chain fatty acids, and innate lymphoid cell 3 (ILC-3)-secreted interleukin-22 (IL-22) through mesenteric lymph vessels. The distribution and activity of ILC-3 in rat small intestine by water intake were evaluated using flow cytometry and RT-PCR. The intragastric administration of distilled water caused significant increases in rat mesenteric lymph flow and in the total flux of cells, albumin, long-chain fatty acids, and IL-22 through the lymph vessels. Intravenously injected Evans blue dye was rapidly transported into rabbit mesenteric lymph vessel and cisterna chyli. The distribution of ILC-3 and the expression of IL-22 mRNA were maximal in the lamina propria cells of the rat jejunum. No significant presence of ILC-3 in the lymph was observed in the control and under water intake conditions. In conclusion, the absorbed water in the jejunum is transported through mesenteric lymph vessels. The higher permeability of albumin in the jejunal microcirculation may play key roles in the transport of consumed water and the reservoir and transporter of long-chain fatty acids. Water intake also accelerates the transfer of IL-22 to the mesenteric lymph, which may contribute, in part, to maintaining and promoting the innate immunity in the body.

NEW & NOTEWORTHY The higher permeability of albumin-mediated transport of water-soluble substances in mesenteric lymph vessels of the jejunum may have a large impact on the classic concept suggesting that water-soluble small molecules travel to the liver via the portal vein. ILC-3 is mainly housed in the lamina propria of the jejunum, especially its upper part. IL-22 released from the ILC-3 is also transported through mesenteric lymph in collaboration with the albumin-mediated movement of consumed water.

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consumed water; ILC-3; IL-22; long-chain fatty acids; mesenteric lymph

INTRODUCTION

The traditional Japanese health care system, known since the Edo period, recommends that a suitable volume of water be consumed every day, e.g., by drinking green tea or eating miso soup (10). However, physiological and immunological mechanisms in support of this traditional practice are still lacking. Meanwhile, the jejunal microcirculation, compared with that in other organs, has specific properties such as movement of large amounts of albumin from the venular walls to tissues and its related higher tissue osmotic pressure at the venular side (5, 6). Jejunum-originated mesenteric lymph flow is also known to be higher than that in other organs (20). Consistent with these properties, mesenteric collecting lymph vessels show heart-like spontaneous contractions (14, 15), helping to actively transport such large amounts of lymph formation related to gastrointestinal peristalsis, abdominal respiration, and venous pressure (14, 15, 20). On the other hand, lymph transport in the upper and lower extremities is dependent mainly on extrinsic forces, such as skeletal muscle activity, arterial pulsation, and central venous pressure (20). However, it is still unclear how these physiological properties of the jejunal microcirculation and lymph circulation contribute to the absorption and transport of the consumed water and water-soluble substances. In addition, the mesenteric lymph was named "white blood" by Hippocrates in ancient Greece (1), as opposed to the lymph originating from other organs. However, detailed mechanisms of the development of a white color in the mesenteric lymph are not fully elucidated.

To investigate the questions of the Japanese traditional health care system, we next focused, from gut immunological points of view, type 3 innate lymphoid cells (ILC-3) to clarify the mechanisms of the water intake-dependent health care, because in the gut immunology, the ILC-3 has recently been found to be localized in the gut walls and to play crucial roles in innate immunity in the body (19). However, the relation-

ships between the localization and activity of ILC-3 in the gut walls and the intake of water are unclear.

Therefore, we attempted to evaluate with animal cells and molecular experiments *1*) the effects of water intake on the jejunum-originated lymph flow, *2*) the concentrations and total flux of albumin and long-chain fatty acids in the lymph, *3*) the concentration and composition of lymphocytes in the lymph, *4*) the distribution and activity of ILC-3 in the gut walls, and *5*) the concentration and total flux of ILC-3-secreted interleukin-22 (IL-22) in the lymph.

MATERIALS AND METHODS

In vivo animal experiments. This study and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Shinshu University.

Rabbit experiments: transport observation of intravenously injected Evans blue. Ten 22- to 26-mo-old male Japanese rabbits were used. The rabbits were fed a standard pellet diet (LRC4; Oriental Yeast, Tokyo, Japan) and water ad libitum. The animals were fasted overnight to reduce the effect of mesenteric lymph flow. Anesthesia was maintained with 2.0–3.0% isoflurane (Dainippon Sumitomo Pharma, Tokyo, Japan) after the lower part of the thyroid gland had been subjected to a tracheostomy. Sterile physiological saline solution (PSS; Otsuka Pharma, Tokyo, Japan) was administered into the external jugular vein during the experiments.

The rabbits were placed on the operating table in a supine position. To record the color change in mesenteric lymph vessels and the cisterna chyli after intravenous administration of 1 ml of Evans blue dye (Wako, Osaka, Japan), the abdomen was opened by cutting along the midline. The retroperitoneum was opened at the upper pole of the left kidney. Mesenteric lymph vessels and the cisterna chyli were exposed by removing the surrounding adipose and connective tissues. The color changes within lymph vessels and the cisterna chyli were recorded via video (Ricoh CX6, Tokyo, Japan). The color changes in collected lymph vessels running along the left external jugular vein were also recorded to be compared with those within the mesenteric lymph vessels and the cisterna chyli. The reason that rabbits were used is that the cisterna chyli in mice and rats are too small and difficult for recording color changes using via video.

To quantitatively measure the color changes within the mesenteric lymph vessels and the cisterna chyli, high-resolution digital photomicrographs were processed using the Scion image analysis program (11). The same area was outlined in each cisterna chyli photomicrograph on a grayscale image and processed for density measurement. The results were expressed in arbitrary units of the mean density per pixel.

Rat experiments. Male Sprague-Dawley rats (10–12 wk old; Japan SLC, Tokyo, Japan) were fed a standard pellet diet (MF; Oriental Yeast, Tokyo, Japan) and water ad libitum. The animals were fasted overnight to reduce the effect of mesenteric lymph flow. The rats were anesthetized with isoflurane and then placed on the operating table in a supine position. A catheter was inserted into the femoral vein to inject PSS. To minimize hemodynamic changes in the jejunal microcirculation, the intravenous infusion of PSS was stopped during the experiments, because we had confirmed with preliminary experiments that the intravenous infusion of PSS caused a significant increase in rat mesenteric lymph flow.

To collect the lymph from mesenteric lymph vessels, the abdomen was opened by cutting along the midline, and mesenteric adipose and connective tissues were removed to expose the mesenteric lymph nodes and efferent lymph vessels. A small polyethylene catheter (0.5–0.6 mm) was inserted centrifugally into an efferent lymph vessel. To evaluate the effects of water intake on the mesenteric lymph flow and the concentrations of cells, albumin, long-chain fatty acids, and IL-22 in the lymph, intragastric administration of distilled water (3 ml) was performed by inserting a needle catheter through the mouth to the stomach. The volume of distilled water was determined by the maximum rat intragastric volume. Distilled water was selected because an earlier study (17) had demonstrated that 5 ml of distilled water, but not PSS, injected into rat stomach produced a significant increase in the lymph flow through thoracic duct. The lymph was collected over set periods of time (15 or 60 min), and the volume was measured.

Measurements of cell, albumin, long-chain fatty acid, and IL-22 concentrations in mesenteric lymph. First, the obtained mesenteric lymph was centrifuged, and the supernatants were used to measure the concentrations of albumin, long-chain fatty acids, and IL-22. The concentration of cells was measured with the Türk reagent, using diluted mesenteric lymph. The diluted lymph was dropped onto a Burker-Türk counter, and the number of cells on the plate was counted under a photomicroscope.

The concentrations of albumin and IL-22 in the lymph were measured using enzyme-linked immunosorbent assay (ELISA) kits, a rat albumin ELISA quantitative kit (catalog no. E111-125; Bethyl, Montgomery, TX) and a mouse/rat IL-22 Quantikine ELISA kit (catalog no. M2200; R&D Systems, Minneapolis, MN), respectively.

