TRADITIONAL & KAMPO MEDICINE

ORIGINAL ARTICLE

Fibroblastic reticular cells and type 3 innate lymphoid cells in Peyer's patches: Target cell candidates for inulooligosaccharides in hochuekkito formula containing Atractylodis rhizoma

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ABSTRACT

Aim: Inulooligosaccharides in a Kampo formula, hochuekkito, have been shown to participate in the regulation of the pulmonary immune system. Difference in chain length distribution of the oligosaccharides strongly affects their immunopharmacological activities. This phenomenon cannot be explained by bifidogenic activity and/or short-chain fatty acids produced by intestinal microflora from the oligosaccharides. Effects of intermediate-size inulooligosaccharides, which have a similar chain length distribution as the oligosaccharides obtained from hochuekkito containing Atractylodis rhizoma, were analyzed on immunocompetent cells in Peyer's patches.

Methods: Effect of intermediate-size inulooligosaccharide preparation (degree of polymerization (d.p.) 3–15) on mRNA expression of genes of markers and functional molecules of stromal cells and type 3 innate lymphoid cells (ILC3) in Peyer's patches was analyzed.

Results: Oral administration of intermediate-size inulooligosaccharide preparation significantly up-regulated expression of mRNAs of genes of not only marker molecules of fibroblastic reticular cells (FRC) and ILC3 but also immunological factors for positive and negative regulatory functions of FRC in addition to genes of auto-immune regulator (AIRE), DEAF-1 transcription factor, and some AIRE-dependent tissue-related antigens (TRA).

Conclusion: FRC and ILC3 are suggested to be target cell candidates in Peyer's patches for inulooligosaccharides in hochuekkito formula containing Atractylodis rhizoma.

KEY WORDS: fibroblastic reticular cells, hochuekkito, inulooligosaccharides, Peyer's patches, type 3 innate lymphoid cells

INTRODUCTION

A Kampo formula, hochuekkito containing Atractylodis lanceae rhizoma (ALR-formula), enhances pulmonary immune system to reinforce antigen-specific antibody response in lung to intranasally inoculated antigen [1]. Meanwhile, the formula containing Atractylodis rhizoma (AtR-formula) did not enhance the antibody response in lung [1]. The further addition of Atractylodis rhizoma to ALR-formula was found to suppress the enhancing activity of ALR-formula on the pulmonary immune system [1]. To clarify why these hochuekkito formulas have different effects on the pulmonary immune system, comparative analysis was conducted for responsible active ingredients in these formulas. Inulooligosaccharides in the decoctions of these formulas were found to have different chain length distributions [1]. Poly(I:C)-induced pulmonary inflammation was improved

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by oral administration of the inulooligosaccharide preparation having a similar chain length distribution as the inulooligosaccharides from AtR-formula [1]. However, inflammation was not affected by the inulooligosaccharide preparation having a similar chain length distribution as the oligosaccharides from ALR-formula [1]. The results strongly postulate that the immunomodulatory activity of the inulooligosaccharides differs in accordance with their chain length distributions.

It has been generally considered that inulooligosaccharides express their immunomodulating activities by encouraging growth of bifidobacterium (bifidogenic activity) and producing short-chain fatty acids (SCFA) as metabolites via intestinal microflora [2]. However, it was difficult to explain the difference in immunopharmacological activity among the inulooligosaccharides having different chain length distributions and differences in bifidogenic activity and SCFA production. Degradation of inulooligosaccharides by digestive enzymes is difficult, and intestinal epithelial cells are unable to incorporate oligosaccharides that have degree of polymerization (d.p.) over 5 [3]. From these observations, it was postulated that the inulooligosaccharides having d.p. > 5 might be incorporated into Peyer's patches, and express their characteristic immunomodulating effects through different actions on immunocompetent cells in the patches according to their chain length distribution. Inulooligosaccharide preparations from ALR- and AtR-hochuekkito formulas administered orally have been shown to have different stimulating activities on cytokine production from T lymphocytes in Peyer's patches [1].

In the present study, we sought candidates of target cells in Peyer's patches for intermediate-size inulooligosaccharide preparation, which contain oligosaccharides possessing a similar chain length distribution as the inulooligosaccharides in AtR-hochuekkito formula.

MATERIALS AND METHODS

Animals

Specific pathogen-free female BALB/c mice (young, 7 weeks old; retired, 3–6 months old) were obtained from Japan SLC (Shizuoka, Japan). The mice were kept on a 12 h light/dark cycle at controlled temperature (23 ± 1°C), and they had free access to standard laboratory chow and water. Animal experiments were approved by the Animal Research Committee of Kitasato University, and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at Kitasato University and the Guidelines for Proper Conduct of Animal Experiments from Science Council of Japan.

Inulooligosaccharides

Intermediate-size inulooligosaccharide preparation, which contains oligosaccharides having d.p. 3–15, was prepared from rhizomes of Jerusalem artichoke, which were harvested in Kamaishi City, Iwate Prefecture, Japan, according to a procedure described previously [1]. The chain length distributions of the oligosaccharide preparations were analyzed by high-performance anion-exchange chromatography equipped with pulsed amperometry detector (HPAEC-PAD) according to methods described previously [1] (Fig. S1).

