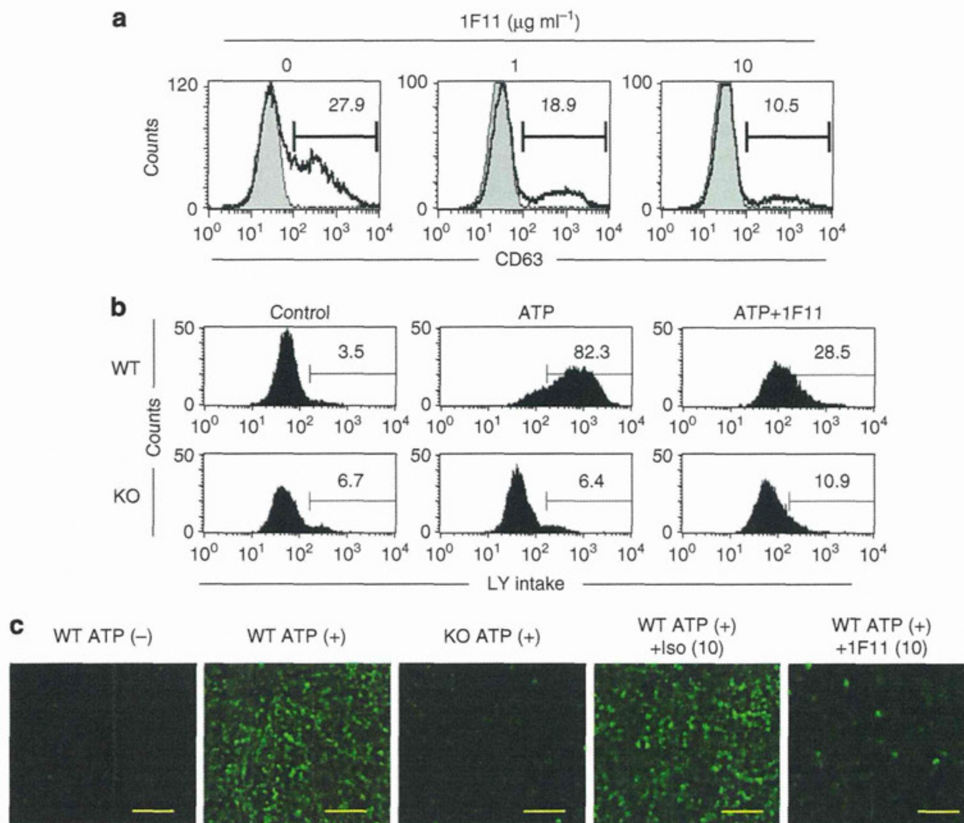

Figure 4 | Inhibition of *in vitro* ATP-mediated MC activation by 1F11 mAb. (**a**) BM-derived MCs, pretreated with various concentrations of 1F11 mAb (0, 1, 10 μg ml^{-1}) for 15 min, were stimulated with 0.5 mM ATP for 30 min. Cells were stained with an anti-CD63 mAb for flow cytometric analysis. Data are representative of three independent experiments. (**b**) BM-derived MCs pretreated with various concentrations of 1F11 mAb or control rat IgG2b (0, 10 μg ml^{-1}) for 15 min were stimulated with 0.5 mM ATP for 30 min in the presence of 1 mg ml^{-1} Lucifer yellow (LY). (**c**) LY uptake was determined by using flow cytometry and fluorescence microscopy. Scale bar, 100 μm. Data are representative of three individual experiments.

ATP stimulation of MCs induced the expression of chemokines, including CCL2, CCL7 and CXCL2 (Fig. 8e–g), and 1F11 mAb treatment or P2X7 deficiency resulted in decreased CCL2 production from MCs activated by ATP but not by IgE plus allergen (Fig. 8g). Furthermore, $Kit^{W\text{-}sh/W\text{-}sh}$ mice showed decreased levels of both CCL2 and IL-1β in the colon tissue, but the production levels of these molecules recovered when the mice were reconstituted with wild-type MCs (Supplementary Fig. S10a). As neutrophils express the corresponding chemokine receptors, it is likely that ATP-dependent MC activation induced inflammatory neutrophil infiltration into the colon from the peripheral blood (Supplementary Fig. S10b,c), given the high level of TNFα production by the neutrophils (Supplementary Fig. S10d). These results indicate that ATP-dependent MC activation has key roles in the induction of inflammatory responses (by inducing inflammatory cytokines) and in the exacerbation of inflammatory responses (by inducing LTs and chemokines to recruit TNFα-producing neutrophils to the colon).

Discussion

Here, we showed that MCs have a critical role in the severity of colitis through their interaction with ATP and P2X7 purinoceptors. These interactions not only induce MC-mediated inflammatory responses but also exacerbate them by promoting neutrophil infiltration. Indeed, MC-deficient mice reconstitution with wild-type, but not $P2x7^{-/-}$, MCs resulted in neutrophil infiltration and severe inflammatory responses, together with increased production of IL-1β, LTs and CCL2 (Figs 5 and 8, and Supplementary Fig. S10). $Kit^{Wsh/Wsh}$ mice spontaneously show elevated levels of neutrophils in their spleens[35]; however, we confirmed that the neutrophil levels were the same as those in the colons of $Kit^{+/+}$, $Kit^{Wsh/+}$ and $Kit^{Wsh/Wsh}$ mice under naïve conditions (Fig. 1h,i). To exclude the possible involvement of other immunological defects in $Kit^{Wsh/Wsh}$ mice, such as the involvement of the *Corin* gene, which is associated with type II transmembrane serine protease[35], we further confirmed the amelioration of intestinal inflammation in conditional MC-deficient mice (Fig. 2d–h). These findings strongly suggest that P2X7 on MCs has a pivotal role in the development of murine and human intestinal inflammation.

P2X7 purinoceptors are expressed on T cells, DCs, macrophages and ECs[9–11,25,36]. In a recent study, ATP/P2X7-mediated signalling inhibited the generation and function of regulatory T cells and ATP stimulation led to their conversion into Th17 cells via an IL-6-dependent pathway; thus, the P2X7 antagonist OxATP inhibited colitis[37]. In that study, ATP/P2X7-mediated regulation of regulatory T cells was involved in the chronic phase of intestinal inflammation, which takes about 4 weeks for disease development[37]. Similarly, ATP-mediated DC activation occurs in the chronic phase of intestinal inflammation through the preferential induction of Th17 cells, although whether this is mediated by P2X7 remains to be seen[38]. In contrast, ATP/P2X7-mediated MC activation in our model was important in the development of T-cell-independent acute colitis, which occurs within 1 week. Thus, our study and those of others[37,38] complement each other by reflecting the complicated pathological aspects and kinetics of the acute and chronic phases of intestinal inflammation mediated by ATP and P2X7.

We also found that the expression level of P2X7 receptors differed depending on the tissue and animal species. First, colonic MCs expressed high levels of P2X7, but skin MCs did not

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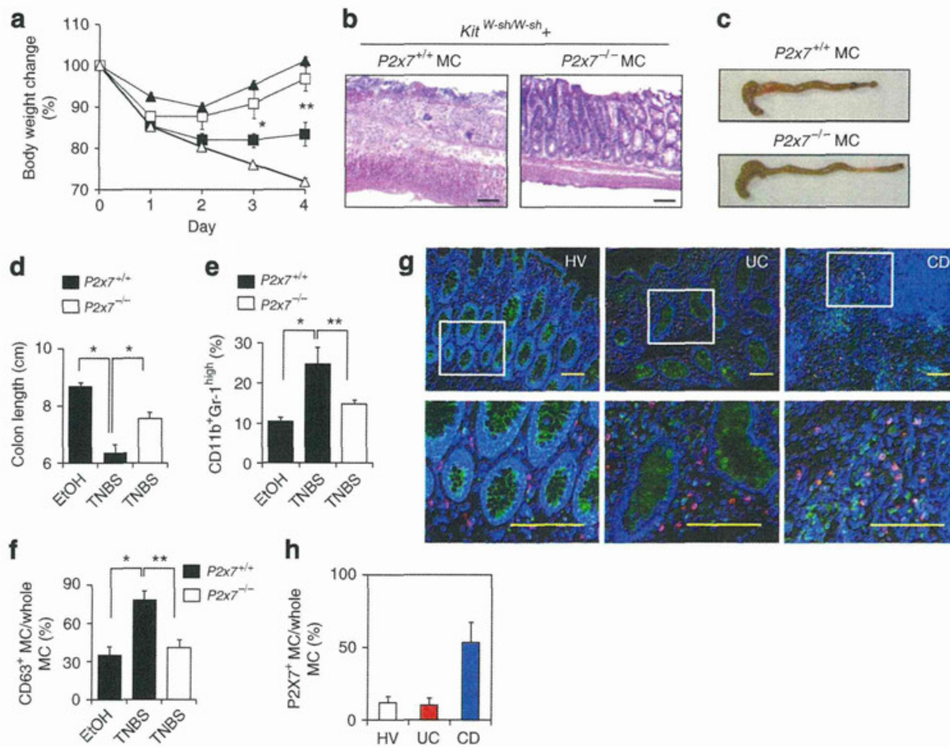

Figure 5 | Inhibitory targeting of P2X7 purinoceptors on MCs leads to amelioration of colonic inflammation. *Kit*$^{W\text{-}sh/W\text{-}sh}$ MC-deficient mice reconstituted with *P2x7*$^{+/+}$, *P2x7*$^{-/-}$ or *caspase-1*$^{-/-}$ BM-derived MCs were applied to a TNBS-induced colitis model. (**a**) Body weight changes were monitored in TNBS-treated *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with *P2x7*$^{+/+}$ (closed squares; $n=9$), *P2x7*$^{-/-}$ (open squares; $n=7$) or *caspase-1*$^{-/-}$ (open triangles; $n=4$). BM-derived MCs were used for TNBS treatment, and *P2x7*$^{+/+}$ BM-derived MC-reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice receiving EtOH served as controls (closed triangles; $n=3$). $^{*}P=0.0264$ (two-tailed Student's *t*-test), $^{**}P=0.0058$ (two-tailed Student's *t*-test). Data are shown as percentages of baseline weights and are means±s.e.m. (**b**) Representative images of haematoxylin and eosin staining are shown. Scale bars represents 100 µm. (**c**) Representative images of whole colons are shown. (**d**) Colon length was measured 4 days after TNBS administration. Data are shown as means±s.e.m. ($n=3$ for *P2x7*$^{+/+}$ EtOH, $n=9$ for *P2x7*$^{+/+}$TNBS, $n=7$ for *P2x7*$^{-/-}$ TNBS), $^{*}P<0.001$ (two-tailed Student's *t*-test). (**e**) Representative flow cytometric data of infiltrated neutrophils (CD11b^{+}Gr-1^{high}) in the colon from three individual experiments. $^{*}P=0.00741$, $^{**}P=0.0009$ (two-tailed Student's *t*-test). Data are shown as means±s.e.m. (**f**) The percentage of CD63^{+} MCs in all c-kit^{+} FcεRIα^{+} MCs was determined with flow cytometry. Data are shown as means±s.e.m. ($n=3$–9), $^{*}P=0.007$ (Welch's *t*-test), $^{**}P=0.0234$ (Welch's *t*-test). (**g**) Colonic tissue sections from a healthy volunteer (HV) and from UC and CD patients were stained with 4′,6-diamidino-2-phenyl indole (blue), MC tryptase (red) and P2X7 (green). Scale bars, 100 µm. (**h**) Cells expressing both P2X7 and MC tryptase were counted in the fields of the tissue sections (four fields for each section). Data are means±s.e.m. ($n=6$).

