responses (5), the immunologic function of dietary PA in the control of IgA production remained to be investigated. In this study, we show that PA-enriched diets enhance intestinal IgA responses both directly and through their metabolic pathways.

Materials and Methods

Mice

Female BALB/c, C3H/HeJ, and C3H/HeN mice were purchased from CLEA Japan (Tokyo, Japan). Chemically defined AIN-93M–based diets containing soybean, palm, or coconut oil or soybean oil plus supplemental purified PA were from Oriental Yeast (Tokyo, Japan). All mice were provided with a sterile diet and water ad libitum. To inhibit serine palmitoyltransferase (SPT) activity, mice were treated with myriocin (1.0 mg/kg i.p. daily; Sigma-Aldrich, St. Louis, MO) for 4 d (21). All mice were maintained in the experimental animal facility at the University of Tokyo and National Institute of Biomedical Innovation; the experiments were approved by the Animal Care and Use Committee of each institute and conducted in accordance with their guidelines.

Cell isolation

To isolate mononuclear cells from PPs, we stirred intestinal tissues in RPMI 1640 medium containing 2% FCS and 0.5 mg/ml collagenase (Wako, Osaka, Japan). Cells were isolated from the intestinal lamina propria (iLP) as previously described (9, 22). Briefly, PPs were removed, and small and large intestines were cut into 2-cm pieces, which were stirred in RPMI 1640 containing 1 mM EDTA and 2% FCS. Then the tissues were stirred in collagenase for 15 min three times (small intestine, 0.8 mg/ml; large intestine, 1.6 mg/ml) before undergoing discontinuous Percoll gradient centrifugation. Lymphocytes were isolated at the interface between the 40 and 75% layers.

Oral immunization

Mice received sodium bicarbonate to neutralize gastric acid before oral immunization (9, 22). Thirty minutes later, the mice were immunized orally concurrently with 1 mg OVA (Sigma-Aldrich) and 10 μg cholera toxin (CT; List Biological Laboratories, Campbell, CA). This oral-immunization procedure was conducted on days 0, 7, and 14.

Detection of total Ig by ELISA and OVA-specific Ab responses by ELISPOT assay

Total Ig levels in serum and fecal extracts were determined by ELISA, as previously described (9, 22). To measure Ab concentration, purified murine isotype-specific Abs (BD Biosciences, San Jose, CA) were used as standards for quantification. For the detection of OVA-specific Abs and Ab-forming cells, fecal extracts and iLP were prepared 7 d after the final immunization. Standard OVA-specific ELISAs and ELISPOT assays were performed as previously described (9, 22).

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described (9, 22). Cells were preincubated with anti-CD16/32 Ab and then stained with

fluorescent Abs specific for B220, IgG1, IgA (all from BD Biosciences), CD19, and CD138 (BioLegend, San Diego, CA). Forward scatter–height and forward scatter–area discrimination was used to exclude doublet cells, and Via-Probe Cell Viability Solution (BD Biosciences) was used to discriminate between dead and living cells. For the BrdU-uptake assay, mice received 1 mg BrdU i.p., and the BrdU signal was detected according to the manufacturer's protocol (BD Biosciences) (9). Concentration-matched isotype Abs were used as negative controls. Flow cytometric analysis and cell sorting were carried out using FACSCanto II and FACSAria (BD Biosciences), respectively. We confirmed that cell purity was ~95%.

In vitro culture of PCs with PA

Stock solutions containing 5 mM PA and 10% BSA were prepared as previously reported (23). Purified IgA$^+$ B220$^-$ PCs isolated from the iLP (10^4 cells/well) or CD19$^+$ CD138$^+$ PCs isolated from the spleen (10^5 cells/well) were cultured with 10 or 100 μM PA for 96 h. The amount of IgA (for iLP) or IgG (for spleen) in the culture supernatant was determined by ELISA, as described earlier.

Measurement of PA in the serum and intestinal tissues

Lipids were extracted from serum and intestinal tissues by using chloroform–methanol and chloroform solutions. The specimens were dried in nitrogen gas and dissolved in 0.4 M potassium methoxide in methanol and 14% boron trifluoride in methanol. The FA concentrations in the solutions were measured (SRL, Tokyo, Japan) using gas chromatography (model GF 17A; Shimazu, Kyoto, Japan).

Statistics

Experimental groups were compared using the Mann–Whitney U test (GraphPad, San Diego, CA).