Measurement of *ex vivo* Peyer's patch-immunomodulating activity of inulooligosaccharide preparations

Ex vivo Peyer's patch-immunomodulating activities of inulooligosaccharides were measured according to a modified procedure described previously [1,4] (Fig. S2a). Briefly, young and retired BALB/c mice ($n = 4$) were orally administered water or aqueous solutions of inulooligosaccharide preparations at 50 and 200 mg/kg/day once a day for 10 days. From day 7 to 9, mice were also orally administered an aqueous solution of FTY720 (1.25 mg/kg, Cayman Chemical Co., Michigan, USA) before 3–4 h of oligosaccharide administration to inhibit efflux of lymphocytes from Peyer's patches [1]. About 24 h after the last administration of the oligosaccharides, Peyer's patches were removed from the mice. Peyer's patch cell suspensions (2×10^6 cells/mL,

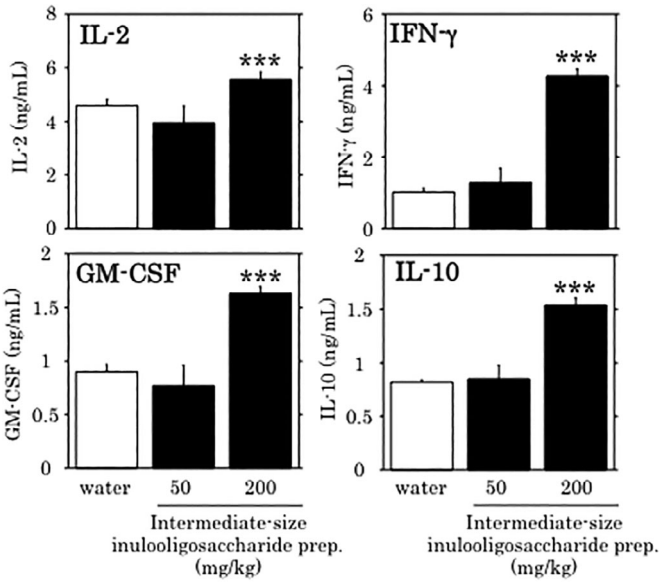

Figure 1 | Effects of oral administrations of intermediate-size inulooligiosaccharide preparation on cytokine production from T lymphocytes in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 50 and 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. The mice were also orally administered FTY720 (1.25 mg/kg) for the last 3 days of oligosaccharide administration. The collected Peyer's patch cells from the mice were cultured for 3 days in the presence of anti-CD3ε antibody, and the concentrations of cytokines in the resulting culture supernatants were analyzed by ELISA. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. ***$P < 0.001$ versus water-administered mice.

500 μL per well) prepared from the treated BALB/c mice were cultured in a 24-well tissue culture plate (FALCON, New York, USA) coated with hamster anti-mouse CD3ε antibody (5 μg per well, BioLegend, San Diego, USA) for 3 days at 37°C. Resulting culture supernatants were kept at −80°C prior to use.

ELISA for cytokines and chemokines

Cincentrations of cytokines in culture supernatants of Peyer's patch cells were measured by enzyme-linked immunosorbent assay (ELISA) kits (DuoSet, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Analyses of mRNA expression

1 Administration and sample preparation: Retired BALB/c mice ($n = 4$) were orally administered water or intermediate-size inulooligosaccharide preparation (200 mg/kg) once a day for 10 days. Peyer's patches were recovered from mice at 6, 24 (after one-time administration), and 72 h (after three-time administration), and at day 7 (after seven-time administration) and day 10 (after 10-time administration) (Fig. S2b), and kept at −80°C prior to use.
2 RNA extraction: RNA from Peyer's patches was extracted by homogenization in Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) with disposable homogenizer (BioMasher II, Nippi Co. Ltd, Tokyo, Japan).
3 Quantitative polymerase chain reaction (PCR): Single-stranded complementary DNA was prepared by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Target messenger RNA (mRNA) expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a housekeeping gene. The primer sequences are listed in Table S1.

Statistical analyses

Data are expressed as mean ± standard error (SE), and statistical comparison between the groups was performed using analysis of variance (ANOVA) supported by Dunnett's post hoc test. $P < 0.05$ was considered to be significant, and $P < 0.1$ was considered to be trending toward significance.

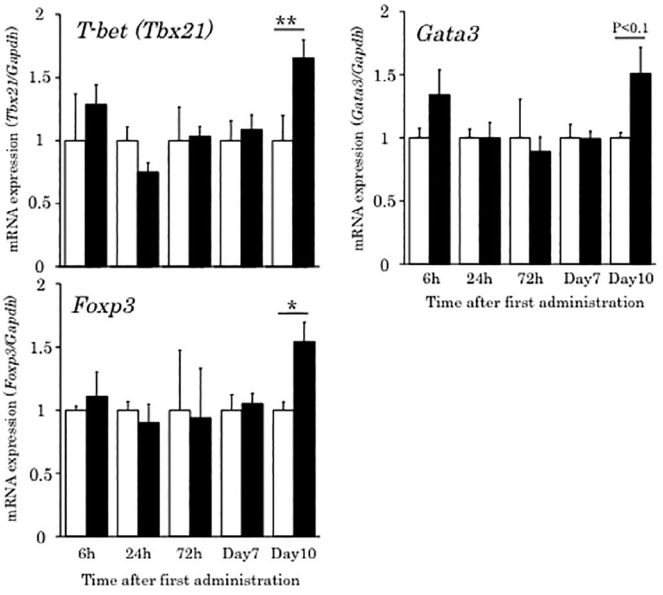

Figure 2 | Effect of oral administration of intermediate-size inulooligosaccharide preparation on mRNA expression of genes of master transcription factors of effector/regulatory T lymphocytes (*T-bet* for Th1, *Gata3* for Th2, *Foxp3* for Treg) in Peyer's patches. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus water-administered mice.