(Fig. 3a). Second, in contrast to MCs, some macrophages (for example, microglia and RAW264.7 cells) expressed higher levels of P2X7 than did colonic macrophages (Fig. 3b and data not shown). Third, among the several types of immunocompetent cell in the colon, MCs expressed the highest levels of P2X7 (Fig. 3a,b). Fourth, we found P2X7 expression on human colonic ECs, but not on murine colonic ECs (Figs 3b and 5g). In addition, as reported previously[36], P2X7 expression on ECs was downregulated in the colons of CD patients; instead, CD patients showed increased numbers of P2X7^{+} MCs in their colons (Fig. 5g,h). It is important to note that, like murine MCs, human lung MCs express functional P2X7 (ref. 39). Therefore, although we must recognize the similarities and differences between mouse and human intestinal inflammation and MC distribution, ATP/P2X7-mediated MC activation seems to have a major role in the development of intestinal inflammation.

We found elevated levels of extracellular ATP in the colons of TNBS-treated mice (Fig. 6a). This high ATP concentration was most likely achieved by a combination or cascade of several ATP production pathways (for example, cell injury or lysis[7], pattern recognition receptor-mediated activation of monocytes[40] and commensal bacteria[38]). In our tissue culture system, we detected elevated release of ATP (40 µM) in the inflamed colon compared with the control (Fig. 6); however, at least 100 µM ATP was required for MC activation *in vitro* in the single cell culture system (Fig. 7b). This disparity likely reflects the differences in the culture conditions. Unlike in the single cell culture system, the concentration of secreted ATP in the tissue culture system could have been diluted in the culture medium, or ATP could have been consumed rapidly by activated inflammatory cells in the tissue. Alternatively, a lack of commensal bacteria-derived ATP in the tissue culture system as a result of the inclusion of antibiotics may have reduced the ATP level. Another possibility is that the abundant endogenous ATP-degrading enzymes (for example, CD39) in the colon tissue may have degraded some of the ATP. In support of this idea, a suppressive role for CD39 in intestinal inflammation has been reported[41].

We found that ADP-reactive P2Y1 and P2Y12 receptors were expressed on colonic MCs (Fig. 7c), but inhibition or knockdown of these receptors did not suppress the CD63 expression (Fig. 7d,e; Supplementary Fig. S8a). In previous studies, stimulation of MCs with ADP (0.05–50 µM) has led to calcium influx via the P2Y1- but not the P2Y12-mediated pathway[42], whereas our results indicate that CD63 expression required a higher concentration of ADP and was not suppressed by a P2Y1 inhibitor (Fig. 7b,d). This finding indicates that P2Y purinoceptors are not involved in the induction of CD63^{+}-activated MCs that is mediated by high concentrations of ADP. However, we found that adenylate kinase and ATP synthase converted ADP back to ATP, which subsequently induced P2X7

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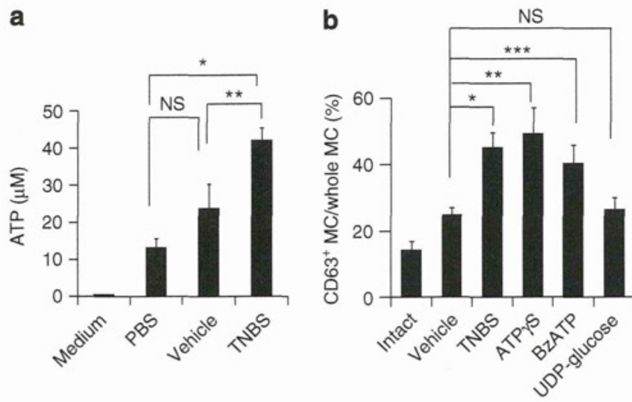

Figure 6 | Enhanced ATP production in intestinal inflammation and MC activation induced by non-hydrolyzable ATP. (**a**) The concentration of ATP released from the colon tissue of mice receiving intrarectally administered phosphate-buffered saline, vehicle or TNBS was measured. Data are shown as means±s.e.m. (n=3–7). *P=0.0004, **P=0.0447 (two-tailed Student's t-test). (**b**) CD63 expression of colonic MCs was measured with flow cytometry after intrarectal administration of vehicle (n=14), TNBS (n=5), non-hydrolyzable ATP (adenosine 5′-O-(3-thio) triphosphate (ATPγS); n=9 or O-(4-benzoyl)benzoyl adenosine 5′-triphosphate (BzATP); n=10) or UDP-glucose (n=6), or in intact mice (n=7). Data are shown as means±s.e.m. *P=0.0002 (two-tailed Student's t-test), **P=0.0135 (Welch's t-test) and ***P=0.0238 (Welch's t-test). NS, not significant.

purinoceptor-dependent MC activation. A similar conversion of ADP to ATP has been reported for endothelial cells[27]. Among adenylate kinases, AK2 was highly expressed on MCs and had a pivotal role in the conversion of ADP to ATP (Supplementary Fig. S9a,b). As another P2Y ligand (UTP) did not induce MC activation (Fig. 7b), our findings suggest that ADP could be converted into ATP by AK2 and ATP synthase, and that this ATP subsequently activates MCs through P2X7 purinoceptors. In addition, colonic MCs do not express ecto-5′-nucleotidase (CD73), an enzyme that degrades ADP into adenosine, which has anti-inflammatory effects in intestinal inflammation[43]. Therefore, our study indicates that MCs express CD39, adenylate kinases and ATP synthase, but not CD73, to preferentially convert ADP to ATP for the exacerbation of inflammatory responses through P2X7 purinoceptors.

Here, we showed that colitis aggravated by P2X7-mediated activation of MCs was independent of the inflammasome pathway, and that P2X7-mediated activation of MCs promoted TNFα production by effector cells to further promote intestinal inflammation[44]. Our findings also suggest that MCs exacerbate inflammation by recruiting neutrophils to produce abundant TNFα, but less IL-10 than is produced by other cells (for example, eosinophils, DCs and macrophages; Supplementary Fig. S10d). This neutrophil recruitment was mediated by the production of IL-1β, LTs and chemokines, which are potential targets for the treatment of colitis. Mice with experimentally induced colitis that lack CXCR2 or 5-LO (a key enzyme for converting arachidonic acid to LTs), as well as mice treated with inhibitors of CCR2, CXCR2 or 5-LO, show reduced inflammation and less neutrophil recruitment in their colons[33,45,46]. Moreover, given that ATP promotes neutrophil migration[47], it is possible that P2X7-dependent LT and chemokine production, as well as ATP generation via AK2 and ATP synthase from MCs, could amplify neutrophil infiltration of the colon. These data collectively indicate that MCs are key factors in the induction of intestinal inflammation and also recruit neutrophils to heighten inflammatory responses. P2X7-dependent MC activation could, therefore, be a target for the treatment of intestinal inflammation.

Methods

Mice and human samples. Female C57BL/6 mice were purchased from CLEA Japan. Rag1$^{-/-}$ and P2x7$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbour, ME, USA). MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice were obtained from Dr H. Suto (Atopy Research Center, Juntendo University, Japan). For the conditional MC-deficient analysis, Mas-TRECK tg mice were injected intraperitoneally with 250 ng of diphtheria toxin for 5 consecutive days and then with 150 ng every other day[18]. *Caspase-1*$^{-/-}$ mice were backcrossed with C57BL/6 mice; F5 mice were used for this experiment[48]. All mice were maintained under specific-pathogen-free conditions at the Experimental Animal Facility of the Institute of Medical Science, the University of Tokyo. All experiments were approved by the Animal Care and Use Committee of the University of Tokyo.

MC reconstitution was performed as described previously[49]. Briefly, BM-derived MCs were obtained from $P2x7^{+/+}$, $P2x7^{-/-}$ or *caspase-1*$^{-/-}$ mice as described previously[22]. BM-derived MCs (5×10^6) were intravenously transferred to $Kit^{W\text{-}sh/W\text{-}sh}$ mice at two time points (0 and 14 days). The reconstituted mice were used 3 months after the last transfer.

Colon specimens from UC and CD patients and healthy volunteers were obtained by endoscopic biopsy at Osaka University Hospital. All subjects provided written informed consent, and the study protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine (no. 08243) and the Institute of Medical Science, The University of Tokyo (no. 20-67-0331).

Experimental colitis. For TNBS-induced colitis, anaesthetized mice (18–22 g) were sensitized with 2.5% TNBS (Sigma-Aldrich) together with acetone and olive oil[50]. After 1 week, after a 3-h fast, the mice were given 100 μl of 2.5% TNBS in 50% ethanol via a flexible feeding tube that maintained their heads in a vertical position for 10 min. The control group received only 50% ethanol. Weight changes were recorded daily, and tissues were collected for histological analysis and isolation of mononuclear cells from the colonic lamina propria. For mAb treatment, mice were injected intraperitoneally with 0.5 mg of mAb (1F11 or an isotype control) 1 day before being given TNBS/EtOH intrarectally. mAb administration was continued for 3 days. For P2Y12 inhibition with clopidogrel sulphate, (Wako, Osaka, Japan), mice received clopidogrel (0.5 mg ml^{-1}) in their drinking water from 3 days before intrarectal administration of TNBS/EtOH until the end of the study[50]. For DSS-induced colitis, mice were given 3.5% DSS (Wako, for C57BL/6) or 2.5% DSS (MP Biomedicals, Illkirch, France, for Mas-TRECK tg mice) in their drinking water for 5 days and their body weights were monitored daily[50]. In some experiments, non-hydrolysable ATP (adenosine 5′-*O*-(3-thio) triphosphate and *O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate) or UDP-glucose (0.25 mg in 50% EtOH) was intrarectally administered and the effects were analysed 2 days later.