The concentration and composition of long-chain fatty acids were measured using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan). Isolation of fatty acids from the mesenteric lymph was performed using a DB-23 column (Agilent Technologies, Santa Clara, CA). Both procedures were carried out according to the manufacturer's instructions.

Cell isolation. Male Sprague-Dawley rats (10- to 12-wk-old; Japan SLC, Tokyo) were fasted overnight and then given water ad libitum for 2 h. The 2-h water intake may be suitable to evaluate the gene expression of ILC-3 (19). The rats were anesthetized with isoflurane, and then the small intestines and mesenteric lymph nodes were isolated. The upper and lower parts of both jejunum and ileum were isolated in quarters along the length of the small intestine. Each preparation was washed with excess PSS to remove stools and mucus, followed by incubation for 30 min at 37°C with vigorous shaking in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution supplemented with 5 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol to remove the epithelial layer, because the distribution of ILC-3 is known to localize mainly in the gut lamina propria (19). To isolate lamina propria cells, the remaining upper and lower parts of the small intestine, were chopped into small pieces with a scalpel and digested for 30 min at 37°C with gentle shaking in RPMI 1640 supplemented with 5% fetal bovine serum, 0.5 mg/ml collagenase IV (Sigma, St. Louis, MO), and 50 U/ml DNase (Wako). Cells were subjected to 40–70% Percoll gradient centrifugation, and the cells from the middle layer were harvested and used for experiments.

Cells from the mesenteric lymph nodes were isolated using the same method after gentle cutting of the nodes. The cells obtained from the mesenteric lymph were also subjected to flow cytometric analysis using pellets obtained after centrifugation of the mesenteric lymph.

Flow cytometry. The following fluorochrome-conjugated antibodies (Abs) were used for flow cytometry: phycoerythrin (PE) mouse anti-rat CD3, PE anti-human/mouse RAR-related orphan receptor-γ (RORγt), and PE rat Ig2A, K isotype control, purchased from eBioscience (San Diego, CA); fluorescein isothiocyanate mouse anti-rat CD45R and PerCP/Cy5.5 anti-rat CD45, purchased from BD PharMingen (Franklin Lakes, NJ). The Ghost Red 780 viability dye was purchased from Tonbo Biosciences (San Diego, CA). Cells were stained on ice in the presence of an anti-CD132 Ab to block Fc-mediated nonspecific staining. To detect rat RORγt, cells were loaded with the anti-human/mouse RORγt Ab and developed with the PE-labeled anti-mouse IgG Ab according to the manufacturer's instructions. Rat T and B lymphocytes were identified using the mouse anti-rat CD3 Ab and mouse anti-rat CD45R Ab, respectively. Fluorescence-activated cell sorting (FACS) analysis was performed using a BD FACS Canto II flow cytometer (BD Bioscience, San Diego, CA).

The gating strategy for isolating ILC-3 from the cells in the lamina propria or the lymph nodes was as follows: *1*) isolation of the live cells,

2) gating and isolation of CD-45 positive cells, and then *3*) gating and isolation of CD-45R- and CD-3-negative cells and RORγt-positive cells.

Quantitative RT-PCR. Expression of IL-22 mRNA, which is one of markers for ILC-3 localization, was evaluated by RT-PCR. Total RNA was extracted from the cells isolated from the lamina propria of the four parts of the small intestine, i.e., the upper and lower parts of the jejunum and ileum, using the Isogen reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. A Superscript First-strand Synthesis kit (Invitrogen, Carlsbad, CA) and 1.0 μg of total RNA were used to synthesize cDNA. The following primers (SIGMA Genosis, Tokyo, Japan) were used for each specific reaction: IL-22, 5′-GATAAAAAACAACACAGATGTCAG-GCTC-3′ (forward) and 5′-GATCGCTTTAATCTCTCCACTCTC-3′ (reverse); β-actin, 5′-CGTGAAAAGATGACCCAGATCA-3′ (forward) and 5′-CACAGCCTGGATGGCTACGT-3′ (reverse). cDNA was diluted fivefold before PCR amplification. Quantitative RT-PCR was performed using a LightCycler (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer's instructions. Negative controls were included in each reaction, and PCR products obtained with each primer pair were subjected to melting curve analysis. Data were analyzed with the LightCycler analysis software.

Statistical analysis. All results are expressed as means ± SE. Statistical analyses were performed using Student's *t*-test for paired or unpaired results or one-way analysis of variance followed by Duncan's post hoc test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Jejunum-originated lymph flow confirmed in rats. First, we injected Evans blue dye (1 ml) into the jejunal, ileal, or gastric mucosal and submucosal layers of walls, observed the pathway of the blue dye through small mesenteric lymph vessels, and measured the time needed for the dye to arrive at the cannulated lymph vessels. Representative photomicrographs recording the pathway of the Evans blue dye through small mesenteric lymph vessels are shown in Fig. 1*A*. The dye injected into the jejunal wall arrived at the cannulated mesenteric lymph vessel within 5 min (Fig. 1, *B* and *C*), but that injected into the gastric or ileal wall did not arrive until 40 or 60 min later (Fig. 1*A*, *D*, and *E*). Thus, the lymph flow recorded in rat mesenteric lymph vessels in these experiments mainly originated from the jejunal microcirculation.

To evaluate its physiological properties under control conditions, the lymph flow in mesenteric lymph vessels was video recorded. Figure 1*B* shows representative photomicrographs of the mesenteric lymph flow extracted from the video. White-colored lymph was observed within the lymph vessel (Fig. 1*B*). The average lymph flow rate was 140.0 ± 11.1 μl/h ($n = 5$) in the controls.