Results

Mice maintained on a diet containing palm oil show enhanced intestinal IgA production

To examine whether dietary PA affects intestinal IgA production, we maintained mice on a diet containing 4% soybean (control) or palm (PA-rich) oil for 2 mo (Fig. 1A) and measured the amounts of fecal IgA. Intestinal IgA production was higher in the mice that received palm oil than in those given soybean oil (Fig. 1B). In contrast, the amounts of serum IgG and IgA were similar between the soybean and palm oil groups (Fig. 1B, Supplemental Fig. 1). Palm oil is high in both PA and oleic acid (Fig. 1A), but mice maintained on a diet containing rapeseed oil, which contained a similar amount of oleic acid as that present in the palm oil, showed no enhancement of intestinal IgA production (data not shown).

We then considered whether other saturated FAs enhance intestinal IgA production. To this end, we used coconut oil, which (like palm oil) is a Palmae plant–based oil but contains large

FIGURE 1. Palm oil enhances intestinal IgA production. (**A**) FA composition of soybean, palm, and coconut oils. (**B**) Mice were maintained on a diet containing soybean, palm, or coconut oil for 2 mo. Feces and serum samples were collected for measurement of the amounts of IgA and IgG, respectively (mean ± 1 SD, n = 10/group).
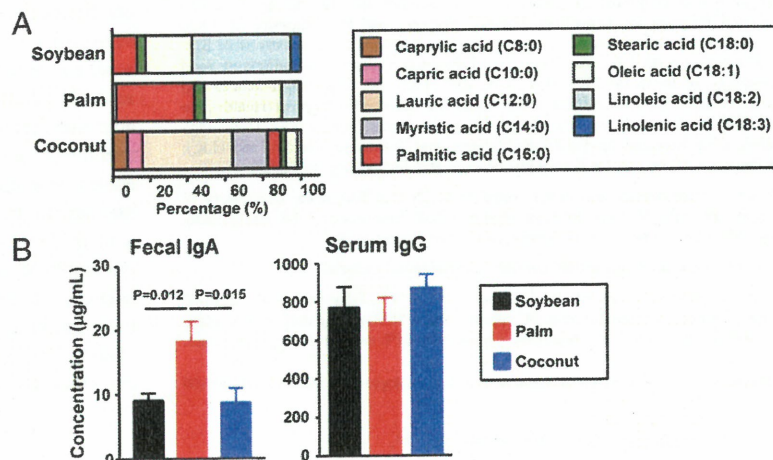
amounts of other saturated FAs, including lauric and myristic acids (Fig. 1A). Unlike palm oil, coconut oil did not alter fecal or serum Ab production in the mice (Fig. 1B). These findings suggest that dietary PA uniquely enhances intestinal IgA production.

PA-enriched dietary oils enhance intestinal IgA production and IgA responses against oral vaccine Ag

To confirm that PA enhances intestinal IgA production, we added PA to soybean oil to adjust its PA concentration to that of palm oil (Fig. 2A, *upper bar*). Fecal IgA levels in mice maintained for 2 mo on a diet containing the PA-enriched soybean oil were increased similarly to those of mice fed a palm oil–containing diet, but serum IgA production was unchanged (Fig. 2A, *lower bar*, Supplemental Fig. 1). These data suggest that PA is a key FA in the enhancement of intestinal IgA production.

We then investigated whether the enhanced production of intestinal IgA induced by dietary PA is reflected in the responses to an orally administered vaccine. To this end, we orally immunized mice concurrently with OVA and the mucosal adjuvant CT. In agreement with levels of naturally produced IgA, OVA-specific fecal IgA responses were enhanced in mice maintained on a diet that contained palm oil (Fig. 3). In addition, similarly increased OVA-specific IgA production was noted in mice maintained on PA-enriched soybean oil (Fig. 3). These findings suggest that dietary PA enhanced not only naturally produced intestinal IgA but also Ag-specific intestinal IgA induced by oral immunization.