***In vitro* MC stimulation and inhibition.** BM-derived MCs (2.5×10^5) were cultured with various concentrations of adenosine, ADP, ATP, UTP or anti-DNP-IgE with DNP-human serum albumin. Adenosine-3-phosphate 5-phosphosulfate (0.25 mM), carbenoxolone (10 μM), flufenamic acid (100 μM), pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (100 μM), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (100 μM), OxATP (0.5 mM), AD2P5 (1 mM), oligo (10 or 100 μM) or UDP (100 μM) was added to the cells for the inhibition assay[27,28,40,51]. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA, purity was ≥95%). 5-LO (BD Pharmingen, Franklin Lakes, NJ, USA) was stained after permeabilization with 0.2% Triton-X100 for 10 min; nuclei were stained with 4′,6-diamidino-2-phenyl indole.

Cell preparation and flow cytometry. ECs and lamina propria mononuclear cells were isolated from the colon, as described previously[52]. For flow cytometric analysis, cells were incubated with 5 μg ml^{-1} of an anti-CD16/32 antibody (10 μg ml^{-1}, Fc block, BD Pharmingen) for 5 min and stained for 30 min at 4 °C with fluorescence-labeled Abs specific for c-kit (0.2 μg ml^{-1}), Gr-1 (0.4 μg ml^{-1}), CD4 (1 μg ml^{-1}), CD11b (0.2 μg ml^{-1}), CD11c (0.4 μg ml^{-1}), CD39 (0.4 μg ml^{-1}), CD45 (0.4 μg ml^{-1}), IgA (10 μg ml^{-1}), B220 (0.4 μg ml^{-1}; BD Pharmingen), CCR3 (2 μg ml^{-1}), CXCR2 (4 μg ml^{-1}; R&D Systems, Minneapolis, MN, USA), FcεRIα (0.4 μg ml^{-1}), CD73 (0.4 μg ml^{-1}), TLR2 (10 μg ml^{-1}; eBioscience, San Diego, CA, USA), F4/80 (20 μg ml^{-1}), CCR2 (10 μg ml^{-1}), P2X7 (Hano43; 2 μg ml^{-1},Serotec, UK) or CCR1 (10 μg ml^{-1}, Abnova, Taiwan). Flow cytometric analysis and cell sorting were performed by using FACSCalibur and FACSAria (BD Biosciences, Franklin Lakes, NJ, USA), respectively. Sorted cells were stained with May-Giemsa stain in some experiments. Colonic MCs and BM-derived MCs were prepared as described elsewhere[22].

Establishment of an anti-P2X7 mAb (1F11) and an anti-CD63 mAb. The procedure used to establish MC-specific mAbs is shown as a flowchart in Supplementary Figure S3. Briefly, c-kit$^+$ FcεRIα$^+$ MCs were obtained as described previously[22] from the colons of mice that exhibited allergic diarrhoea. Purified colonic MCs (10^6 cells) were injected into the footpads of Sprague Dawley rats seven times, as described previously[53]. Lymphocytes were isolated from the spleen and inguinal lymph nodes and fused with P3X63-AG8.653 myeloma cells (CRL-1580; American Type Culture Collection, Manassas, VA, USA). The reactivity of each hybridoma to the colonic MCs was examined by means of flow cytometry. To identify antigens

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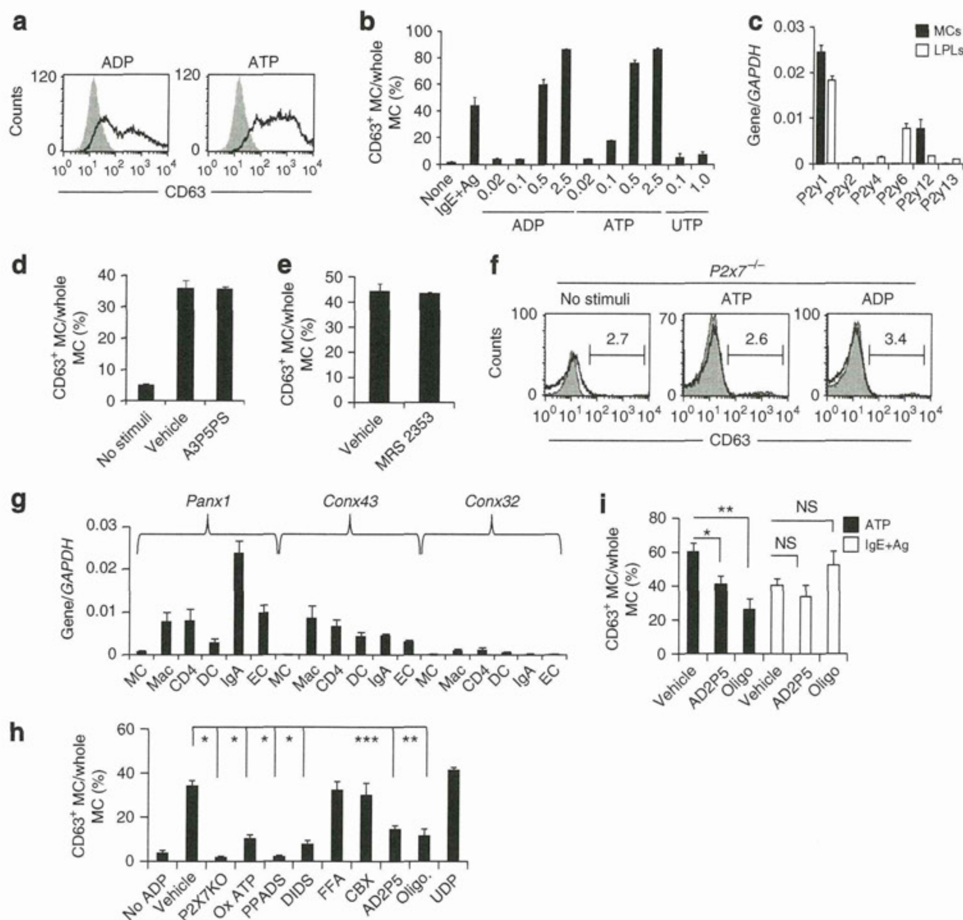

Figure 7 | The ecto-adenylate kinase pathway mediates ADP-dependent MC activation through P2X7 purinoceptors. (**a**) BM-derived MCs treated with ADP or ATP at 0.5 mM for 30 min and examined for CD63 expression. (**b**) BM-derived MCs treated with IgE plus relevant allergen or various concentrations of ADP, ATP or UTP for the analysis of CD63 expression. Data are representative of four experiments. (**c**) Expression of mRNA encoding each P2Y receptor in colonic lamina propria lymphocytes (LPLs) and MCs was analysed by quantitative reverse transcription (RT)–PCR ($n=3$). (**d**,**e**) BM-derived MCs pre-treated with 0.25 mM P2Y1 inhibitor (adenosine-3-phosphate 5-phosphosulfate (A3P5PS)) (**d**) or 0.01 mM P2Y12 inhibitor (MRS2353) (**e**), stimulated with ADP and examined for CD63 expression ($n=3$). (**f**) BM-derived MCs from *P2x7*$^{-/-}$ mice stimulated with ATP or ADP; CD63 expression was determined with flow cytometry. Data are representative of four experiments. (**g**) Expression of pannexin-1 (Panx1), connexin-43 (Conx43) and Conx32 on colonic MCs, macrophages (Mac), CD4$^+$ T cells (CD4), DCs, IgA$^+$ cells (IgA) and ECs was measured by quantitative RT–PCR ($n=4$). (**h**) BM-derived MCs were pretreated with inhibitors of P2X receptors [OxATP, 0.5 mM; pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS); 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)], connexins [flufenamic acid (FFA)], Panx-1 [carbenoxolone (CBX)], ecto-adenylate kinase [diadenosine pentaphosphate (AD2P5)], ATP synthase (oligomycin) or nucleoside diphosphokinase (UDP) and subsequently stimulated with 0.25 mM ADP. CD63 expression was determined with flow cytometry. ($n=3$) $^*P<0.01$, $^{**}P<0.05$ (two-tailed Student's *t*-test). All data are shown as means±s.e.m. (**i**) BM-derived MCs were treated with AD2P5, oligomycin or UDP and stimulated with 0.5 mM ATP or IgE plus allergen. CD63 expression was determined with flow cytometry ($n=5$). $^*P<0.0001$ (two-tailed Student's *t*-test), $^{**}P=0.0008$ (two-tailed Student's *t*-test) and $^{***}P=0.0008$ (Welch's *t*-test). NS, not significant.

recognized by the mAbs, immunoprecipitation was performed with the mAbs, followed by Liquid chromatography–mass spectrometry analysis, as described previously[53]. Antigen specificity was confirmed by transfecting CHO cells with plasmids that encoded the murine P2X7 receptor and CD63.

Measurements of membrane permeability and inflammatory mediators. To assess membrane permeability, BM-derived MCs were washed twice with phosphate-buffered saline (PBS) and incubated with 1 mg ml^{-1} Lucifer yellow (Sigma-Aldrich) containing 250 μM sulfinpyrazone (Sigma-Aldrich). The MCs were then stimulated with 0.5 mM ATP (Sigma-Aldrich) for 15 min, as described elsewhere[12]. In the inhibition assay, 1 or 10 μg ml^{-1} of 1F11 mAb or the control antibody (Rat IgG2b) was added before ATP stimulation. The fluorescence signal of Lucifer yellow was determined by using fluorescence microscopy (BZ9000, Keyence, Osaka, Japan) and flow cytometry.

To measure the production of cytokines, chemokines and LTs from MCs, BM-derived MCs (2.5×10^5) were stimulated with 2.5 mM ATP for 30 min, after which the supernatants were collected. Chemokine and cytokine production was detected with an inflammatory cytokine kit (BD Pharmingen). For IL-1β measurement, BM-derived MCs from wild-type, *P2x7*$^{-/-}$ and *caspase-1* $^{-/-}$ mice were stimulated with 0.1 μg ml^{-1} of LPS for 4 h, followed by ADP or ATP stimulation. LT C4/D4/E4 production was detected by use of an enzyme-linked immunosorbent assay (GE Healthcare Bio-Science, NJ, USA). For ATP, cytokine and chemokine measurements from the colon tissue, the colon tissues were isolated from mice 2 days after intrarectal administration of TNBS. Released ATP was measured by culturing colon tissues at 100 mg of tissue per 100 μl of RPMI1640 medium for 3 h and using a luminescence ATP detection system (PerkinElmer, Norwalk, CT, USA).

Immunoprecipitation and western blotting. Cell lysates obtained from BM-derived MCs or CHO transfectants (mouse P2X7 variants a, c and d and flag-mP2X7s, cloned from C57BL/6 mice) were analysed by western blotting and immunoprecipitation with 1F11 mAb or the control Ab. Membranes were probed with an anti-flag and a polyclonal rabbit anti-P2X7 antibody (Sigma-Aldrich).