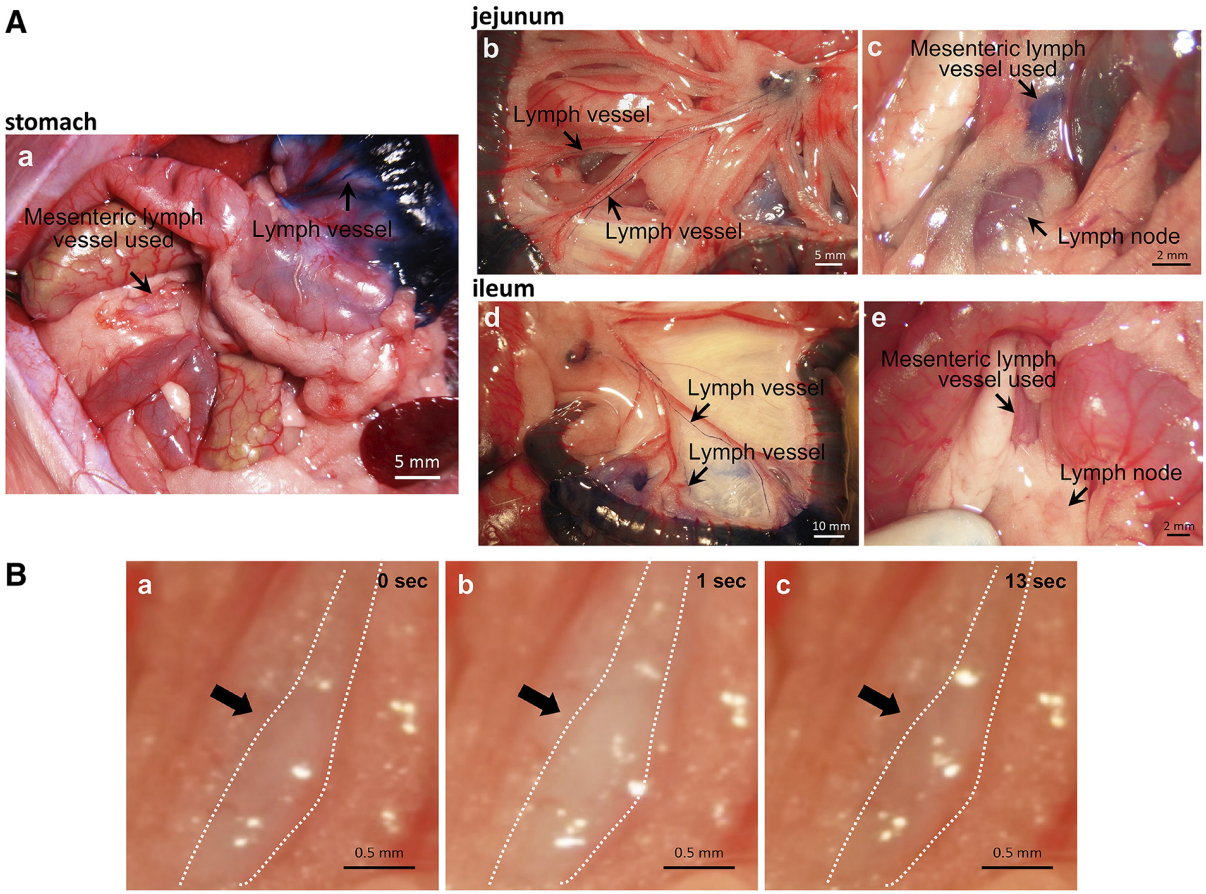

Fig. 1. *A*: representative photomicrographs of rat stomach (*a*), jejunum (*b* and *c*), and ileum (*d* and *e*) with Evans blue dye injected into mucosal and submucosal layers of walls. Photomicrographs of the jejunum were taken ~5 min after injection (*c*), and those of the stomach (*a*) and ileum (*e*) were taken ~40 and 60 min after injection, respectively. *B*: representative photomicrographs extracted from a 7-s recorded video of a jejunal mesenteric lymph vessel (*a*, 0 s; *b*, 1 s; *c*, 7 s). Arrows point to the lymph vessel. white-colored lymph was observed in physiological conditions.

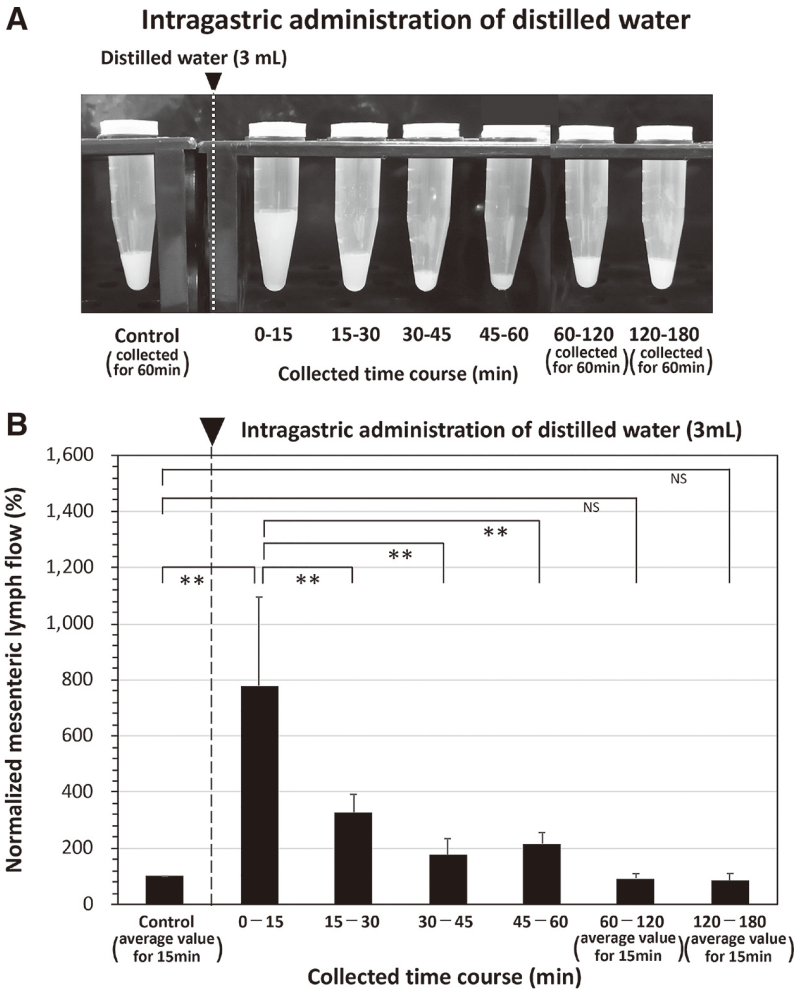

Fig. 2. *A*: effects of intragastric administration of distilled water (3 ml) on the volume of the lymph collected over consecutive periods of 60 or 15 min each after administration. Control shows the volume of the lymph collected during 60, but not 15, min before administration of distilled water from the jejunal lymph vessel. Maximal lymph volume was obtained during the first 15 min after administration, which then decreased in a time-dependent manner until 60 min. Lymph volumes within the tubes shown at both 60–120 and 120–180 min in the panel were collected for 60 min, respectively. *B*: time course of changes in normalized volumes of lymph collected over 15-min periods. Control value (ordinate, 100% = 45.7 ± 4.7 μl/15 min; $n = 4$) was calculated by dividing by 4 the volume of lymph collected for 60 min before administration. Values of lymph volume shown in 60–120 and 120–180 min were also obtained in the same manner as that obtained in the control, respectively. $^{**}P < 0.01$; NS, not significant.

Water intake significantly accelerated jejunum-originated lymph flow. Figure 2*A* shows the effects of intragastric administration of distilled water (3 ml) on the volume of the lymph collected into tubes over several consecutive periods of 15 or 60 min. The tube under the control condition (collected for 60 min) clearly shows white-colored lymph. To analyze the time-dependent changes in the volume of the lymph collected in the first hour after administration of distilled water, the mesenteric lymph was collected over consecutive periods of 15 min each for 1 h. The highest volume of the lymph was collected in the first 15 min after administration of distilled water (Fig. 2*A*). Figure 2*B* shows the summarized data with lymph volume collected for 15 min. Thus, the control, 60- to 120-, and 120-to 180-min lymph volumes were calculated by dividing by 4 the volumes of the lymph collected during 60 min each, respectively. All values were normalized to the calculated control value (100% = 45.7 ± 4.7 μl/15 min; $n = 4$) (0–15 min: 778.5 ± 287.7%, $P < 0.01$ vs. control; 15–30 min: 326.1 ± 60.0%; 30–45 min: 177.9 ± 51.1%; 45–60 min: 216.3 ± 36.2%; $n = 4$ each).

Effects of intragastric administration of distilled water on the concentration of albumin in the lymph and total flux of albumin through lymph vessels. Plasma albumin is known to move from the venular walls to tissues in the jejunum (3, 21). Thus, to examine the effects of intragastric administration of water on the concentration of albumin in the lymph and the total flux of albumin through lymph vessels, we first measured the time-dependent changes in the concentration of albumin in the lymph after administration. The summarized data are shown in Fig. 3*A*. The concentration of albumin in the lymph significantly decreased at 1 and 2 h after intragastric administration of distilled water (1 h: 62.3 ± 16.1% of control, $P < 0.05$; 2 h: 63.1 ± 13.4% of control, $P < 0.05$; $n = 4$ each) compared with the control value of 3.0 ± 0.4 g/dl.

We next calculated the total flux of albumin per hour through a lymph vessel by multiplying the concentration of albumin by the volume of the lymph collected during each hour. The data are shown in Fig. 3*B*. The total flux of albumin transported through the lymph vessel significantly increased only for 1 h after the administration of distilled water (250.4 ± 32.5% of control, $P < 0.01$; $n = 4$).