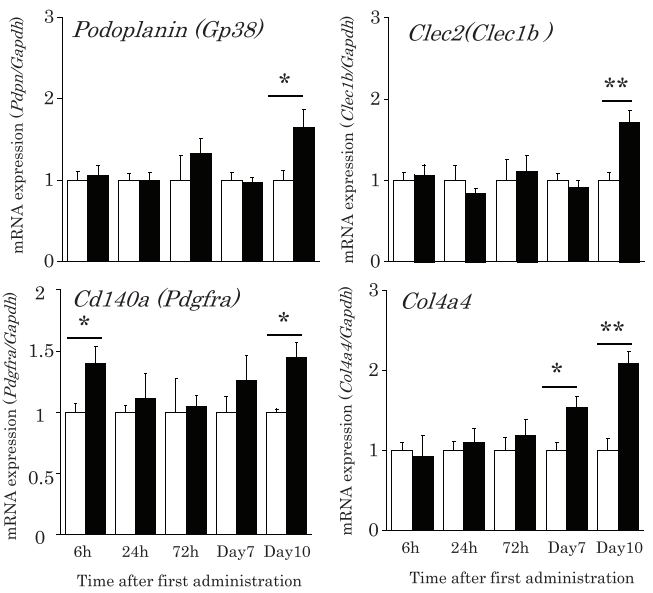

Figure 3 | Effect of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of genes of marker molecules of fibroblastic reticular cells (FRC) (*Gp38*, *Cd140a*, *Col4a4*) and dendritic cells (*Clec1b*) in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus water-administered mice.

RESULTS

Effect of intermediate-size inulooligosaccharide preparation on cytokine production from T lymphocytes in Peyer's patches

In a previous study, inulooligosaccharides obtained from ALR-hochuekkito and AtR-hochuekkito formulas showed a different effect on cytokine production from T lymphocytes in Peyer's patches of retired mice [1]. Therefore, retired BALB/c mice were used in the present study for analysis of target immunocompetent cells of intermediate-size inulooligosaccharides. Intermediate-size inulooligosaccharide preparation (50 and 200 mg/kg/day) or water were each orally administered to retired BALB/c mice for 10 days. All the mice were also orally administered FTY720 prior to administration of the inulooligosaccharide preparation during the last 3 days to prevent efflux of lymphocytes from Peyer's patches (Fig. S2a). Peyer's patch cells were recovered 24 h after the last administration of the inulooligosaccharide preparation, and cultured in the presence of anti-CD3ε antibody to stimulate T lymphocytes for 3 days (Fig. S2a). Concentrations of cytokines were compared in the resulting culture supernatants of Peyer's patch cells. Concentrations of interleukin (IL)-2, interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-10 were increased by administration of the preparation at 200 mg/kg, but not at 50 mg/kg (Fig. 1). To analyze effect of the intermediate-size inulooligosaccharide preparation on induction of subsets of T lymphocytes in Peyer's patches, the preparation was administered for consecutive 10 days at 200 mg/kg, and mRNA expression of genes of master transcription factors for T-lymphocyte subsets in Peyer's patches was analyzed using the patches collected at day 10 as shown in Figure S2b by quantitative PCR. Administration of the intermediate-size inulooligosaccharide preparation significantly increased mRNA expression of genes of the master transcription factors of Th1 (*T-bet*) and regulatory T lymphocytes (*Foxp3*), and those (*GATA3*) for Th2 lymphocytes tended to increase at day 10 (Fig. 2).

These results suggest that intermediate-size inulooligosaccharides are incorporated into Peyer's patches, and induce effector and regulatory T lymphocytes in the patches through unknown action mechanism.

Analysis of action of intermediate-size inulooligosaccharides to stromal cells in Peyer's patches

DNA microarray analysis was performed on cDNA obtained from Peyer's patches of BALB/c mice, which were administered the intermediate-size inulooligosaccharide preparation (200 mg/kg) for 3 days. The analysis indicated 214-fold up-regulation of mRNA expression of a gene (*Col4a4*) of collagen, type IV, alpha 4 (data not shown). This protein is known to be a marker molecule of fibroblastic reticular cells (FRC) as stromal cells in peripheral lymph nodes such as Peyer's patches [5]. Therefore, effect of 10-day oral administration of the

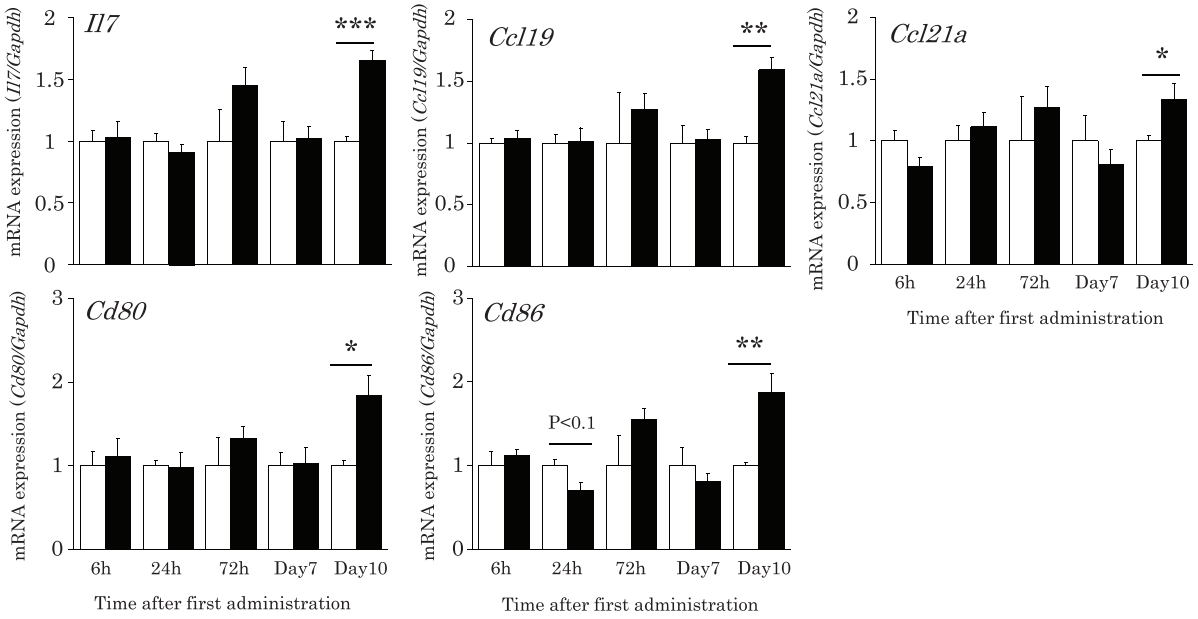

Figure 4 | Effect of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of genes of immunological factors for positive regulatory function of fibroblastic reticular cells (FRC) in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$,***$P < 0.001$ versus water-administered mice.