PA content is increased in the intestinal, but not systemic, compartment

We next measured the amounts of PA in the intestines and serum of the mice. PA concentrations in the small and large intestines were higher in the mice maintained on PA-rich soybean oil compared with soybean oil alone (Fig. 4). In contrast, PA amounts in serum were comparable between groups (Fig. 4). These findings indicate that dietary PA affects the amount of PA locally in the intestine without any influence on serum PA concentration.
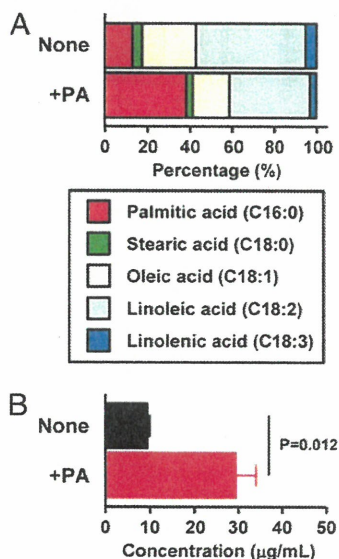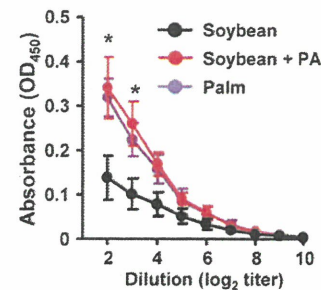

FIGURE 3. Ingestion of PA-enriched oil enhances intestinal IgA responses against orally immunized Ag. After mice were maintained for 2 mo on a diet containing soybean oil, soybean oil + PA, or palm oil, they were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, fecal extracts were collected for the determination of OVA-specific intestinal IgA by ELISA. Data are mean $\pm$ 1 SD (n = 4/group), and similar results were obtained from two independent experiments. *$p < 0.05$.

PA directly stimulates intestinal PCs to produce IgA

We then examined whether PA directly affected IgA production from PCs. To address this issue, we purified IgA[+] PCs from the intestine and cultured them with PA for 4 d. The amount of IgA in the intestinal PC culture supernatants increased in a dose-dependent manner (Fig. 5A). ELISPOT assays showed that PA did not increase the number of IgA-forming cells (Fig. 5B), suggesting that PA instead enhances Ab production from PCs. In addition, IgG production from CD19^{+} CD138^{+} PCs from the spleen was increased in the presence of PA (Supplemental Fig. 2), indicating that PA enhances Ab production, regardless of the Ig subtype.

PA acts as a ligand for TLR4 (24), prompting our investigation into whether TLR4 mediated the PA-induced direct activation of IgA PCs. To address this issue, we used C3H/HeN and C3H/HeJ mice. Although TLR4-mediated signaling differs between these two strains because of a spontaneous point mutation in the *Tlr4* gene of the C3H/HeJ mice (25, 26), intestinal PCs from these mouse strains produced identical levels of PA-induced IgA (Fig. 5C). Therefore, the direct effect of PA on IgA production from PCs likely is independent of the TLR4 pathway.

The PA-induced increase in IgA PCs in the large intestine is dependent on SPT

We used flow cytometry to determine the frequency of IgA[+] PCs in the small and large intestines. Although no significant difference between the control and PA-enriched diets was noted in the small intestine, the frequency of IgA[+] B220[−] PCs was increased in the

FIGURE 2. Dietary PA alone is sufficient to enhance intestinal IgA production. (**A**) Purified PA was added to soybean oil to achieve the same PA content as that in palm oil. (**B**) Mice were maintained for 2 mo on a diet containing soybean oil, with or without supplemental PA. The IgA levels of fecal extracts were measured (mean $\pm$ 1 SD, n = 16/group).
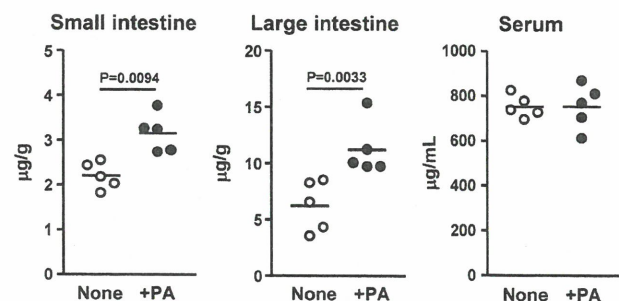

FIGURE 4. Increased PA content in the intestine of mice maintained on PA-enriched oils. Mice were maintained for 2 mo on a diet containing soybean oil, with or without PA, and small and large intestinal tissues and serum were collected for measurement of their PA contents. The graphs show data from individual mice, and the horizontal lines indicate the means; similar results were obtained from two independent experiments.