Effects of intragastric administration of distilled water on the concentration of long-chain fatty acids in the lymph and their total flux through lymph vessels. We measured the time-dependent changes in the concentration of long-chain fatty acids in the lymph and their total flux through lymph vessels after the intragastric administration. Similar to the findings on albumin, the concentrations of long-chain fatty acids in the lymph decreased at 1 and 2 h after the intragastric administration (1 h: 80.8 ± 4.3% of control, $P < 0.05$; 2 h: 79.3 ± 2.7%

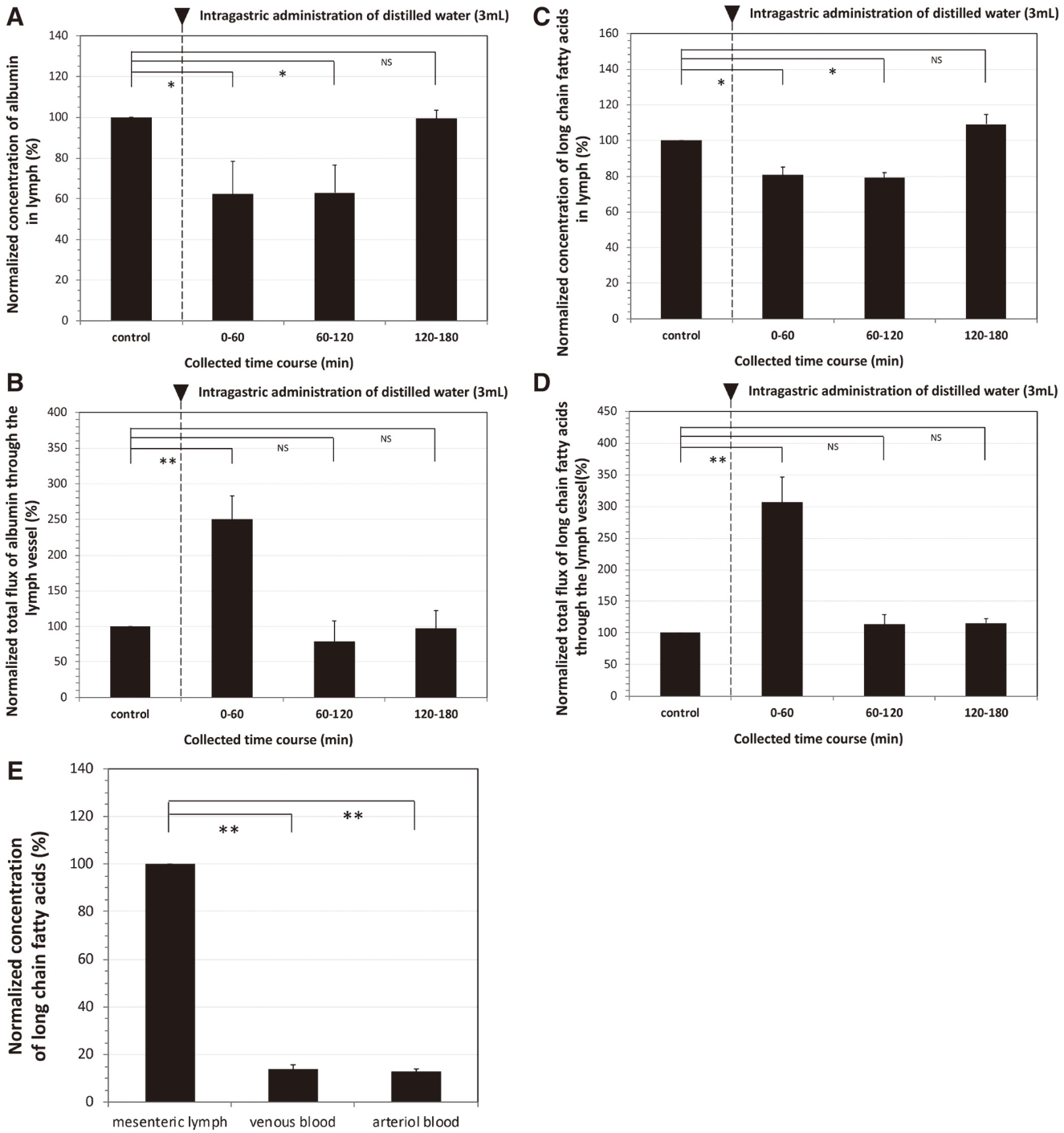

Fig. 3. *A*: effects of intragastric administration of distilled water (3 ml) on normalized concentrations of albumin in mesenteric lymph collected over 60-min periods from the jejunal lymph vessel (ordinate, 100% = 3.0 ± 0.4 g/dl). *$P < 0.05$; NS, not significant. *B*: effects of intragastric administration of distilled water (3 ml) on normalized total flux of albumin through the lymph vessel, calculated as concentration of albumin × volume of lymph collected over a respective 60-min period (the ordinate). **$P < 0.01$. *C*: effects of intragastric administration of distilled water (3 ml) on normalized concentration of long-chain fatty acids in lymph collected over 60-min periods from the jejunal lymph vessel (ordinate, 100% = 5,041.2 ± 304.9 μg/ml; $n = 4$). *$P < 0.05$. *D*: effects of intragastric administration of distilled water (3 ml) on normalized total flux of long-chain fatty acids, calculated as concentration of long-chain fatty acids × volume of lymph collected over a respective 60-min period (the ordinate). **$P < 0.01$. *E*: normalized concentrations of long-chain fatty acids, including palmitic, stearic, oleic, linoleic, arachidonic, docosahexaenoic, and other acids in mesenteric lymph, as well as in venous and arterial blood, under control conditions (ordinate, 100% = 4,733.5 ± 477.6 μg/ml; $n = 5$). **$P < 0.01$.

of control, $n = 4$ each; Fig. 3*C*) compared with the control value of 5,041.2 ± 304.9 μg/ml. In contrast, the total flux of long-chain fatty acids through the lymph vessel significantly increased at 1 h after the intragastric administration (Fig. 3*D*; 306.8 ± 39.5% of control, $P < 0.01$, $n = 4$). As shown in Fig. 3*E*, under control conditions of overnight fasting, the concentration of long-chain fatty acids in the mesenteric lymph was approximately seven times higher than those in the venous and arterial blood. The main long-chain fatty acids contained in the lymph were palmitic, stearic, oleic, and linoleic acids. Significant amounts of ω-3 fatty acids, i.e., arachidonic and docosahexaenoic acids, were also contained in the mesenteric lymph. The composition of long-chain fatty acids in the lymph was not significantly changed by the intragastric administration of distilled water.

Effects of intragastric administration of distilled water on the movement of intravenously injected Evans blue through rabbit mesenteric lymph vessels and cisterna chyli. To confirm the crucial role of the plasma albumin movement in the water intake-mediated increase in the mesenteric lymph flow, we measured the time for the lymph color to change within mesenteric lymph vessels and the cisterna chyli in rabbits pretreated with intravenous administration of Evans blue dye, which is known to rapidly bind to plasma albumin (8). Figure 4*A* shows representative photomicrographs of time-dependent changes in the blue color of the lymph within rabbit mesenteric lymph vessels and the cisterna chyli in the control. Two minutes after intravenous administration of Evans blue dye, the lymph in rabbit mesenteric lymph vessels became slightly blue and then gradually became more intensely colored. Approximately 15 min after Evans blue dye administration, the rabbit cisterna chyli was stained blue. The intragastric administration of distilled water (3 ml) shortened the time for these changes. The data using Scion image analysis are summarized in Fig. 4*B*.

However, no significant color changes of the lymph occurred in the cervical collecting lymph vessel, running along the external cervical vein, at 1 h after intravenous administration of Evans blue dye.