intermediate-size inulooligosaccharide preparation on mRNA expression of genes (*Gp38*, *Cd140a*, and *Col4a4*) of marker molecules of FRC was analyzed [5,6]. Intermediate-size inulooligosaccharide preparation was administered to retired mice for 10 days, and Peyer's patches were recovered at 6 h, 24 h, 72 h, day 7, and day 10 (Fig. S2b). Expression of all mRNA of genes (*Gp38*, *Cd140a*, and *Col4a4*) of marker molecules of FRC was significantly up-regulated at day 10 (Fig. 3). It has been reported that GP-38 (podoplanin) on FRC interacts with CLEC2 on dendritic cells to modulate differentiation of naïve T lymphocytes [7]. The mRNA expression of a gene (*Clec1b*) of CLEC2 was also up-regulated by administration of the intermediate-size inulooligosaccharide preparation synchronously with up-regulation of mRNA of *Pdpn* (Fig. 3). These results suggest that the intermediate-size inulooligosaccharides enhance proliferation of FRC in Peyer's patches.

FRC have been known to possess positive and negative regulatory functions for T lymphocytes [8]. IL-7, C-C motif chemokine ligand 19/21 (CCL19/21), and cluster of differentiation 80/86 (CD80/86) are reported to be positive regulators of FRC [8]. Meanwhile, negative regulatory factors of FRC are known to include indoleamine-2,3-dioxygenase (IDO), cyclooxygenase 2 (COX2), inducible nitric oxide synthase 2 (NOS2), and programmed cell death 1 ligand 1 (PD-L1) as co-inhibitory molecules [8]. mRNA expression analysis of genes of these positive and negative regulatory factors revealed that the mRNA expression of all the genes except *Cox2* was significantly up-regulated by administration of the intermediate-size inulooligosaccharide preparation at day 10 (Figs 4,5). Therefore, intermediate-size inulooligosaccharides are strongly proposed to potentiate both positive and negative regulatory functions to T lymphocytes of FRC, and it is postulated that this potentiation may lead to inductions of Th1, Th2, and regulatory T lymphocytes in the patches as shown in Figures 1,2.

It also has been reported that FRC in peripheral lymph nodes expresses a master transcription factor, deformed epidermal autoregulatory factor 1 (DEAF-1), and that DEAF-1 controls induction of tissue-restricted antigens (TRAs) in FRC [8]. TRAs are derived into antigenic oligopeptides (TRA-oligopeptides) in FRC, and the oligopeptides are mounted on major histocompatibility complex (MHC) class I of FRC [8]. Auto-reactive T lymphocytes, which are recruited into Peyer's

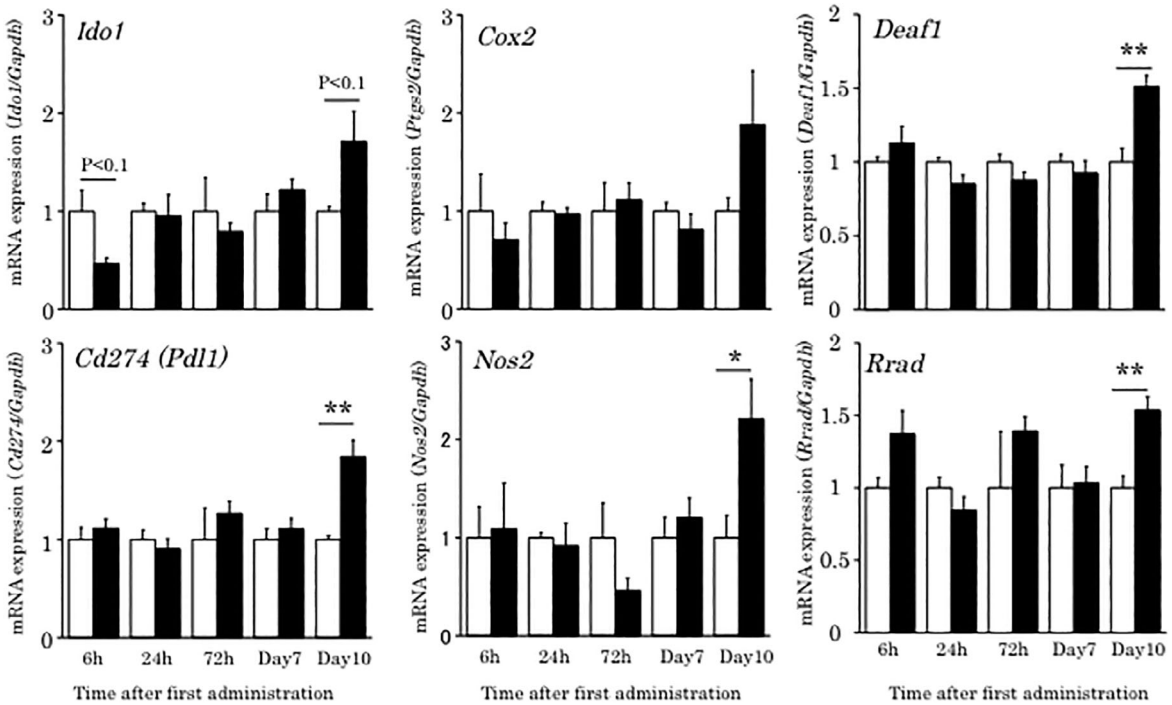

Figure 5 | Effect of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of genes of immunological factors for negative regulatory function and of DEAF-1 transcription factor (*Deaf1*) and Ras-related associated with diabetes (*Rrad*) as DEAF-1-dependent tissue specific antigen (TRA) of fibroblastic reticular cells (FRC) in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus water-administered mice.