FIGURE 5. PA directly stimulates IgA-producing PCs via a TLR4-independent mechanism. IgA$^+$ B220$^-$ PCs were isolated from the small intestine of BALB/c (**A** and **B**) and C3H/HeN or C3H/HeJ mice (**C**) and cultured with 10 or 100 μM (A) or 100 μM (B and C) PA. The amount of IgA in the culture supernatant (A and C) and the number of IgA$^+$ Ab–forming cells (IgA$^+$-AFCs) (B) were determined by ELISA and ELISPOT, respectively. The graphs show data from individual samples, and the horizontal lines indicate the means; similar results were obtaine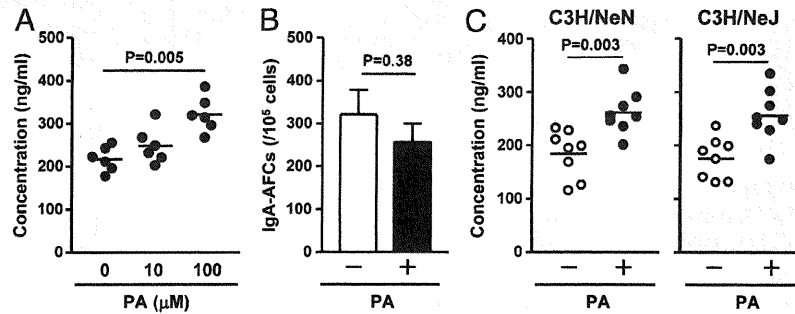d from two independent experiments (A and C). Data in (B) are mean ± 1 SD (n = 10/group from two separate experiments).

large intestine of the PA-enriched diet group (Fig. 6A). Similarly, the number of OVA-specific IgA-forming cells was increased in the large, but not small, intestine of mice receiving oral immunization and the PA-enriched diet (Fig. 6B). In agreement with the lack of a PA-associated effect on small intestinal IgA, B cell differentiation into IgA$^+$ cells in the PPs, a lymphoid tissue for the initiation of small intestinal IgA responses (3), was unchanged in mice maintained on a PA-enriched diet (Supplemental Fig. 3).

PA can be converted into sphingolipids, including ceramide, sphingosine, and sphingosine 1 phosphate (S1P) (Fig. 6C); all of these

lipids all known to promote cell proliferation, survival, and trafficking (27, 28). Therefore, we supposed that these PA-derived sphingolipids might be involved in the regulation of intestinal IgA PCs in the large intestine. To test this possibility, mice were treated with myriocin, an inhibitor of SPT, which is a key enzyme in the conversion of PA into sphingolipids (Fig. 6C). The PA-mediated increase in IgA PCs did not occur in the large intestine of mice that received myriocin (Fig. 6D), and myriocin had little effect on the number of IgG1$^+$ cells in the spleen (Supplemental Fig. 4), suggesting that SPT activity is required for this effect on large intestinal IgA PCs.
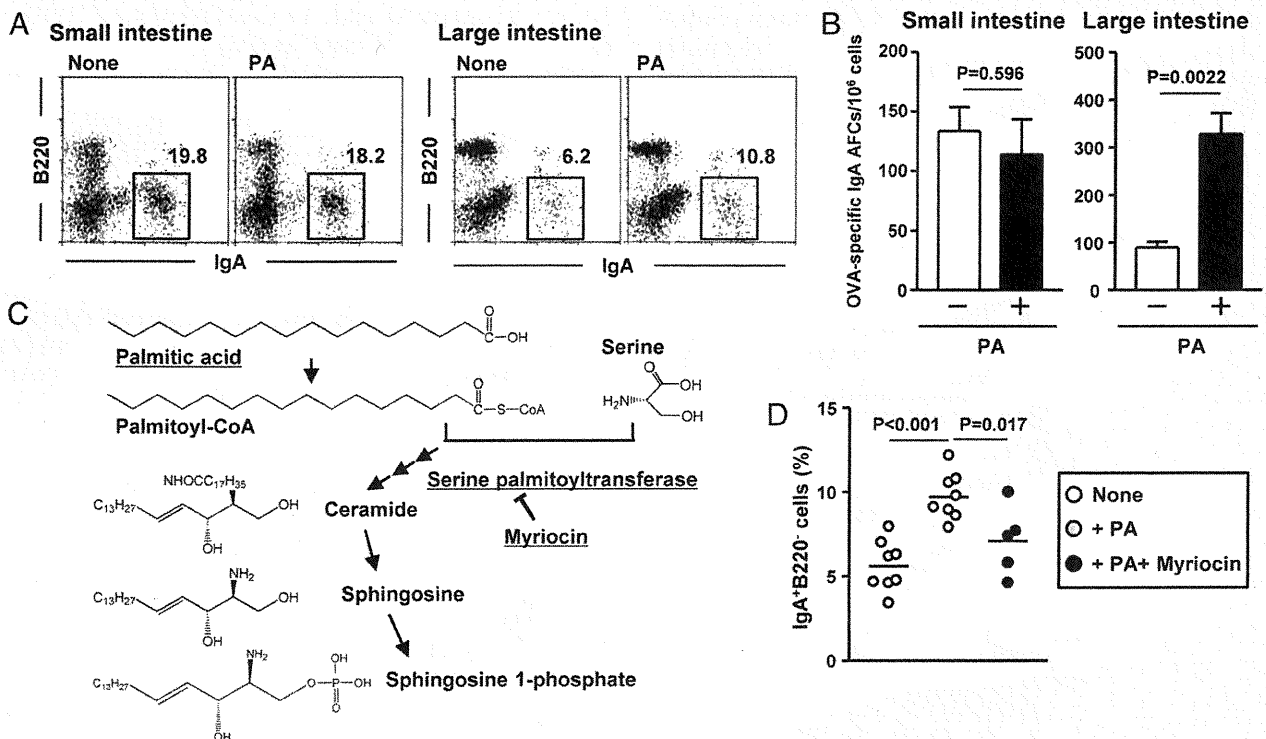