Effects of intragastric administration of distilled water on the cell concentration in the mesenteric lymph and total cell flux through lymph vessels. To evaluate the effects of water intake on the transport of cells by the mesenteric lymph, we measured the time-dependent changes in the concentration of cells in the lymph and the total flux of cells through a lymph vessel after intragastric administration of distilled water. In contrast to the findings on albumin and long-chain fatty acids, the intragastric administration of water resulted in a significant

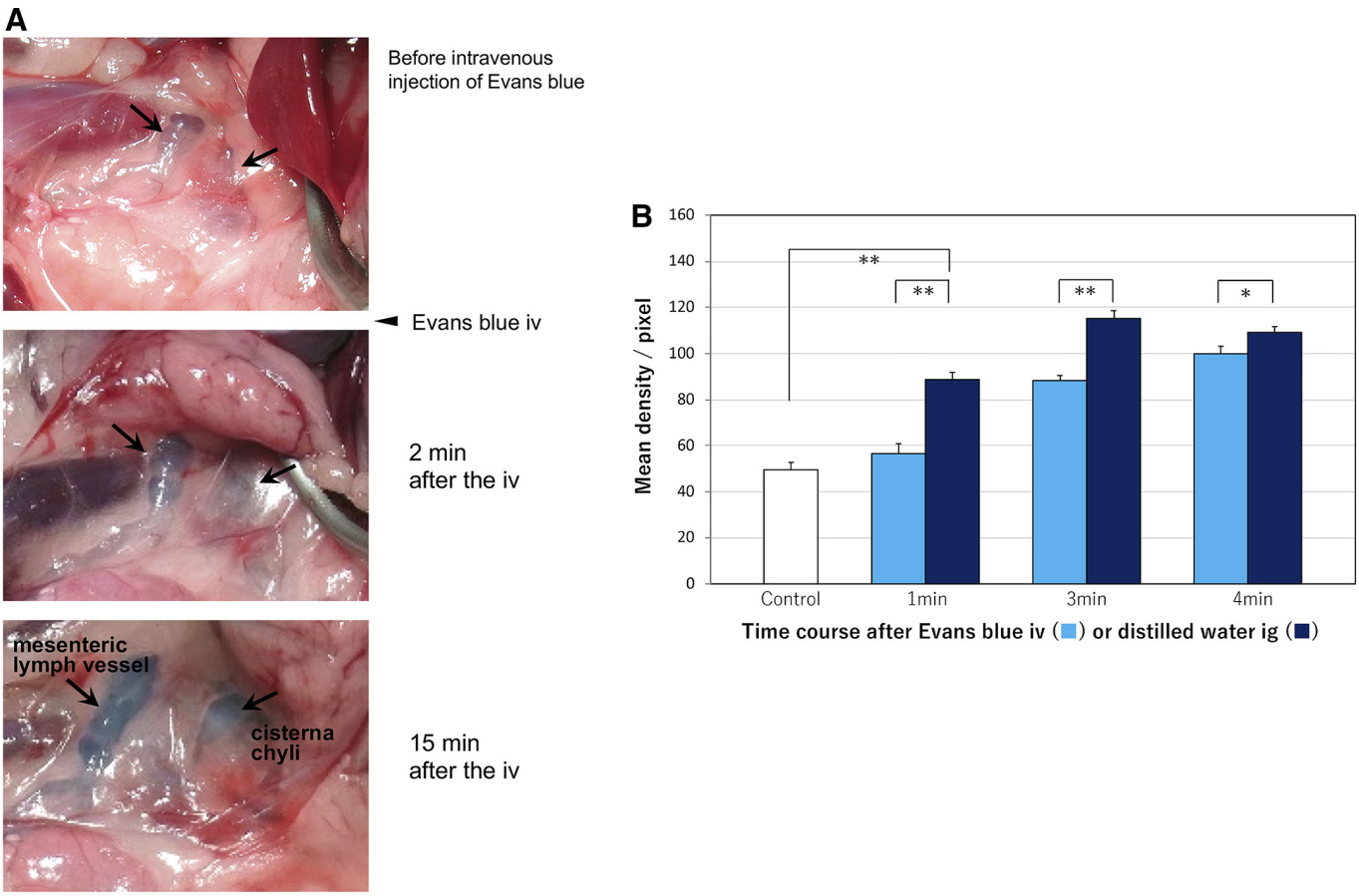

Fig. 4. *A*: representative photomicrographs of color changes in rabbit mesenteric lymph vessels and cisterna chyli upon administration of Evans blue dye (0.3 ml/kg iv) under control conditions. *B*: summary of time-dependent color changes in rabbit mesenteric lymph vessels in control and after intragastric administration of distilled water (3 ml). The ordinate shows mean density of each photomicrograph, obtained using Scion image analysis software. The abscissa shows the time course after iv administration of Evans blue dye without or with intragastric administration of distilled water. *$P < 0.05$, **$P < 0.01$; NS, not significant.

increase in the concentration of cells in the lymph (1 h: 254.1 ± 65.4% of control, $P < 0.05$, $n = 4$) compared with the control value of 7.35 ± 0.71 × 10^6 cells, as well as in the total flux of cells through the lymph vessel (1 h: 230.0 ± 90.4% of control, $P < 0.01$, $n = 4$; Fig. 5, *A* and *B*).

Figure 5*C* shows the effects of the intragastric administration on the ratio between T and B cells in the mesenteric lymph. No significant time-dependent changes in the ratio between T and B cells were observed in the lymph. In the control, the proportions of B and T cells were 18.7 ± 2.5 and 81.1 ± 2.0% ($n = 5$), respectively.

Effects of 2-h water intake on the ratio between T and B lymphocytes in the lamina propria of jejunum and ileum and in mesenteric lymph nodes. To evaluate the effects of 2-h water intake on the composition of lymphocytes in the lamina propria of the jejunum and ileum and in mesenteric lymph nodes, we determined the composition of lymphocytes using flow cytometry. As shown in Fig. 5*D*, in the lamina propria of jejunal walls from both upper and lower parts of the jejunum, the numbers of B cells were higher than those of T cells in the control (upper part: 55.6 ± 0.8 and 23.4 ± 1.1%, respectively; lower part: 62.5 ± 1.5 and 19.6 ± 6.5%, respectively, $n = 5$ each). The ratio between B and T cells in the jejunal walls did not change at 2 h after water intake (B cells: 53.8 ± 2.3%; T cells: 21.1 ± 1.8%, $n = 5$ each) compared with that in the control. In contrast, the numbers of T cells in lymph nodes in the control were slightly higher than those of B cells (54.4 ± 0.7 and 43.5 ± 1.1%, respectively, $n = 5$ each). At 2 h after water intake, a significant increase was observed in the proportion of T cells (58.6 ± 0.8%, $P < 0.05$ vs. control, $n = 5$) but not of B cells in the lymph node.

Effects of 2-h water intake on the distribution and dynamics of ILC-3 in jejunal lamina propria, mesenteric lymph nodes, and mesenteric lymph. To assess whether water intake could play a crucial role in gut-mediated innate immunity, we evaluated the distribution and dynamics of ILC-3 in the lamina propria of the jejunal and ileal walls, mesenteric lymph nodes, and the mesenteric lymph. Figure 6*A* shows representative flow cytometry data on RORγt-positive ILC-3. As shown in this figure, there was a marked heterogeneity in the distribution of ILC-3 between jejunum and ileum. Thus, ILC-3 were localized mainly in the lamina propria of the upper part of the jejunum (8.4 ± 1.0 vs. 2.1 ± 0.4% and 1.0 ± 0.2% in the upper and lower parts of the ileum, respectively, $P < 0.01$, $n = 5$).