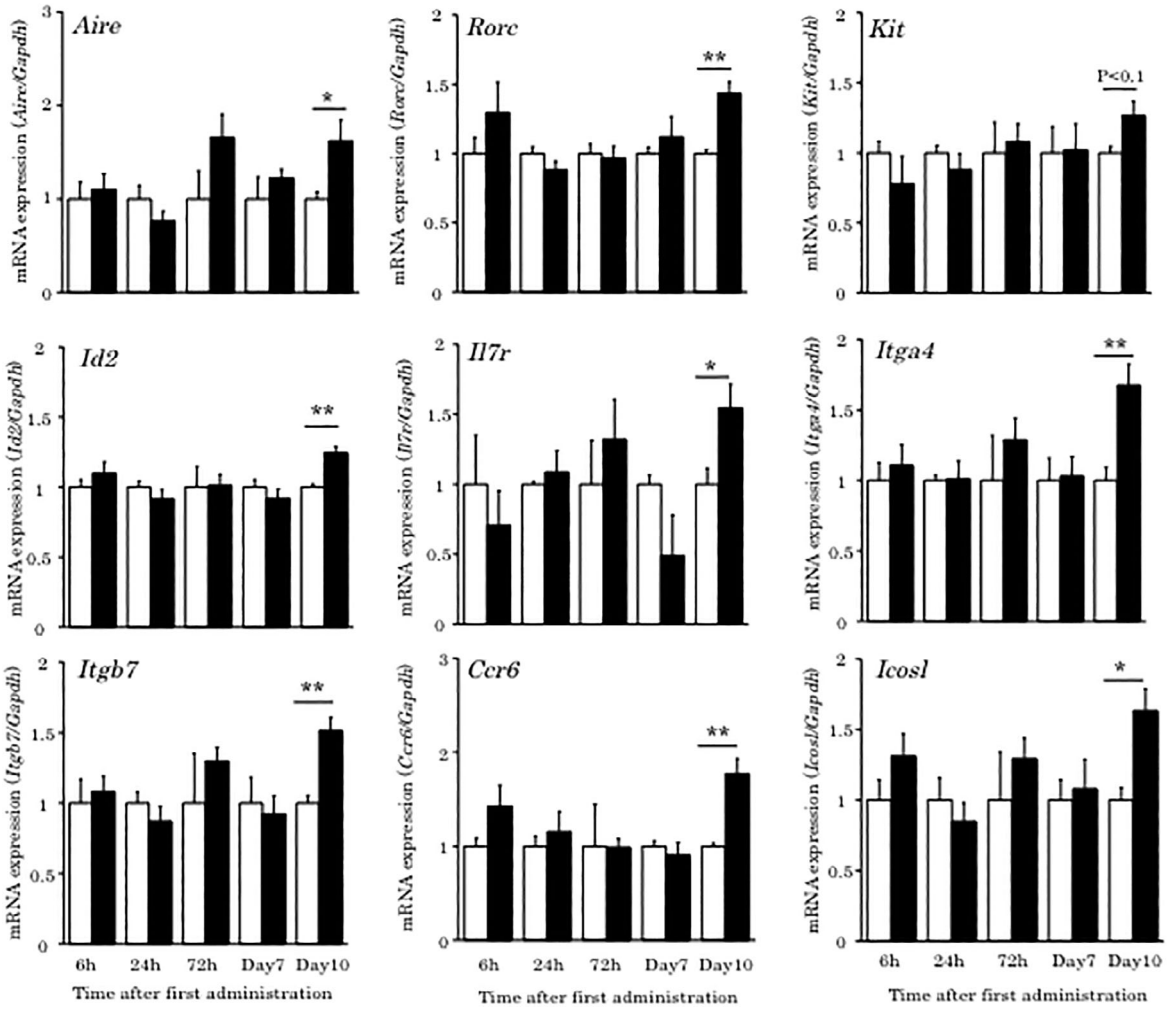

Figure 6 | Effect of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of genes of auto-immune regulator (*Aire*) and of marker molecules of type 3 innate lymphoid cells (ILC3) (*Rorc*, *Kit*, *Id2*, *Il7r*, *Itga4*, *Itgb7*, *Ccr6*, *Icosl*) in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus water-administered mice.

patches from peripheral blood stream, are induced apoptosis by interaction with TRA oligopeptide-mounted MHC on FRC [8]. Effect of oral administration of the intermediate-size inulooligosaccharide preparation on mRNA expression of DEAF-1 gene (*Deaf1*) in Peyer's patches of retired mice was analyzed. As shown in Figure 5, mRNA expression of *Deaf1* in Peyer's patches was significantly up-regulated at day 10 by administration of the inulooligosaccharide preparation. mRNA expression of a gene of Ras-related associated with diabetes (*Rrad*) as one of DEAF-1-dependent TRAs [8] increased synchronously with up-regulation of mRNA of *Deaf1* (Fig. 5). These results suggest that the intermediate-size inulooligosaccharides also lead to potentiation of DEAF-1-dependent deletion of auto-reactive T lymphocytes in Peyer's patches.