Histology. Colonic tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin solution, as described previously[22]. For the detection of MCs and P2X7

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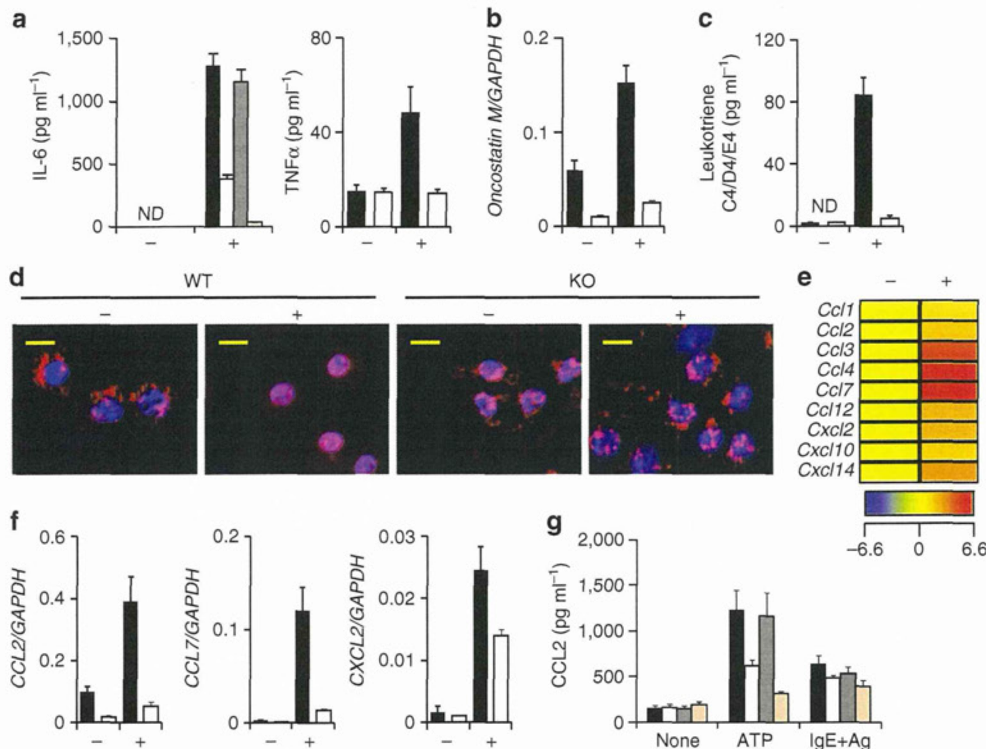

Figure 8 | Critical role of the intestinal MC-associated ATP-P2X7 purinoceptor pathway for induction of neutrophil infiltration. *P2x7*$^{+/+}$ and *P2x7*$^{-/-}$ BM-derived MCs were treated with 0.25 mM ATP (+) or left untreated (−). (**a**) Production of IL-6 (left panel; isotype mAb-treated MC, closed column; 1F11 mAb-treated MC, open column; *P2x7*$^{+/+}$, grey column; and *P2x7*$^{-/-}$, beige column) and TNFα (right panel) in culture supernatant (*P2x7*$^{+/+}$, closed column; *P2x7*$^{-/-}$, open column) was determined after 24 h stimulation. ND, not detected. Data are shown as means±s.e.m. (n=3). (**b**) Oncostatin M mRNA expression was measured 30 min after stimulation of *P2x7*$^{+/+}$ (closed column) and *P2x7*$^{-/-}$ (open column) MCs with ATP. Data are shown as means±s.e.m. (n=3). (**c**) LT C4/D4/E4 production from ATP-stimulated (+) or -unstimulated (−) *P2x7*$^{+/+}$ (closed column) or *P2x7*$^{-/-}$ BM-derived MCs (open column) in culture supernatants was measured by using enzyme-linked immunosorbent assay (ELISA). Data are shown as means±s.e.m. (n=3). ND, not detected. (**d**) *P2x7*$^{+/+}$ and *P2x7*$^{-/-}$ BM-derived MCs were stimulated with 0.5 mM ATP. Cells were fixed and stained with an anti-5LO antibody (red) and 4′,6-diamidino-2-phenyl indole (blue). Scale bar, 10 μm. Data are representative of two experiments. (**e**) Representative data of a chemokine gene array are shown. Increased levels of each chemokine are shown as a heat map. (**f**) mRNA expression of CCL2 (left), CCL7 (middle) and CXCL2 (right) was measured by using quantitative reverse transcription-PCR. Data are shown as means±s.e.m. (n=3). (**g**) CCL2 production was enumerated by using ELISA 24 h after stimulation of BM-derived MCs with ATP or IgE plus antigen (IgE+Ag). Isotype mAb-treated MC, closed column; 1F11 mAb-treated MC, open column; *P2x7*$^{+/+}$ MC, grey column; and *P2x7*$^{-/-}$ MC, beige column). Data are shown as means±s.e.m. (n=3).

expression in human specimens, colonic tissue sections were stained with antibodies for MC tryptase and P2X7 purinoceptors (Alomone Laboratories, Jerusalem, Israel).

shRNA plasmid construction and lentiviral transduction. For the construction of shRNA expression lentivirus vector plasmids, a series of oligonucleotide pairs were synthesized, as listed below. Each oligo pair was annealed and cloned into pmU6[54]. Each mU6-shRNA cassette was then subcloned into the ΔU3 sequence of the 3′-LTR of the lentivirus vector plasmid pLCG to generate pLCG-shCD63 (sense: 5′-TTTGATTCTTGCTGCATCAACATAGCTTCCTGTCACTATGTTGATGCAG CAAGAATCTTTTTTG-3′, antisense: 5′-AATTCAAAAAAGATTCTTGCTGCA TCAACATAGTGACAGGAAGCTATGTTGATGCAGCAAGAAT-3′), pLCG-shP2Y12 (sense: 5′-TTTGATCTACTAATGATTCTAACTGCTTCCTGTCACAGTTAGAAT CATTAGTAGATCTTTTTTG-3′, antisense: 5′-AATTCAAAAAAGATCTACTAA TGATTCTAACTGTGACAGGAAGCAGTTAGAATCATTAGTAGAT-3′) and pLCG-shAK1 (sense: 5′-TTTGCGAGAAGATTGTACAGAAATGCTTCCTGTCA CATTTCTGTACAATCTTCTCGCTTTTTTG-3′, antisense: 5′-AATTCAAAAAA GCGAGAAGATTGTACAGAAATGTGACAGGAAGCATTTCTGTACAATCTT CTCG-3′) and pLCG-shAK2 (sense: 5′-TTTTGGAGCTAATTGAGAAGAATTGC TTCCTGTCACAATTCTTCTCAATTAGCTCCATTTTTTG-3′, antisense: 5′-AATTCAAAAAATGGAGCTAATTGAGAAGAATTGTGACAGGAAGCA ATTCTTCTCAATTAGCTCC-3′).

To obtain lentivirus-encoding green fluorescent protein (as a reporter gene) and shRNA for CD63, 293FT cells (6×10^5) were transfected with pLP1, pLP2, pLP-VSVG and pLCG-shRNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol (Invitrogen). After 24- and 48-h incubations, lentivirus-encoding shRNA was collected.

BM-derived MCs (1×10^6) or MC/9 cells were transduced with shRNA expression lentivirus stock in the presence of 8 μg ml^{-1} Polybrene (Sigma-Aldrich)[55]. After 24 h, the cells were washed and green fluorescent protein-positive cells were sorted by FACSAria and used for subsequent experiments.

Quantitative real-time-PCR. Total RNA was prepared by using TRIzol (Invitrogen) and reverse transcribed by use of Superscript VILO (Invitrogen), as described. Quantitative reverse transcription–PCR was performed with the LightCycler 480 II (Roche, Mannheim, Germany) and the Universal Probe Library (Roche). Primer sequences are listed in Supplementary Table S1.

Statistical analysis. Statistical analysis was performed by using the unpaired two-tailed Student's t-test and Welch's t-test. The data are presented as means±s.e.m.

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Author contributions

Y.K. conducted the research, performed experiments and wrote the manuscript; T.A. and K.F. performed gene expression and animal experiments; T.N. conducted the mAb experiment; H.T., H. Iba, T.H., M.K. and S.S. contributed to the experimental design and data analysis; S.N. and H. Iijima obtained clinical samples and J.K. and H.K. supervised the project and wrote the manuscript. JK should be contacted for material requests.

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A Pivotal Role of Vitamin B9 in the Maintenance of Regulatory T Cells *In Vitro* and *In Vivo*

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Abstract

Dietary factors regulate immunological function, but the underlying mechanisms remain elusive. Here we show that vitamin B9 is a survival factor for regulatory T (Treg) cells expressing high levels of vitamin B9 receptor (folate receptor 4). In vitamin B9-reduced condition *in vitro*, Treg cells could be differentiated from naïve T cells but failed to survive. The impaired survival of Treg cells was associated with decreased expression of anti-apoptotic Bcl2 and independent of IL-2. *In vivo* depletion of dietary vitamin B9 resulted in the reduction of Treg cells in the small intestine, a site for the absorption of dietary vitamin B9. These findings provide a new link between diet and the immune system, which could maintain the immunological homeostasis in the intestine.

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Introduction

To achieve immunosurveillance and immunological homeostasis at the interface between the interior and exterior of the gastrointestinal tract, the intestinal immune system tightly balances states of immune activation and quiescence [1]. Thus, gastrointestinal tissues contain numerous kinds of T cells, such as Th1, Th2, Th17, forkhead box P3 (Foxp3)$^+$ regulatory T (Treg) cells, IL-10–producing Foxp3$^-$ T regulatory type 1 cells, and T cells expressing $\gamma\delta$ T cell receptor, which together create the appropriate immunological environment.

Th17 and Treg cells are observed most frequently in the intestine, and their preferential differentiation is achieved by a unique cytokine environment created by transforming growth factor β (TGF-β), IL-6, and IL-23 [2]. In addition to these host-derived factors, the development and function of the immune system are influenced by crosstalk with environmental factors [3]. For example, stimulation by segmented filamentous bacteria results in the preferential induction of Th17 cells, whereas colonic Treg cells are induced by crosstalk between epithelial cells and Clostridium clusters IV and XIVa [4,5,6].

Nutritional molecules are also considered to be essential environmental factors for the development, maintenance, and regulation of gut immune responses. Thus, deficient or inappropriate nutritional intake increases the risk of infectious, allergic, and inflammatory diseases [7,8]. Among various dietary factors, vitamins are important participants in the regulation of immune responses. For example, vitamin A is converted into retinoic acid (RA) by gut-associated dendritic cells; RA induces the expression of gut-homing molecules (e.g., $\alpha4\beta7$ integrin and CCR9) on activated T and B cells [9,10] and promotes the preferential differentiation of Treg cells and the simultaneous inhibition of Th17 cells [11,12,13,14]. Vitamin B6 is required for the metabolic pathway of sphingosine 1-phosphate, a lipid mediator that regulates cell trafficking [15]; disruption of vitamin B6 function results in aberrant T-cell differentiation and cell trafficking in both systemic and intestinal compartments [16,17,18].