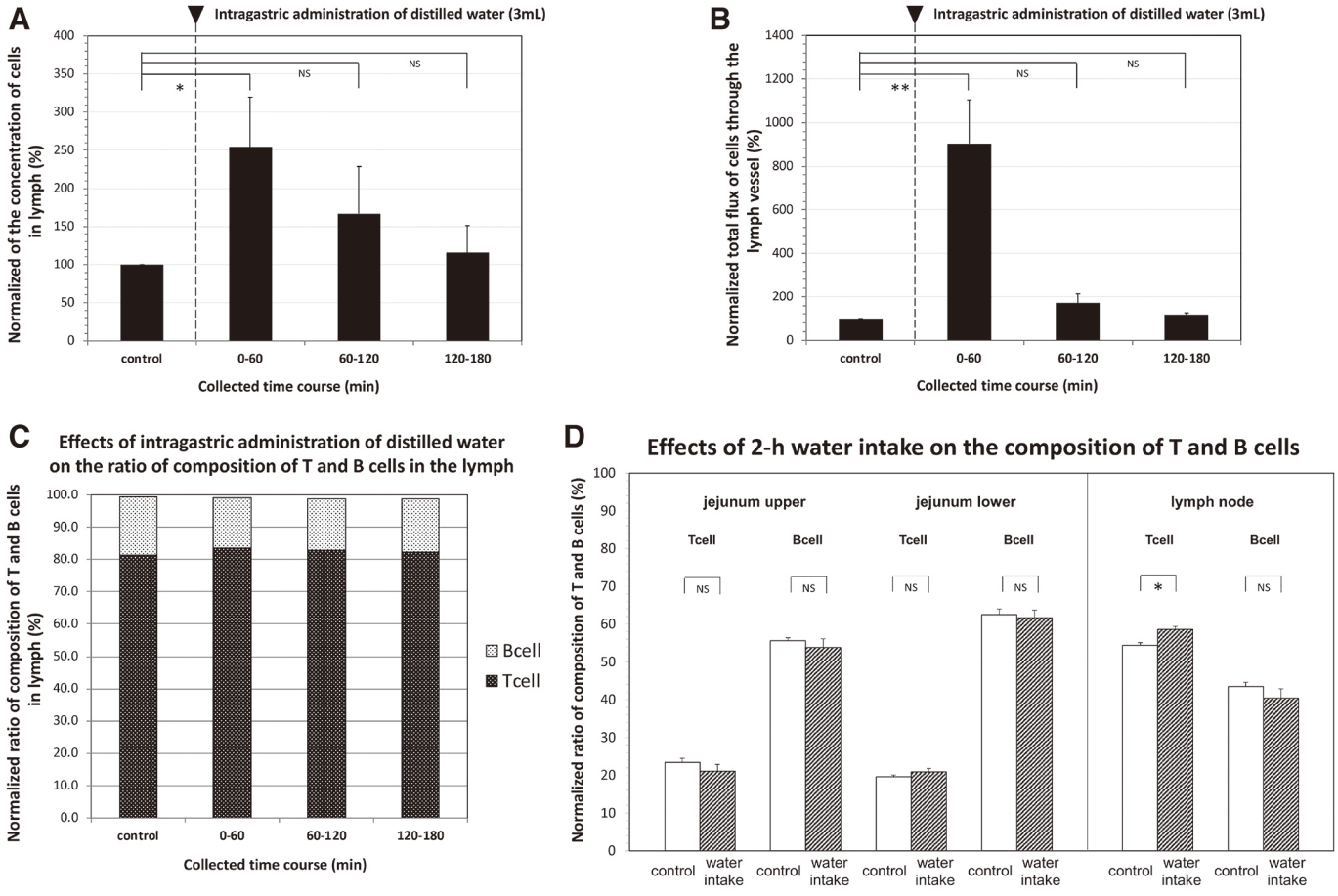

Fig. 5. *A*: effects of intragastric administration of distilled water (3 ml) on normalized concentration of cells in mesenteric lymph collected over 60-min periods (ordinate, 100% = 7.35 ± 0.71 × 10^6 cells; $n = 4$). *$P < 0.05$; NS, not significant. *B*: effects of intragastric administration of distilled water (3 ml) on normalized total flux of cells through the lymph vessel, calculated as concentration of cells × volume of lymph collected over a respective 60-min period (the ordinate). **$P < 0.01$. *C*: effects of intragastric administration of distilled water (3 ml) on time-dependent changes in the ratio of composition of T and B cells in mesenteric lymph collected over 60-min periods (the ordinate). *D*: changes in the ratio of composition of T and B cells in upper and lower parts of rat jejunum and in mesenteric lymph nodes without (open bars) and with (dappled bars) 2 h after water intake ($n = 5$). *$P < 0.05$.

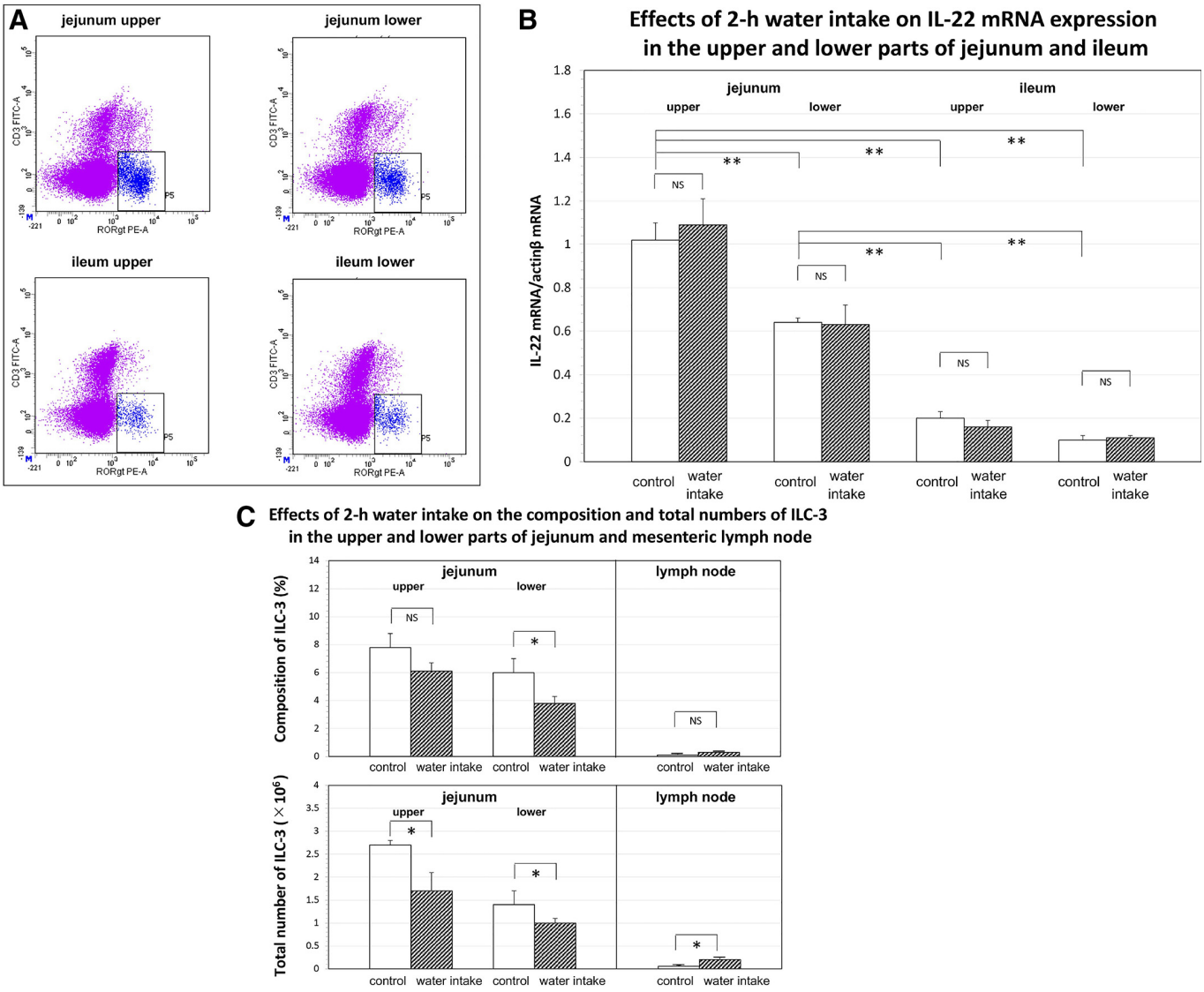

Fig. 6. *A*: representative flow cytometry recordings of RAR-related orphan receptor-γ (RORγt)-positive cells (blue) in lamina propria of upper and lower parts of jejunum and ileum in the control. *B*: effects of 2-h water intake on expression of IL-22 mRNA in the lamina propria of upper and lower parts of jejunum and ileum. Expression levels of IL-22 mRNA were normalized relative to those of β-actin (the ordinate, $n = 5$). Open and dappled bars show control and treatment with 2-h water intake, respectively. $^{**}P < 0.01$; NS, not significant. *C*: effects of 2-h water intake on composition (*top*) and total number (*bottom*) of innate lymphoid cell 3 (ILC-3; $n = 5$) in upper and lower parts of the jejunum and in mesenteric lymph nodes (the ordinate). Open and dappled bars show control and treatment with 2-h water intake, respectively. $^{*}P < 0.05$.