Analysis of effect of intermediate-size inulooligosaccharides in type 3 innate lymphoid cells (ILC3)

It is well known that a transcription factor, auto-immune regulator (AIRE), also plays a similar key role as DEAF-1 for removal of auto-reactive T lymphocytes through induction of TRAs in peripheral lymph nodes such as Peyer's patches [9]. Because expression of DEAF-1 in Peyer's patches was suggested be up-regulated by the intermediate-size inulooligosaccharides in the present study, it was expected that the oligosaccharides also

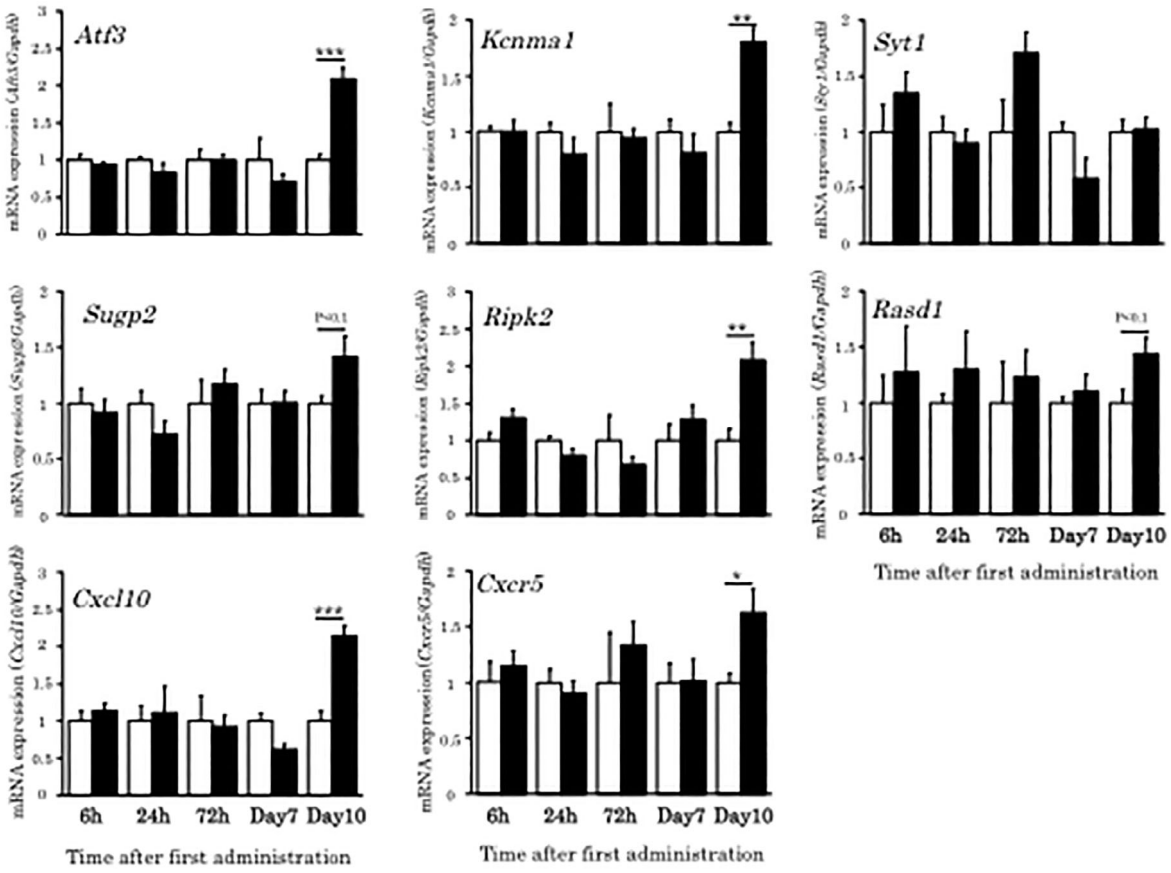

Figure 7 | Effect of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of genes of activating transcription factor 3 (*Atf3*), potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (*Kcnma1*), synaptotagmin (*Syt1*), SURP and G patch domain containing 2 (*Sugp2*), receptor (TNFRSF)-interacting serine–threonine kinase 2 (*Ripk2*), RAS, dexamethasone-induced 1 (*Rasd1*), chemokine (C–X–C motif) ligand 10 (*Cxcl10*) and chemokine (C–X–C motif) receptor 5 (*Cxcr5*) as AIRE-dependent tissue-specific antigens (TRA) in Peyer's patches of retired BALB/c mice. Retired BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar, oligosaccharide-administered mice. Data are given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$, **$P < 0.01$ versus water-administered mice.

affect AIRE expression in the patches. As shown in Figure 6, expression of mRNA of the gene of AIRE (*Aire*) was significantly enhanced at day 10 in Peyer's patches of retired mice by oral administration of the intermediate-size inulooligosaccharide preparation. Type 3 innate lymphoid cells (ILC3)-like cells have been identified as one of AIRE-expressing cells in lymph nodes [10]. Marker molecules of ILC3-like cells have been clarified in lymph nodes and Peyer's patches [10]. Effect of oral administration of the intermediate-size inulooligosaccharide preparation on mRNA expression of genes of the marker molecules of ILC3-like cells was analyzed. mRNA of genes of the marker molecules of ILC3-like cells (*Rorc*, *Kit*, *Id2*, *Il7r*, *Itga4*, *Itgb7*, *Ccr6*, *Icosl*) were found to up-regulate synchronously with increment of *Aire* expression by the inulooligosaccharide preparation (Fig. 6). Forty-four kinds of TRAs, expressions of which are controlled by AIRE, have been identified in lymph node cells such as ILC3 [10]. Among eight kinds of AIRE-dependent TRAs tested in the present study, oral administration of the intermediate-size inulooligosaccharide preparation increased mRNA expression of seven TRA genes (activating transcription factor 3 (*Atf3*), potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (*Kcnma1*), SURP and G patch domain containing 2 (*Sugp2*), receptor (TNFRSF)-interacting serine–threonine kinase 2 (*Ripk2*), RAS, dexamethasone-induced 1 (*Rasd1*), chemokine (C–X–C motif) ligand 10 (*Cxcl10*) and chemokine (C–X–C motif) receptor 5 (*Cxcr5*)) synchronously with up-regulation of *Aire* expression in Peyer's patches at day 10 except synaptotagmin I (*Syt1*) (Fig. 7).