Vitamin B9 (also known as folate and folic acid) is a water-soluble vitamin derived from both diet and commensal bacteria [19]. Vitamin B9 is essential for the synthesis, replication, and repair of nucleotides for DNA and RNA and is thus required for cell proliferation and survival [20]. Methotrexate (MTX) acts as a vitamin B9 antagonist and blocks vitamin B9–mediated nucleotide synthesis, making MTX useful as an anti-tumor [21] and anti-rheumatoid arthritis agent [22]. Vitamin B9 deficiency also reduces the proliferative responses of lymphocytes and natural killer cell activity [23,24]. Additionally, the vitamin B9 receptor folate receptor 4 (FR4) is both a marker of Treg cells and is immunologically functional [25]; however, how it functions in the intestinal immune system is largely unknown. In this study, we examined the role of vitamin B9 in the regulation of Treg cell *in vitro* and *in vivo*.

Materials and Methods

Mice and experimental treatment

Female Balb/c mice (7–9 wk of age) were purchased from Japan Clea (Tokyo, Japan). Vitamin B9–deficient and control

diets composed of chemically defined materials (Oriental Yeast, Tokyo, Japan) were used within 3 months. All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines (Approval #20–28).

Lymphocyte isolation

Lymphocytes were isolated from the lamina propria (LP), as previously described [18,26]. Briefly, lymphocytes were isolated from dissected PPs by enzymatic dissociation using collagenase (Wako, Osaka, Japan). To isolate lymphocytes from the LP of jejunum/duodenum, PPs were removed and the remaining intestinal tissue was cut into 2-cm pieces and stirred in RPMI 1640 medium containing 1 mM EDTA and 2% fetal calf serum (FCS). The tissue pieces were then stirred in 0.5 (for small intestine) or 1.0 (for large intestine) mg/mL collagenase, and the dissociated cells were subjected to centrifugation through a discontinuous Percoll gradient. Lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described [18,26]. Cells were pre-incubated with anti-CD16/32 antibodies and then stained with fluorescent antibodies specific for CD4, ICOS, and GITR (BD Biosciences, San Jose, CA) and FR4 (Biolegend). A Via-probe solution (BD Biosciences) was used to discriminate between dead and living cells. Intracellular staining of Foxp3 (eBioscience, San Diego, CA), phosphorylated STAT5, Ki67 and Bcl2 (BD Biosciences) was performed in accordance with the manufacturers' instructions. Flow cytometry and cell sorting were carried out using the FACSCantoII and FACSAria systems (BD Biosciences), respectively.

Vitamin B9 measurement

To measure vitamin B9 concentrations, intestinal washes were collected by washing 12 cm of jejunum/duodenum or whole colon with 1 mL of PBS. The vitamin B9 concentration in intestinal washes and serum was measured with a RIDASCREEN enzyme immunoassay kit (R-Biopharm AG, Darmstadt, Germany) in accordance with the manufacturer's instructions. To measure the amounts of intracellular vitamin B9, 5×10^6 purified cells were washed twice with PBS, and a cell lysate was obtained by homogenizing cells in PBS containing 0.01% NP-40. After cell debris was removed by centrifugation, vitamin B9 amounts in the supernatant were measured with a RIDASCREEN enzyme immunoassay kit.

In vitro culture

For the induction of Treg cells from naïve T cells, CD62L^{hi}CD4$^+$ naïve T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody and 1 μg/mL of an anti-CD28 antibody (BD Biosciences) plus 2 ng/mL of human TGF-β (PeproTech, Rocky Hill, NJ) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS. To examine the maintenance of differentiated Treg cells, purified CD25$^+$CD4$^+$ T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody with or without 1000 units/mL of IL-2 (Peprotech) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS in the presence or absence of 100 nM MTX.

Statistics

Results were compared with the Student's *t*-test by using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was established at $P<0.05$.

Results

Vitamin B9 is required for the survival of Foxp3$^+$ Treg cells

Foxp3$^+$ Treg cells express high levels of FR4, which is essential for their maintenance [25]. We therefore examined whether vitamin B9 is required for the differentiation of Treg cells from naïve T cells, the survival of differentiated Treg cells, or both. To address this, we initially performed an *in vitro* T-cell differentiation assay. Purified naïve CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in complete or vitamin B9–reduced medium. Although a small amount of vitamin B9 is supplied from fetal calf serum (FCS) even in vitamin B9–null medium (0.2 ppb, compared with 25 ppb in normal medium), the total cell number was decreased in the condition with reduced vitamin B9 compared to the control; however, Foxp3$^+$ Treg cells were generated at a normal frequency (Fig. 1A).

To investigate the effects of vitamin B9 on differentiated Treg cells, we cultured CD25$^+$ Treg cells with anti-CD3 antibodies. The total cell number was significantly lower in the vitamin B9–reduced condition than in the control condition (Fig. 1B). The reduction in cell number occurred predominantly among the Foxp3$^+$CD4$^+$ Treg cells (Fig. 1B). The reduction of FR4^{hi}Foxp3$^+$ T cells was dependent on the dose of vitamin B9 (Fig. 1C).

We then measured the expression of Ki67 and anti-apoptotic Bcl-2 to investigate whether decreased number of Foxp3$^+$CD4$^+$ Treg cells in vitamin B9–reduced medium was due to the defects of cell proliferation, survival, or both. We found that both Ki67 and Bcl2 were decreased in Foxp3$^+$CD4$^+$ Treg cells cultured in vitamin B9 vitamin B9–reduced medium, but magnitude of Bcl2 reduction was higher than Ki67 reduction (Fig. 2A and B). These findings suggest that vitamin B9 is preferentially but not exclusively required for the survival of Treg cells *in vitro*.

Vitamin B9 carrier-mediated pathway is not specifically involved in the survival of Treg cells

Because vitamin B9 is highly hydrophilic, mammalian cells must actively mediate the entry of vitamin B9 into cells by carrier- or receptor-mediated pathways [27]. Carriers include the proton-coupled folate transporter and the reduced folate carrier [27]. To examine whether a carrier-mediated pathway is involved in maintaining Treg cells, we employed MTX, an antagonist of vitamin B9 that is transported mainly via the reduced folate carrier and rarely via folate receptors [28,29]. MTX treatment reduced the numbers of both Treg and non-Treg cells (Fig. 3), suggesting that the carrier-mediated pathway does not specifically maintain Treg cells.

Vitamin B9 is an IL-2–independent survival factor for Treg cells

Treg cells could vigorously proliferate in some circumstances (e.g., antigen-specific activation through their highly sensitive TCR signaling [30] and IL-2-mediated activation [31]), which led to a hypothesis that Treg cells simply require large amounts of vitamin B9 as a source of nucleotides, and thus Treg cells might express FR4 as an additional means of acquiring vitamin B9. If so, FR4^{hi} Treg cells should contain a larger amount of vitamin B9 in the intracellular compartments; however, the amount of intracel-

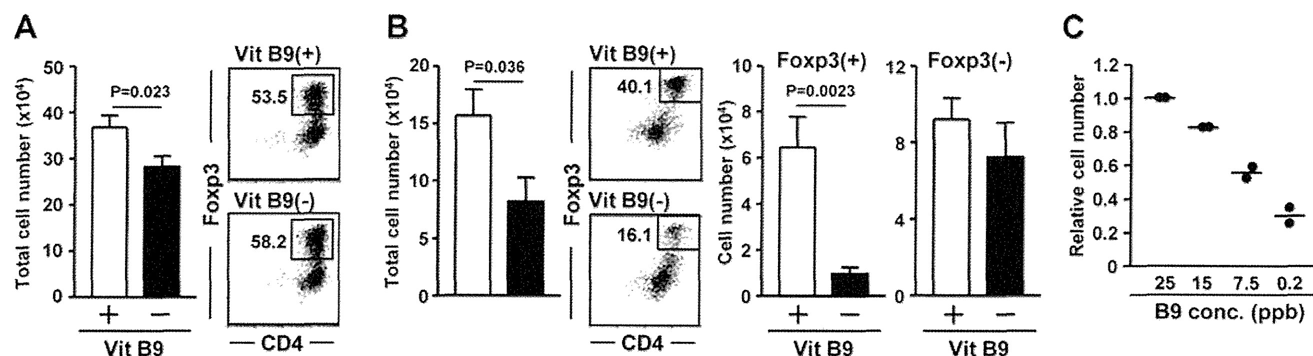

Figure 1. Requirement of vitamin B9 for the maintenance of Treg cells. (A) Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in the presence of normal [Vit B9(+)] or reduced [Vit B9(−)] amounts of vitamin B9. After 4 days, total cell numbers were calculated, and the differentiation into $Foxp3^+$ Treg cells was examined by flow cytometry. Data are means ± SEM (n = 4). (B) $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Cont or B9(−) medium. The frequencies of $Foxp3^+$ and $Foxp3^-CD4^+$ T cells (B) were determined by flow cytometry. Cell numbers were calculated using the total cell number and flow cytometric data. Data are means ± SEM (n = 6). (C) Experiments similar to that shown in (B) were performed with different concentrations of vitamin B9. The relative cell number of $Foxp3^+$ Treg cells is expressed as a ratio to the cell number in control medium. The values and means are indicated with dots and lines, respectively. Similar results were obtained from 2 independent experiments.
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lular vitamin B9 was equivalent between $FR4^{hi}$ Treg and $FR4^{low/-}$ non-Treg cells (Fig. 4A). Thus, FR4 might have an additional specific function for the survival of Treg cells.

IL-2 stimulation enhance the survival of Treg cells [31,32,33]. The FR4-mediated vitamin B9 signal might undergo crosstalk with IL-2-mediated signaling to maintain the survival of $FR4^{hi}Foxp3^+$ Treg cells. To test this, Treg cells were cultured with an anti-CD3 antibody together with IL-2. Although the absolute cell numbers were low in the reduced vitamin B9 condition, the magnitude of the IL-2-mediated enhancement of Treg cell growth was similar in the control and vitamin B9-reduced conditions (Fig. 4B). Consistent with this finding, comparable expression of phosphorylated STAT5 was noted in the control and vitamin B9-reduced conditions (Fig. 4C).