Similar to the flow cytometry data on ILC-3, the expression of IL-22 mRNA, as one of the markers for ILC-3 localization, showed a marked heterogeneity between the jejunum and ileum (Fig. 6*B*). Thus, the expression of IL-22 mRNA in both upper and lower parts of the jejunum (1.02 ± 0.08 and 0.64 ± 0.02, respectively, $n = 5$) was significantly higher than that in the upper and lower parts of the ileum (0.20 ± 0.03 and 0.10 ± 0.02, respectively, $n = 5$). No significant effect of 2-h water intake was observed on the IL-22 mRNA expression in the jejunum and ileum.

On the other hand, 2-h water intake resulted in a significant decrease ($P < 0.05$ vs. control) in the total numbers of ILC-3 in both upper and lower parts of the jejunum ($1.7 \pm 0.4 \times 10^6$ and $1.0 \pm 0.1 \times 10^6$, respectively, $n = 5$ each; Fig. 6*C*). In contrast, the total numbers of ILC-3 in mesenteric lymph nodes were significantly increased by 2-h water intake ($0.20 \pm 0.05 \times 10^6$, $P < 0.05$ vs. control, $n = 5$).

No significant presence of ILC-3 in the lymph was observed under both control and time-dependent water intake conditions.

Effects of intragastric administration of distilled water on the concentration of IL-22 in mesenteric lymph and total flux of IL-22 through lymph vessels. To evaluate the effects of intragastric administration of distilled water on the activity of ILC-3 in the lamina propria of the rat jejunum, we measured the concentration of IL-22 in the lymph collected over 60-min periods. Similar to the findings on albumin and long-chain fatty acids, the concentration of IL-22 in the lymph significantly decreased for 1 h after intragastric administration of distilled water (1 h, 171.5 ± 17.7 vs. control, 260.1 ± 14.0 pg/ml, $P < 0.01$, $n = 4$; Fig. 7*A*).

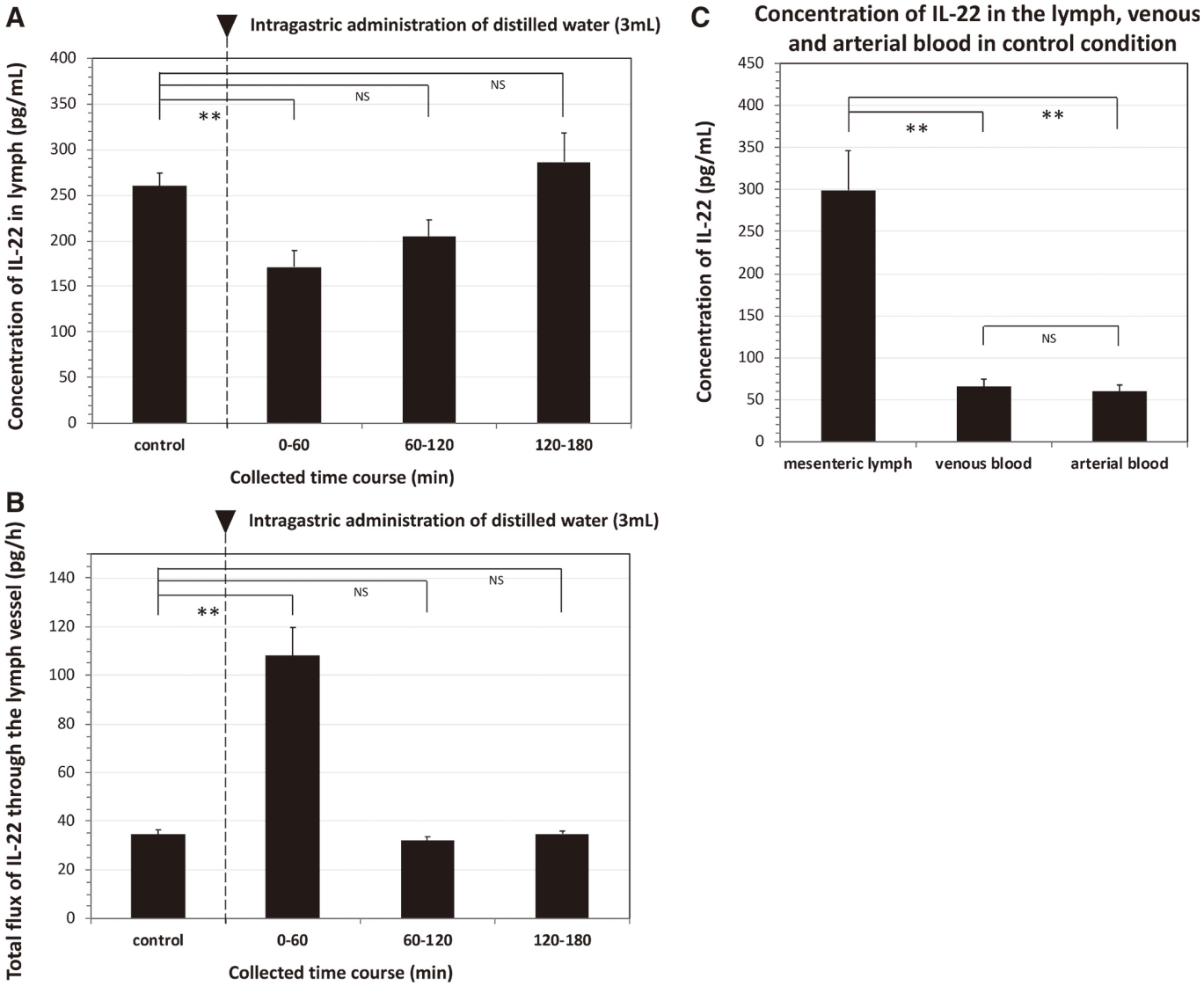

Fig. 7. *A*: effects of intragastric administration of distilled water (3 ml) on concentrations of IL-22 in lymph collected over 60-min periods ($n = 4$). $^{**}P < 0.01$; NS, not significant. *B*: effects of intragastric administration of distilled water (3 ml) on total flux of IL-22, calculated as concentration of IL-22 × volume of lymph collected over respective 60-min period ($n = 4$). $^{**}P < 0.01$. *C*: concentrations of IL-22 in mesenteric lymph and in venous and arterial blood under control conditions ($n = 5$). $^{**}P < 0.01$.

On the other hand, the total flux of IL-22 through the lymph vessel, calculated by multiplying the concentration of IL-22 in the lymph by the lymph volume per hour, significantly increased at 1 h after the administration (1 h, 108.3 ± 11.4 vs. control, 34.4 ± 2.0 pg/h, $P < 0.01$, $n = 4$; Fig. 7*B*).

The concentration of IL-22 in the control lymph was 299.5 ± 46.9 pg/ml ($n = 5$), which was significantly higher than the values obtained for the arterial and venous blood (66.1 ± 8.4 and 60.4 ± 7.2 pg/ml, respectively, $n = 5$ each; Fig. 7*C*).