FIGURE 6. SPT is required for the increased number of IgA PCs in the large intestine. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo. (**A**) Mononuclear cells were isolated from the small and large intestines for the flow cytometric analysis of IgA$^+$ B220$^-$ PCs. Data are representative of three independent experiments. (**B**) Mice were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, mononuclear cells were isolated from the small and large intestine for the detection of OVA-specific IgA Ab–forming cells by ELISPOT assay. Data are mean ± 1 SD (n = 6/group from two separate experiments). (**C**) The metabolic pathway mediated by SPT to generate sphingolipids from PA. (**D**) The frequency of IgA$^+$ B220$^-$ PCs in the large intestine of mice maintained on a PA-enriched diet and myriocin. The graphs show data from individual samples, and the horizontal lines indicate the means. Similar results were obtained from two independent experiments.

The increased proliferation of IgA PCs in the large intestine of mice maintained with a PA-enriched diet

We performed BrdU-uptake assays to examine the effects of PA on the proliferation and survival of IgA PCs in the large intestine. Soon after BrdU administration (day 1), the proportions of $BrdU^+$ and $BrdU^-$ IgA^+ cells in the large intestine were higher when mice were maintained on a PA-enriched diet than when they were maintained on the control diet, but the increase ratio of IgA^+ cells was higher in $BrdU^+$ IgA^+ cells than in $BrdU^-$ IgA^+ cells (Fig. 7A). These findings suggest that PA metabolites induced the proliferation of IgA^+ cells in the large intestine. In contrast, on day 4, both the control and PA-enriched groups showed similar levels of $BrdU^+$ IgA^+ cells (Fig. 7B). These data collectively suggested that PA metabolites primarily induced the proliferation of IgA^+ cells in the large intestine rather than prolonged their survival.

Discussion

In the current study, we extended our knowledge of lipid-mediated immune regulation by showing the immunologic function of dietary PA in the enhancement of intestinal IgA responses. Unlike the sterile environment of systemic immune compartments (e.g., spleen), intestinal tissues are continuously exposed to exogenous factors, including commensal bacteria and dietary materials and actively use them to establish a homeostatic inflammatory condition (6). Indeed, in contrast to the massive inflammatory responses induced by the systemic injection of bacterial products, such as LPS (e.g., sepsis), the intestinal immune system requires bacterial stimulation for its maturation (29). Our current study demonstrates that dietary PA can augment intestinal IgA responses when an appropriate amount of dietary oil (4%) is supplied. This effect contrasts sharply with the deleterious plasma PA levels that are induced by a high-fat diet and are considered to be a risk factor for inflammation and diabetes (30). Therefore, like bacterial products, PA has the opposite immunologic effect on intestinal and systemic immune compartments and actually plays a beneficial role in the maturation of the intestinal immune system.

The enhancement of intestinal IgA responses by dietary PA is mediated by at least two distinct pathways. One is PA's direct effect on IgA-producing PCs, and the other is mediated by PA-derived metabolites, sphingolipids. In addition to these two pathways, PA affects APCs (e.g., macrophages and dendritic cells) to promote Ag presentation and the production of cytokines, including IL-6 and TNF-α; this effect is at least partly mediated by TLR4 (31, 32) and represents a plausible third pathway to enhancing intestinal IgA production. Unlike the effect of PA on APCs, PA promoted

IgA production from PCs in a TLR4-independent manner in our current study. In line with this finding, several groups reported that TLR2 acts as a receptor for PA or that PA enters cells where it induces signal transduction for the consequent production of inflammatory cytokines (33–36).