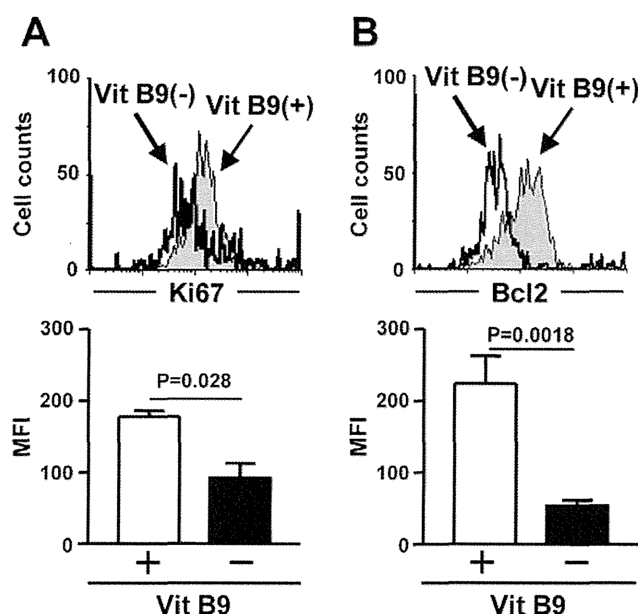

Figure 2. Vitamin B9 is essential for the survival of Treg cells. $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Vit B9(+) or Vit B9(−) medium. The expression of Ki67 (A) and Bcl2 (B) in $Foxp3^+CD4^+$ T cells were determined by flow cytometry (top panels) and graphs show the means fluorescent intensity (MFI; bottom panels). Data are means ± SD (n = 3). Data are representative of 4 independent experiments.
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Dietary vitamin B9 maintains $Foxp3^+$ Treg cells in the small intestine

To examine whether vitamin B9 affects Treg cells *in vivo*, we maintained mice on a vitamin B9–depleted diet for 8 wk. Mice maintained with vitamin B9(−) diet showed less vitamin B9 in the small-intestinal wash than controls (Fig. 5A). In contrast, the amounts of vitamin B9 in the large-intestinal wash and serum were not different in those mice (Fig. 5A), presumably due to vitamin B9 production from commensal bacteria [19].

We then focused on Treg cells in the mice maintained with vitamin B9(−) diet. Consistent with our *in vitro* data, the small intestines of mice maintained with vitamin B9(−) diet had fewer $Foxp3^+$ Treg cells than those of control mice (p = 0.018), and there was no statistical difference (p = 0.3022) in the number of $Foxp3^-CD4^+$ non-Treg cells (Fig. 5B). The number of Treg and

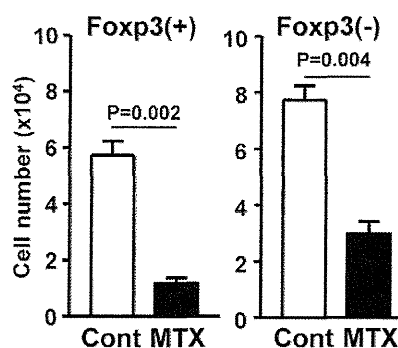

Figure 3. Vitamin B9 carrier-mediated pathway is not specific pathway in the maintenance of T cell survival. $CD25^+$ $CD4^+$ T cells were cultured with an anti-CD3 antibody in complete medium containing 100 nM methotrexate (MTX), and the frequency and absolute cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells were determined. Data are means ± SEM (n = 4). Data are representative of two independent experiments.
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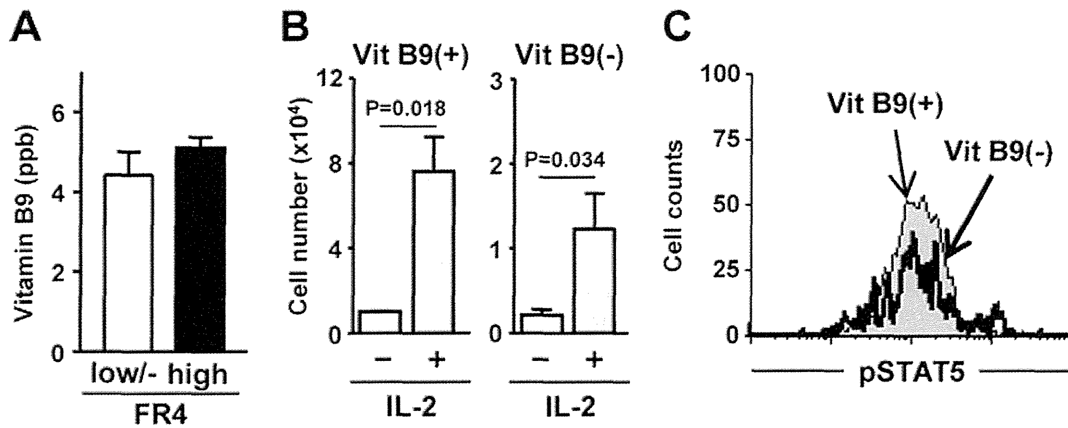

Figure 4. Vitamin B9 is IL-2-independent survival factor for Treg cells. (A) The amounts of intracellular vitamin B9 were measured using purified $CD4^{+}FR4^{hi}$ Treg or $CD4^{+}FR4^{low/-}$ non-Treg cells. Data are means ± SEM (n = 4). (B, C) Experiments similar to those shown in Fig. 1B were performed in the presence of anti-CD3 antibody stimulation with or without IL-2 stimulation. Cell number of $Foxp3^{+}CD4^{+}$ T cells (B) and the expression of phosphorylated STAT5 (pSTAT5) in $Foxp3^{+}CD4^{+}$ T cells (C) were determined. Data in (B) are means ± SEM (n = 6). Similar results were obtained from 3 separate experiments.
doi:10.1371/journal.pone.0032094.g004

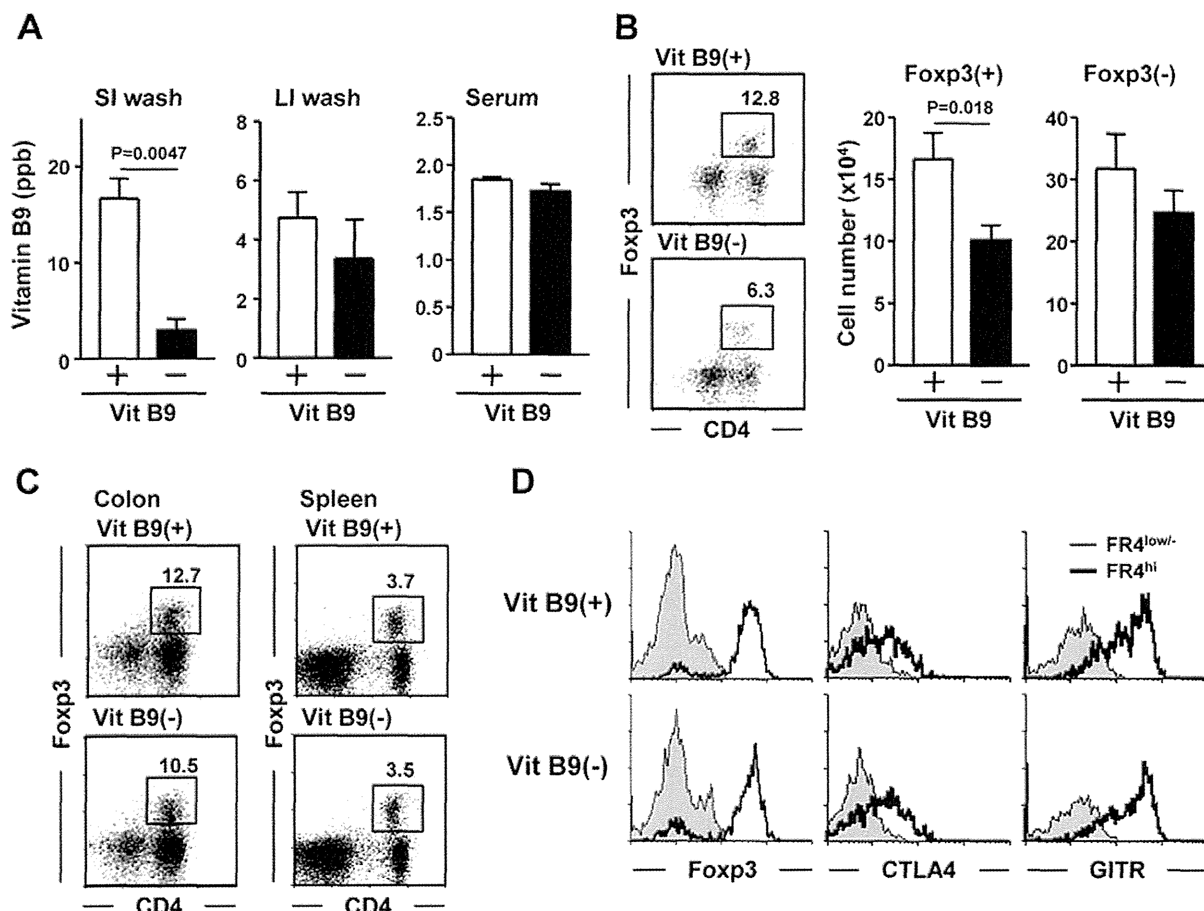

Figure 5. Depletion of dietary vitamin B9 selectively reduces Treg cells in the small intestine. Mice were maintained on a control [Vit B9(+)] or vitamin B9-depleted [Vit B9(−)] diet for 8 wk. (A) Vitamin B9 concentrations were measured in intestinal washes of the small intestine (SI), large intestine (LI), and serum. The data are mean ± SEM (n = 6). (B, C) The frequency and cell numbers of $Foxp3^{+}$ and $Foxp3^{-}$ $CD4^{+}$ T cells in the small intestine (B), colon, and spleen (C) were calculated using the total cell number and flow cytometric data (mean ± SEM, n = 6). (D) Flow cytometric analysis was performed to determine the expression levels of Foxp3, CTLA4, and GITR on the surface of $FR4^{low/-}$ (thin line) and $FR4^{hi}$ (thick line) $CD4^{+}$ T cells in the LP. Similar results were obtained from 3 separate experiments.
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non-Treg cells was not significantly changed in the colon and spleen of mice maintained with vitamin B9(−) diet (Fig. 5C), which could be explained by the similar concentration of vitamin B9 in the large-intestinal washes and sera of both groups of mice. We also found that Foxp3 and the inhibitory molecules CTLA4 and GITR, which are specifically expressed on Treg cells, were comparable between those mice (Fig. 5D).

Discussion

We have shown that vitamin B9 is crucial for the maintenance of Treg cells. Intriguingly, vitamin B9 was required for the survival of differentiated Treg cells, but was not necessary for the differentiation of naïve T cells into Treg cells. This selective effect of vitamin B9 on Treg cells is opposite to the effect of RA, a vitamin A metabolite, which enhances the differentiation of naïve T cells into Treg cells [11,12,13,14]. RA also induces the expression of gut-homing molecules (e.g., $\alpha 4\beta 7$ integrin and CCR9) on B and T cells activated by gut dendritic cells [9,10]. Because CCR9 was expressed normally on Treg cells in the LP of mice maintained with vitamin B9(−) diet (data not shown), the deficiency of dietary vitamin B9 did not affect the RA-mediated expression of gut-homing molecules and, predictably, the induction of Treg cells in the small intestine.