DISCUSSION

Mesenteric lymph vessels act as an absorptive route for consumed water and water-soluble substances. The most important result of the present study is the clear demonstration that intragastric administration of distilled water produced a significant increase in the mesenteric lymph flow. The lymph flow originated mainly from the jejunal microcirculation, with little or no lymph flow from the ileal and gastric microcirculations. The lymph flow increase started immediately after intragastric administration of water and was maximized by the first 15 min. These findings are consistent with the data from an earlier study showing that 5 ml of distilled water injected into the stomach produced a significant increase in lymph flow through the thoracic duct in awake rats (17). However, the mechanisms of the previous findings remained unknown. In the present study, we propose that specific physiological properties of the jejunal microcirculation (16, 18), i.e., higher permeability for plasma albumin, may mainly contribute to the water intake-mediated increase in the mesenteric lymph flow. Thus, the higher permeability of albumin in jejunal microcirculation produces a higher tissue colloid osmotic pressure, which results in the accelerated transport of albumin-binding distilled water into the lacteal vessels in jejunal villi. In the present

study, the total flux of albumin through mesenteric lymph vessels significantly increased within 1 h after intragastric administration of distilled water, although the concentration of albumin in the lymph decreased during the first hour after the administration. In addition, intravenously administered Evans blue dye, which primarily binds to albumin in the blood (8), also appeared in rabbit cisterna chyli within several minutes after the administration under physiological conditions, and the time for the dye appearance rapidly decreased with intragastric administration of distilled water. Incorporating the finding, Granger et al. (4, 5) demonstrated that the massive movement of plasma proteins into and out of the mucosal interstitium in the small intestine may be advantageous for the removal of protein-bound substances to mesenteric lymph vessels. The existence of specialized lymph capillaries, lacteal vessels in jejunal villi, may be consistent with these functional properties of microcirculation and lymph circulation in the jejunum. In conclusion, the higher permeability of albumin-mediated absorption of water-soluble substances into mesenteric lymph vessels of the jejunum may have a large impact on the classic concept suggesting that water-soluble small molecules, i.e., glucose, amino acids, and polypeptides, travel to the liver via the portal vein (7). However, the possibility cannot be ruled out that the absorption and transport of water-soluble molecules into mesenteric lymph produce the modification to transport such molecules through the portal vein. We should be evaluating that possibility in the future.

Higher permeability of albumin serves as a reservoir and transporter of long-chain fatty acids in the jejunum. Another important result of the present study is that intragastric administration of distilled water produced a significant increase of total flux of long-chain fatty acids in rat mesenteric lymph. The findings may suggest that the higher permeability of albumin in villi of the jejunum may be its involvement as a reservoir and transporter of long-chain fatty acids, because in physiological conditions dietary lipid is firstly induced lipolysis and then produced micelle formation, requiring pancreatic lipase and conjugated bile acids in the duodenum. The micelle is next absorbed and forms chylomicron in the epithelial cells of the jejunum. The chylomicron is mainly combined with apoprotein and then moved into mesenteric lymph vessels (2). Taking into consideration the physiological evidence, the finding obtained with the present experiments may strongly support the hypothesis that a part of the chylomicron absorbed is physiologically restored incorporation with apoprotein and albumin in the jejunal wall, because apoprotein B is usually produced in the jejunal wall (2). The great bulk flow produced by the combination of absorbed water and the higher permeability of albumin may accelerate to transport the restored chylomicron connected with apoprotein in the jejunal wall into the mesenteric lymph vessels.

In addition, the concentration of long-chain fatty acids, including ω-3 fatty acids, was several times higher in the lymph than in the arterial and venous blood. The higher concentration of long-chain fatty acids in the lymph may be also be related to the differential presence and activity of endothelial lipoprotein lipase between lymphatic and blood vessel endothelial cells (9). On the other hand, the possibility cannot be ruled out that differences in long-chain fatty acid turnover, as well as metabolites such as the degradation between lymph and blood, are related to the present finding that long-chain fatty acids are sequestered in the lymph. Further investigation will be needed in the future to clarify these possibilities.

In addition, the white color of the mesenteric lymph may be due to large amounts of albumin and long-chain fatty acids in the lymph. Consistent with this conclusion, the white color of the mesenteric lymph was more intense at 1 h after the administration of distilled water.

Intragastric administration of distilled water increased the transport of ILC-3-secreted IL-22 through mesenteric lymph vessels. The other important aspect of the present study is that there was marked heterogeneity in the distribution of ILC-3 between the jejunum and the ileum. Thus, ILC-3 were localized mainly in the upper part of the jejunum. Consistent with these findings, IL-22 mRNA, one of markers for ILC-3 localization, was also expressed mainly in the lamina propria of the upper part of the jejunum. These results may suggest that ILC-3 is mainly localized to the lamina propria of the jejunum, especially its upper part. These findings may be the first such demonstration in the field of gut immunology, although the role of ILC-3 in the small intestine is known to be greater than that in the large intestine (12).

In addition, intragastric administration of distilled water produced a significant decrease in the total number of ILC-3 in the lamina propria of the jejunum. Consistent with this finding, the total numbers of ILC-3 in mesenteric lymph nodes slightly increased, but no change in the number of ILC-3 was observed in the mesenteric lymph. These findings may suggest that ILC-3 are usually housed in the lamina propria of the jejunum and are not moved upon water intake. However, the great bulk flow produced by the combination of absorbed water and higher permeability of albumin may accelerate the passive movement of ILC-3 from the lamina propria of the jejunum to lacteal canals on jejunal villi. This mechanism may also contribute, in part, to the water intake-mediated increase in the concentration of lymphocytes in the mesenteric lymph. On the other hand, other mechanisms may also be involved, based on the evidence that, when the albumin within efferent lymph vessels of lymph nodes increases, the output of nonselective lymphocytes from lymph nodes is accelerated dose dependently (13). However, the mechanisms of water intake-induced changes in the ratio between T and B cells in the lamina propria of the jejunum, mesenteric lymph nodes, and lymph are not fully understood. Further investigation is needed to clarify the mechanisms.

The present study also demonstrated clearly that intragastric administration of distilled water produced significant increases in the total flux of ILC-3-secreted IL-22 through mesenteric lymph vessels. The dynamics of the total flux of IL-22 through the lymph vessels, induced by the administration of distilled water, was quite similar to those of albumin and long-chain fatty acids through the lymph vessels. Thus, the IL-22 secreted from ILC-3 in the lamina propria of the jejunum, which may usually be combined with transported albumin, was moved to the mesenteric lymph by intragastric administration of distilled water. In conclusion, water intake stimulated the transport of IL-22 to the mesenteric lymph and then to the venous blood through the thoracic duct. This finding may be compatible with the finding that the concentration of IL-22 in the mesenteric lymph was significantly higher than those in the venous and arterial blood. Thus, the water intake-mediated and higher

permeability of albumin-dependent movement of IL-22 from the mesenteric lymph to the blood may contribute to maintaining and promoting the innate immunity in the body. However, further investigation will be needed in the future to elucidate the detailed mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.N., D.M., H.S., T.W.-A., S.T., Y.K., and T.O. conceived and designed research; S.N., K.A., D.M., R.K., M.K., and Y.Y. performed experiments; S.N., K.A., D.M., H.S., R.K., M.H., M.K., and Y.Y. analyzed data; S.N., K.A., D.M., M.H., T.W.-A., S.T., Y.K., and T.O. interpreted results of experiments; S.N., K.A., M.K., Y.Y., and T.O. prepared figures; S.N., K.A., D.M., H.S., R.K., M.H., T.W.-A., M.K., Y.Y., S.T., Y.K., and T.O. approved final version of manuscript; Y.K. and T.O. drafted manuscript; T.O. edited and revised manuscript.

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