From these results, we propose that the intermediate-size inulooligosaccharides also affect ILC3-like cells in addition

to FRC, and the oligosaccharides enhance expression of AIRE in ILC3-like cells to stimulate TRA induction for deletion of TRAs-related auto-reactive T lymphocytes.

DISCUSSION

It generally has been proposed that inulooligosaccharides (inulin) express a variety of immunopharmacological activities through bifidogenic action and production of short-chain fatty acids (SCFAs) as metabolites via microbiota [2]. These activities have been observed in previous studies by ingestion of high dose of inulin preparation (1–5 g/kg/day, about 25–125 mg per mouse per day), and sufficient production of SCFAs was to be expected by these high doses [11,12]. Decocted extracts of some Kampo formulas such as hochuekkito formula containing Atractylodis rhizoma or Atractylodis lanceae rhizoma contain intermediate-size and long inulooligosaccharides [1]. However, the daily dose (about 30–50 mg/kg) of the inulooligosaccharides in the formulas was not so high compared with the doses in previous inulin studies [1]. However, these inulooligosaccharides in hochuekkito formula have been found to play a key role in the regulation of the pulmonary immune system [1]. Furthermore, the present study strongly suggests that oral administration of intermediate-size inulooligosaccharides increases the number of ILC3-like cells in Peyer's patches. Meanwhile, it has been reported that oral administration of butyric acid as one of the major SCFAs derived from inulin leads to a decrease in the number of ILC3 in ileal Peyer's patches [13]. Therefore, these findings postulate that the lower dose (4–5 mg per mouse per day) of inulooligosaccharides may induce their immunopharmacological effects through mechanisms other than bifidogenic activity and the produced SCFAs.

The present study strongly suggests that intermediate-size inulooligosaccharides affect both FRC and ILC3-lile cells in Peyer's patches. Peyer's patches are composed of T-cell zone and B-cell follicle, and FRC are known to locate in T-lymphocyte zone to interact with T lymphocytes [14]. Type 3 innate lymphoid-like cells (ILC3-like cells) have been observed in peripheral lymph nodes such as Peyer's patches, and the ILC3-like cells locate in inter-follicular regions surrounded by B-cell follicles in lymph nodes; it has been proposed that ILC3-like cells influence T–B lymphocyte interaction [14].

The present study strongly suggests that the intermediate-size inulooligosaccharides enhance proliferation of FRC. It has been reported that FRC forms a structural network (so called conduit structure) in T-lymphocyte-rich zone of lymph nodes, and the conduit structure of FRC provides a guidance path for migration of T lymphocytes in the patches [15]. FRC in conduit structure has been known to differentiate naive T lymphocytes to effector and regulatory T lymphocytes through production and expression of several immunological factors and co-stimulatory/inhibitory molecules as their positive and negative regulatory functions [8,16]. From these, it was postulated that intermediate-size inulooligosaccharides accelerate proliferation of FRC and stimulate construction of conduit structure, then enhance expression of positive/negative regulatory factors of FRC during 10-day administration of the oligosaccharides (Fig. S3). Finally, naïve T lymphocytes are thought to accelerate differentiation into IL-2-, IFN-γ-, and GM-CSF-producing effector T lymphocytes and IL-10-producing regulatory T lymphocytes in Peyer's patches by interaction with FRC in conduit structure.

The present study suggests that intermediate-size inulooligosaccharides also enhanced expression of transcription factor *Aire* in ILC3-like cells and DEAF-1 in FRC of Peyer's patches. It has been well known that AIRE is a key transcription factor for expression of TRAs in thymus and peripheral lymph nodes for removal of auto-reactive T lymphocytes, which are newly generated from bone marrow and recruited in peripheral blood stream [9]. The present study showed up-regulation of mRNA expression of genes of seven TRAs synchronously with up-regulation of *Aire* mRNA in Peyer's patches by administration of the intermediate-size inulooligosaccharides. These observations indicate that deletions of auto-reactive T lymphocytes are accelerated in Peyer's patches by the intermediate-size inulooligosaccharides. Retired mice were used in the present study, and a similar up-regulation of *Aire* mRNA was also found in Peyer's patches of young (8-week-old) mice by administration of the inulooligosaccharides (Fig. S4). Enhancement of *Aire* expression in young mice was observed at 6 h after single administration of the oligosaccharides in addition to at day 10, and this early up-regulation at 6 h was not observed in retired mice (Fig. S4). It is known that innate lymphoid cells (ILCs) originate from fetal liver, and are recruited to peripheral tissues such as lymphoid organs [17]. ILCs expand in peripheral tissues by self-renewal system until adulthood, and the numbers of ILCs in lymphoid tissues decrease with aging [16]. Therefore, Peyer's patches in young mice, but not in retired mice, are thought to contain a notable number of ILC3. Meanwhile, some ILCs in peripheral tissues are supplied from bone marrow until late adulthood [16]. It is also expected that ILC3 is supplied into Peyer's patches in retired mice. Different effect of the intermediate-size inulooligosaccharides on *Aire* expression in ILC3 between young and retired mice may be explained by difference in the amount of ILC3 in both mice. Further detailed study is required to elucidate the effect of the intermediate-size inulooligosaccharides on conduit structure of FRC and ILC3 by immunohistochemical and cytometrical analyses.

CONFLICT OF INTEREST

The authors declare no conflicts of interest for this article.