Treatment with the vitamin B9 antagonist MTX affected survival of both Treg cells and non-Treg cells, suggesting that the carrier-mediated pathway maintains sufficient amounts of intracellular vitamin B9 for cell survival regardless of the T-cell subset. The indiscriminate effects of MTX could be explained by the ubiquitous expression of the folate carrier [29,34]. As the mechanism of FR4-mediated Treg-cell maintenance, we considered initially that the proliferative activity of Treg cells could require large amounts of vitamin B9 as a source of nucleotides for DNA and RNA. However, the amounts of intracellular vitamin B9 were identical between Treg and non-Treg cells, implying that FR4 specifically recognizes extracellular vitamin B9 for the maintenance of Treg cell survival, consistent with a report that FR4 expressed on Treg cells contributes to their immune function and survival [25]. Additionally, the specific biological functions of vitamin B9 receptors (FR1, FR2, and FR4) have been predicted on the basis of their ~70% amino acid sequence identity, but the expression of each receptor is rigidly restricted, with narrow tissue and cell specificity [35,36]. Because FR1, FR2, and FR4 are glycosyl phosphatidylinositol–anchored proteins [37], adapter molecules may assist FR4 in the maintenance of Treg cell survival. We found that vitamin B9/FR4 was not associated with IL-2–mediated signaling in Treg cells. We will continue to study how FR4-mediated vitamin B9 regulates the survival of Treg cells.

Mammals must obtain vitamin B9 from the diet or from commensal bacteria. The absorption of vitamin B9 from the diet occurs mainly in the small intestine, whereas the uptake of microbial vitamin B9 predominantly occurs in the colon [38]. This explains why depletion of dietary vitamin B9 specifically decreased Treg cells in the small intestine, but not in the colon. It has been proposed that bacterial vitamin B9 absorbed in the colon affects the vitamin B9 status of the host [39,40], which may explain the lack of changes in vitamin B9 in the serum and splenic Treg cells in mice maintained with vitamin B9(−) diet. *Bifidobacterium*, one of the most important genera of commensal bacteria to be used as a probiotic, is well-studied as a vitamin B9 producer [41], and colonic Treg cells are specifically induced by immunological crosstalk with commensal bacteria, especially *Clostridium* clusters IV and XIVa [5]. Although whether *Clostridium* clusters IV and XIVa produce vitamin B9 remains unclear, our current findings suggest that vitamin B9 is an essential survival factor for Treg cells and, in vivo situation, diet vitamin B9 establishes an immunological network in the maintenance of Treg cells specifically in the small intestine.

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Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: JK EH II. Analyzed the data: JK EH II. Wrote the paper: JK HK.

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Alcaligenes is commensal bacteria habituating in the gut-associated lymphoid tissue for the regulation of intestinal IgA responses

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Secretory-immunoglobulin A (S-IgA) plays an important role in immunological defense in the intestine. It has been known for a long time that microbial stimulation is required for the development and maintenance of intestinal IgA production. Recent advances in genomic technology have made it possible to detect uncultivable commensal bacteria in the intestine and identify key bacteria in the regulation of innate and acquired mucosal immune responses. In this review, we focus on the immunological function of Peyer's patches (PPs), a major gut-associated lymphoid tissue, in the induction of intestinal IgA responses and the unique immunological interaction of PPs with commensal bacteria, especially *Alcaligenes*, a unique indigenous bacteria habituating inside PPs.

Keywords: Peyer's patch, IgA, commensal bacteria

INTRODUCTION

Secretory-immunoglobulin A (S-IgA) is predominantly observed in the intestine where it participates in immune defense (Mestecky et al., 2005; Brandtzaeg, 2010). S-IgA inhibits adherence of pathogens to host epithelial cells in the intestinal lumen and neutralizes pathogenic toxins by binding to the toxins' biologically active sites. Based on the immunological importance of S-IgA in immunosurveillance in the intestine, the development of oral vaccines has focused on the induction of antigen-specific S-IgA responses (Kunisawa et al., 2007). In addition to the immunosurveillance in the intestine, S-IgA antibody contributes to the establishment of beneficial gut commensal microbiota and thus dysfunction of S-IgA formation resulted in the alteration of normal bacterial flora (e.g., the reduction of Lactobacillus and increase of segmented filamentous bacteria, SFB; Suzuki et al., 2004).

Peyer's patches (PPs) are major gut-associated lymphoid tissue (GALT) where intestinal IgA responses are initiated and regulated by unique immunological crosstalk via cytokines [e.g., interleukin-4 (IL-4), IL-6, IL-21, and transforming growth factor-β (TGF-β)] and cell–cell interactions (e.g., via CD40/CD40 ligand interactions) among dendritic, T, and B cells (Kunisawa et al., 2008; Fagarasan et al., 2010). Thus, oral delivery of antigens to PPs is considered an important strategy for the effective induction of antigen-specific intestinal IgA responses (Kunisawa et al., 2011).

In addition to host-derived factors, microbial stimulation is also required for the maximum production of S-IgA in the intestine (Cebra et al., 2005). Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structure of GALT when compared with mice housed under SPF or conventional conditions (Weinstein and Cebra, 1991). Although it was reported that some commensal bacteria [e.g., SFB and altered Schaedler flora (ASF), a combined eight culturable bacteria] and bacterial products (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Talham et al., 1999; Butler et al., 2005), it is obscure which bacteria is involved in this process indigenously. Because predominant commensal bacteria in the intestine is uncultivable, it was difficult to determine by culture-based method which bacteria regulated specific immune responses. However, recent advances in the genomic analysis allowed us to identify the uncultivable bacteria, which revealed key bacteria in the regulation of specific immune responses (Ivanov et al., 2009; Atarashi et al., 2011) as well as the development of immune diseases (Chow et al., 2010; Hill and Artis, 2010). Using genomic and immunological methods, we recently found that the microbial community inside PPs is different from those on the epithelium of PPs or in the intestinal lumen (Obata et al., 2010).

In this review, we discuss initially the immunological features of PPs in the induction and regulation of intestinal IgA responses. In the later part, we focus on the unique cross-communication between PPs and habitat commensal bacteria, *Alcaligenes*, a unique indigenous bacteria habituating inside PPs and regulating dendritic cells (DCs) for the efficient production of intestinal IgA.

IMMUNOLOGICAL FEATURES OF PEYER'S PATCHES

In the intestine, GALT comprise several different, organized lymphoid structures (Spencer et al., 2009; Fagarasan et al., 2010). Among them, PPs are the largest and most well-characterized sites

for the initiation of intestinal IgA responses, especially responses to T cell-dependent antigens (Kunisawa et al., 2008; Fagarasan et al., 2010). There are generally 8–10 PPs in the mouse small intestine and hundreds in the human small intestine. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as the interfollicular region (**Figure 1**).

Inside PPs, antigen-sampling M cells located in the follicle-associated epithelium transport luminal antigens to DCs situated in the subepithelium region (Neutra et al., 2001), which then form clusters with T-, B-, and stromal cells in the germinal centers and promote μ-to-α-class-switch recombination of B cells with the help of cytokines such as IL-4, IL-21, and TGF-β (Fagarasan et al., 2010). Upon immunoglobulin class-switching from μ to α, IgA-committed B cells (IgA^+ B cells) begin to express type 1 sphingosine-1-phosphate receptor, CCR9, and α4β7 integrin, allowing them to depart from the PPs and subsequently traffic to the intestinal lamina propria (Mora et al., 2006; Gohda et al., 2008). In the intestinal lamina propria, they further differentiate into IgA-secreting plasma cells under the influence of terminal differentiation factors (e.g., IL-6; Cerutti et al., 2011). DCs play a key role in these processes. For instance, nitric oxide, TGF-β, APRIL, and BAFF produced by TNF-α/iNOS-producing DCs (Tip-DCs) promotes IgA production (Tezuka et al., 2007). Also, DCs in the PPs metabolite vitamin A and produce retinoic acid, which induces the expression of gut-homing receptors (CCR9, and α4β7 integrin) on activated B and T cells (Iwata et al., 2004; Mora et al., 2006). Retinoic acid also induces the preferential differentiation into regulatory T (Treg) cells (Hall et al., 2011), and some of Treg cells differentiated into follicular helper T cells to promote IgA production in the PPs (Tsuji et al., 2009).

The identification of the molecular pathway of PP organogenesis allowed the establishment of PP-deficient mice through the loss of any part of this pathway (Nishikawa et al., 2003). Notably, disruption of the PP organogenesis pathway by blockade of tissue genesis cytokine receptor signaling [IL-7R and/or lymphotoxin-β receptor (LTβR)] during a limited fetus time period results in the selective loss of PPs without affecting other lymphoid tissue organogenesis (Yoshida et al., 1999). Experiments with PP-deficient mice showed that the dependency on PPs in the induction of antigen-specific IgA responses depends on the form of the antigen. For instance, the PP-deficient mice failed to develop antigen-specific IgA responses against orally administered antigens in particle form, but retained their ability to respond to soluble forms of antigens (Yamamoto et al., 2000; Kunisawa et al., 2002). It was also reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the induction of intestinal IgA production (Martinoli et al., 2007). This is consistent with another finding that DCs in the PPs are responsible for intestinal IgA production (Fleeton et al., 2004). Therefore, PPs are considered to be one of the major sites for the initiation of intestinal antigen-specific IgA responses.

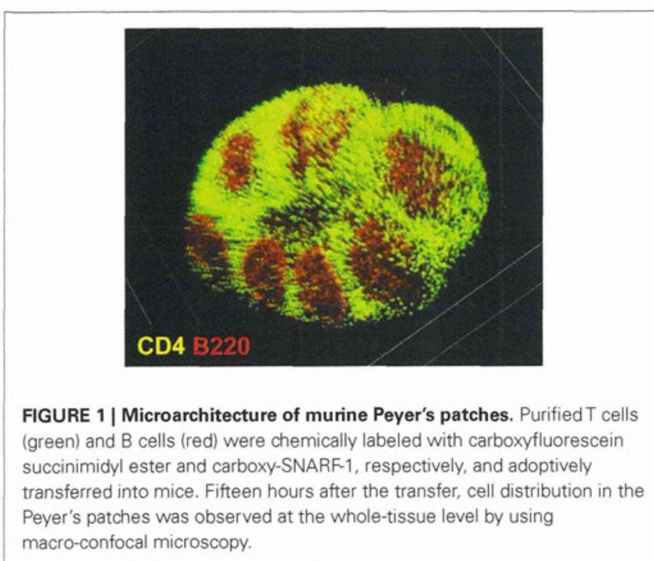

FIGURE 1 | Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and carboxy-SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole-tissue level by using macro-confocal microscopy.