In addition to PA's direct effect on IgA-producing PCs, the SPT-mediated metabolism of PA is involved in the PA-mediated enhancement of intestinal IgA responses. SPT is an essential enzyme for the de novo pathway of sphingolipid synthesis, in which palmitoyl-CoA and serine act as substrates of SPT to generate 3-keto-dihydosphingosine, with subsequent conversion into other sphingolipids, such as sphingomyelin, ceramide, sphingosine, and S1P (37, 38). Sphingolipids are a class of membrane lipids that also are known to have biologic functions (27, 28). For example, ceramide regulates cytoskeletal changes, cell cycle, and apoptosis (27, 28). Accordingly, perhaps an increase in ceramide concentrations induced the proliferation or prolonged the survival of IgA PCs, subsequently increasing the number of IgA-producing PCs in the large intestine. In addition, in the extracellular compartment, S1P controls cell trafficking by recruiting cells toward regions with high concentrations of S1P (39). Of note, we previously reported that S1P regulates intestinal IgA responses by controlling trafficking of IgA^+ cells from inductive sites (e.g., PPs and peritoneal cavity) into the iLP (22, 40). Therefore, another possibility is that the PA-enriched diet induced an increase in the intestinal extracellular S1P concentration, resulting in the effective recruitment of IgA PCs into the intestine. Our current findings suggest that at least one of the enhancing effects of the PA-enriched diet was mediated by the induction of proliferating IgA^+ PCs in the large intestine.

PA was not only included in the diet but was also generated through de novo lipogenesis, whereby carbohydrates are converted to PA. The de novo pathway achieves stable concentrations of PA in vivo. This pathway can explain the specificity of the effect of dietary PA enrichment on the PA content in various tissues. In this study, we found that the increases in PA in the PA-enriched diet group were specific to the intestinal tissues and not the serum. This tissue specificity is consistent with the specific effect of dietary PA, which increased intestinal IgA responses without affecting serum Ab production. Mice fed high-fat diets show increased serum PA levels, which are a risk factor for inflammation and diabetes (30). However, our current findings indicate that the increased proportion of PA in the dietary oil did not affect serum PA levels when the overall amount of oil consumed was normal (4%).

Intestinal tissues contain higher levels of sphingolipids than do other tissues (41), and we found that the effect of an SPT inhibitor was selective for the large, but not small, intestine. One of the major differences between the small and large intestines is the amount of commensal bacteria. We recently reported that some lipid metabolic pathways are uniquely mediated by commensal bacteria (42), raising the possibility that commensal bacteria may affect sphingolipid metabolism. However, germ-free and specific pathogen–free rats have comparable levels of sphingolipids in the intestine (43). Therefore, it is plausible that commensal bacteria are unlikely to participate in this pathway. In contrast, the expression pattern of enzymes involved in the generation of sphingolipids differs between intestinal compartments (44, 45), indicating that the differences in sphingolipid metabolism between the small and large intestines determine their differing dependence on SPT in PA-mediated intestinal IgA responses.

Taken together, our current findings demonstrate that dietary PA and its metabolites play a critical role in the enhancement of intestinal IgA responses. This information can be applied to the development of mucosal adjuvants.
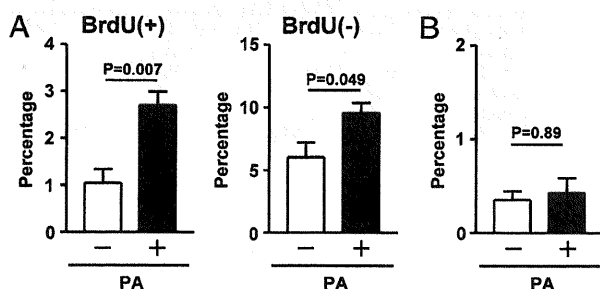

FIGURE 7. A PA-enriched diet induces the proliferation of IgA PCs. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo, after which BrdU was injected i.p. (day 0). On day 1 (**A**) and day 4 (**B**), mononuclear cells isolated from the large intestine were analyzed by flow cytometry to determine the proportion of $BrdU^+$ IgA^+ PCs. Data are mean ± 1 SD ($n = 4$). Similar results were obtained from two independent experiments.

Disclosures

The authors have no financial conflicts of interest.

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