REFERENCES

1. Idehara M, Tanaka Y, Hosaka H *et al.* Inulooligosaccharides in Atractylodis lanceae rhizoma and Atractylodis rhizoma of hochuekkito formula are essential for regulation of the pulmonary immune

system of immuno-compromised mice. *Tradit. Kampo Med.* 2020; **7**: 96–107.
2. Delzenne NM. Oligosaccharides: State of the art. *Proc. Nutr. Soc.* 2003; **62**: 177–182.
3. Eiwegger T, Stahl B, Paul Schmitt HJ *et al*. Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. *Pediatr. Allergy Immunol.* 2010; **21**: 1179–1188. https://doi.org/10.1111/j.1399-3038.2010.01062.x.
4. Sakai Y, Sato M, Funami Y *et al*. Peyer's patch-immunomodulating glucans from sugar cane enhance protective immunity through stimulation of the hemopoietic system. *Int. J. Biol. Macromol.* 2019; **124**: 505–514.
5. Malhotra D, Fletcher A, Turley S. Stromal and hematopoietic cells in secondary lymphoid organs: Partners in immunity. *Immunol. Rev.* 2013; **251**: 160–176.
6. Alvarenga H, Marti L. Multifunctional roles of reticular fibroblastic cells: More than meets the eye? *J. Immunol. Res.* 2014; **2014**: 402038. https://doi.org/10.1155/2014/402038.
7. Fletcher A, Acton S, Knoblich K. Lymph node fibroblastic reticular cells in health and disease. *Nat. Rev. Immunol.* 2015; **15**: 350–361.
8. Siegert S, Luther S. Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes. *Front. Immunol.* 2012; **285**: 1–10.
9. Zhang R, Turka LA. A breath of fresh Aire. *Immunity* 2013; **39**: 427–429.
10. Yamano T, Dobeš J, Vobořil M *et al*. Aire-expressing ILC3-like cells in the lymph node display potent APC features. *J. Exp. Med.* 2019; **216**: 1027–1037.
11. Zhu L, Qin S, Zhai S, Gao Y, Li L. Inulin with different degrees of polymerization modulates composition of intestinal microbiota in mice. *FEMS Microbiol. Lett.* 2017; **364**: 1–7.
12. Li LL, Wang YT, Zhu LM, Liu ZY, Ye CQ, Qin S. Inulin with different degrees of polymerization protects against diet-induced endotoxemia and inflammation in association with gut microbiota regulation in mice. *Sci. Rep.* 2020; **10**: 978. https://doi.org/10.1038/s41598-020-58048-w.
13. Kim S, Cho B, Kiyono H, Jang Y. Microbiota-derived butyrate suppresses group 3 innate lymphoid cells in terminal ileal Peyer's patches. *Nat. Sci. Rep.* 2017; **7**: 3980. https://doi.org/10.1038/s41598-017-02729-6.
14. Sonnenberg GF, Hepworth MR. Functional interactions between innate lymphoid cells and adaptive immunity. *Nat. Rev. Immunol.* 2019; **19**: 599–613.
15. Perez-Shibayama C, Gil-Cruz C, Ludewig B. Fibroblastic reticular cells at the nexus of innate and adaptive immune responses. *Immunol. Rev.* 2019; **289**: 31–41.
16. Brown FD, Turley SJ. Fibroblastic reticular cells: Organization and regulation of the T lymphocyte life cycle. *J. Immunol.* 2015; **194**: 1389–1394.
17. Maya E, Locksley R. Why innate lymphoid cells? *Immunity* 2018; **48**: 1081–1090.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1 Elution patterns of inulooligosaccharide fractions from decoctions of ALR-hochuekkito formula and AtR-hochuekkito formula, and of intermediate-size inulooligosaccharide preparation on high-performance anion-exchange chromatography.
Figure S2 Experimental schedule of administration of intermediate-size inulooligosaccharide preparation for analyses of (a) effect of the oligosaccharide administration on cytokine production from T lymphocytes in Peyer's patches, and (b) effect of the oligosaccharide administration on mRNA expression of genes of marker transcription factors for T-lymphocyte subsets and of molecules related to marker and functional molecules of fibroblastic reticular cells (FRC) in Peyer's patches of retired mice.
Figure S3 Hypothetical diagram of action of intermediate-size inulooligosaccharides on fibroblastic reticular cells (FRC) in Peyer's patches during 10-day administration of the inulooligosaccharides. Proliferation of FRC is accelerated by the action of oligosaccharides, and construction of FRC network structure (conduit structure) is potentiated during 10-day administration. FRC in conduit structure is retiredd by the action of oligosaccharides, and production and expression of immunological factors and co-stimulatory/inhibitory molecules are enhanced. Effector/regulatory T lymphocytes are induced by interaction of naïve T lymphocytes with FRC in the conduit structure. Furthermore, expression of transcription factor DEAF-1 is also enhanced in FRC of conduit structure during 10-day administration of the oligosaccharide to induce tissue-restricted antigens (TRA), and auto-reactive T lymphocytes are deleted by presentation of TRA.
Figure S4 Effects of oral administration of intermediate-size inulooligiosaccharide preparation on mRNA expression of a gene of auto-immune regulator (*Aire*) in Peyer's patches of young and retired BALB/c mice. Retired and young BALB/c mice were orally administered 200 mg/kg of intermediate-size inulooligosaccharide preparation for 10 days. Peyer's patches were collected from the mice at the timing indicated in Figure S2, and total RNA was prepared from the patches. mRNA expression was analyzed by quantitative PCR. White bar, water-administered mice; black bar: oligosaccharide-administered mice. Data were given as mean ± SE ($n = 4$), and differences between groups were analyzed via Dunnett's test. *$P < 0.05$ versus water-administered mice.
Table S1 Primers for real-time PCR.
Appendix S1 Supporting information