EFFECT OF MICROBIAL STIMULATION ON THE PRODUCTION OF INTESTINAL IgA

It is well known that microbial stimulation is required for the full production of S-IgA in the intestine. Indeed, GF mice have decreased intestinal IgA responses when compared with mice housed under SPF or conventional conditions (Cebra et al., 2005). Studies using mono-associated GF mice with SFB have demonstrated that only a minor proportion of the total intestinal IgA is reactive to mono-associated bacteria (Talham et al., 1999). In addition, bacterial products produced by commonly expressed on commensal bacteria (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Butler et al., 2005). In contrast, a recent study using reversible colonization of GF mice with genetically engineered *E. coli* showed that intestinal IgA induced in those mice bound to parent strain but not other bacteria (Hapfelmeier et al., 2010). Therefore, it remains unclear whether intestinal IgA responses induced by commensal bacteria is mediated by polyclonal stimulation and/or by B cell receptors specific for microbial antigens.

As one mechanism of impaired IgA production of GF mice, it was reported that GF mice have structurally immature GALT (e.g., PPs and ILFs) when compared with SPF mice (Weinstein and Cebra, 1991; Hamada et al., 2002). In the PPs, several key pathways for the IgA production require microbial stimulation. For example, Tip-DCs enhance the IgA production by producing nitric oxide, TGF-β, APRIL, and BAFF, which requires microbial stimulation through innate receptors (Tezuka et al., 2007). Indeed, the number of Tip-DCs was much reduced in the intestine of GF and MyD88-deficient mice (Tezuka et al., 2007). Another cell involved the microbe-dependent IgA production is non-hematopoietic follicular DCs (FDCs). It was reported that microbial stimulation of FDCs resulted in expressing chemokine CXCL13, BAFF, and TGF-β for the germinal center formation and B cell class-switching from IgM to IgA (Suzuki et al., 2010).

ALCALIGENES IS A UNIQUE INDIGENOUS BACTERIA INSIDE PPs

Recent advances in genomic technology make it possible to detect commensal bacteria in the intestine, allowing identification of key bacteria involved in the regulation of specific immune responses. For example, SFB was identified as commensal bacteria inducing

Th17 cells (Ivanov et al., 2009), whereas colonic regulatory T cells were induced by *Clostridium* clusters IV and XIV (Atarashi et al., 2011). These commensal bacteria localize at the surface of intestinal epithelium, but we supposed that the immunological crosstalk between host and commensal bacteria might establish in the regulation of intestinal IgA responses in the GALT. In this issue, we analyzed the composition of the microbial community inside PPs and identified *Alcaligenes* as a major commensal bacteria uniquely locating inside PPs (Obata et al., 2010).

By using the 16S rRNA clone library method, SFB are the predominant commensal bacteria co-habitat on FAE of PPs as like small intestinal epithelium. Although the FAE consisted with antigen-sampling M cells, SFB was not found inside of PPs. Instead, *Alcaligenes* are predominant bacteria inside PPs. The result obtained by the 16S rRNA analysis was further confirmed by fluorescence *in situ* hybridization (FISH) method and thus *Alcaligenes* are present exclusively inside PPs, not on the FAE of PPs, and intestinal villous epithelium and intestinal lamina propria (**Figure 2**). Of note, the preferential presence of *Alcaligenes* was observed not only in mouse but also in monkey and human (Obata et al., 2010). One of interesting but unresolved questions is the species specificity of *Alcaligenes*. We are now investigating whether *Alcaligenes* isolated from human or monkey colonize in the PPs to promote IgA production when they are orally fed to GF mice. Inside PPs, a proportion of the *Alcaligenes* seemed to be alive in mice. The presence and growth of *Alcaligenes* were detected in the PPs of GF mice after adoptive transfer of PP homogenates containing *Alcaligenes* from SPF mice. These findings suggest that *Alcaligenes* are indigenous bacteria ubiquitously living inside the PPs of various mammalian species.

ANTIBODY-MEDIATED RECIPROCAL INTERACTION BETWEEN *ALCALIGENES* AND THE HOST IMMUNE SYSTEM

As mentioned above, M cells located on the FAE of PPs transport luminal bacteria into DCs locating at the subepithelial region of FAE (Neutra et al., 2001). 16S rRNA clone library methods consistently revealed that DCs in the PPs predominantly contain *Alcaligenes*, whereas these bacteria are rarely detected in DCs isolated from other lymphoid tissues (e.g., spleen and mesenteric lymph nodes; Obata et al., 2010). We examined the immunological effects of *Alcaligenes* on DCs and found that the production of IgA-enhancing cytokines such as IL-6, TGF-β, and BAFF was increased when DCs isolated from the PPs of GF mice were stimulated with *Alcaligenes* (Obata et al., 2010). Several lines of evidence have revealed that immunological functions of DCs are different between intestinal and other lymphoid tissues (reviewed in Rescigno, 2010), we are now investigating whether immune stimulatory functions of *Alcaligenes* is specific for the PP DCs or not.

In agreement with the uptake of *Alcaligenes* and subsequent production of IgA-enhancing cytokines by DCs, *Alcaligenes*-specific IgA-forming cells were frequently observed in PPs, and consequent IgA production was noted in the intestinal lumen of SPF mice, but not GF mice (Obata et al., 2010). Although biological role of *Alcaligenes*-specific IgA antibody remains to be elucidated, the antibody might be involved in the creation of intra-tissue co-habitation of *Alcaligenes* in PPs. To this end, the number of *Alcaligenes* inside PPs is decreased in B cell-deficient CBA/N xid and IgA-deficient mice compared with wild-type mice (Obata et al., 2010). Therefore, it is interesting to suggest that *Alcaligenes*-specific IgA antibody mediates the uptake and presence of *Alcaligenes* in the PPs. Since M cells express IgA receptors

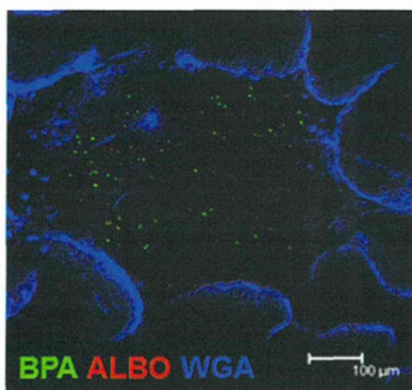

FIGURE 2 | Microarchitecture of murine Peyer's patches. Whole-mount fluorescence *in situ* hybridization was performed to visually analyze the presence of *Alcaligenes* inside PPs. Both BPA and ALBO34a were used as specific probes for *Alcaligenes*. Wheat germ agglutinin (WGA), an *N*-acetylglucosamine-specific lectin, was used to detect epithelial cells. Scale bar indicates 100 μm.

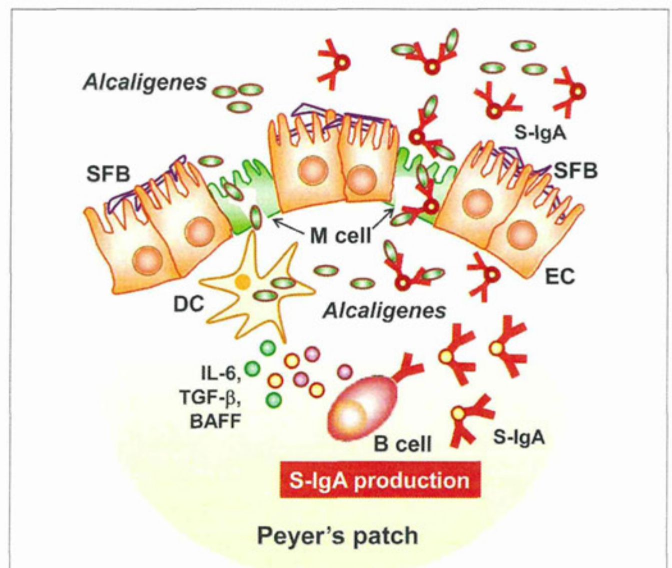

FIGURE 3 | *Alcaligenes* mediates symbiotic communication inside Peyer's patches. On the follicle-associated epithelium of PPs, segmented filamentous bacteria (SFB) is predominantly observed. In contrast, *Alcaligenes* specifically localizes inside Peyer's patches, where some are taken up by dendritic cells (DCs). Stimulation by *Alcaligenes* prompts the DCs to produce IgA-enhancing cytokines [e.g., interleukin-6 (IL-6), transforming growth factor-β (TGF-β)], and B cell activating factor (BAFF), which enhance the intestinal IgA response. The intestinal IgA includes *Alcaligenes*-specific IgA, which might mediate the preferential uptake and presence of *Alcaligenes* in the PPs. The uptake is presumably mediated by M cells.

(Mantis et al., 2002), one possibility is that *Alcaligenes* coated with the *Alcaligenes*-specific antibody are taken up into PPs through M cells. Further, the antigen-specific IgA coating on *Alcaligenes* might be beneficial for the bacteria to create the co-habitation niche since IgA antibody has been shown to non-inflammatory antibody (Mestecky et al., 2005).

CONCLUSION

In this review, we discussed a new concept of symbiotic communication in PPs that is mediated by commensal bacteria-specific IgA antibody. *Alcaligenes*-specific antibodies may mediate the uptake and the presence of *Alcaligenes* in the PPs, and the co-habitation of *Alcaligenes* within the PPs is one of the key factors to promote the intestinal IgA production by enhancing the production of IgA-enhancing cytokines from DCs (**Figure 3**). We still have various questions regarding this co-habitation of *Alcaligenes* in the PPs. For example, it remains unclear whether the presence of *Alcaligenes* inside of PPs is physiologically beneficial or harmful for the host immune system. In this issue, we are now addressing the microbial community in the PPs of mice and human patients suffering from intestinal immune diseases (e.g., intestinal inflammation and allergy). The biological roles of intra-tissue habitation of *Alcaligenes* in the PPs in the appropriate regulation of mucosal immune responses need to be elucidated. The current goal is to elucidate the mechanisms behind the co-habitation of *Alcaligenes* within PPs, and the exact contribution of *Alcaligenes* to educate and guide mucosal immunocompetent cells especially DCs in the PPs for the development, maturation and maintenance of the appropriate host immune system. These studies will provide novel molecular and cellular mechanisms of symbiotic communication with commensal bacteria in the regulation of host immunity.

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Review

Immunological Function of Sphingosine 1-Phosphate in the Intestine

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Abstract: It has been shown that dietary materials are involved in immune regulation in the intestine. Lipids mediate immune regulation through a complex metabolic network that produces many kinds of lipid mediators. Sphingosine-1-phosphate (S1P) is a lipid mediator that controls cell trafficking and activation. In this review, we focus on the immunological functions of S1P in the regulation of intestinal immune responses such as immunoglobulin A production and unique T cell trafficking, and its role in the development of intestinal immune diseases such as food allergies and intestinal inflammation, and also discuss the relationship between dietary materials and S1P metabolism.

Keywords: intestinal immunity; lipid; IgA antibody; intraepithelial T lymphocytes; food allergy

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1. Introduction

It is generally accepted that dietary components are involved in immune regulation. The intestinal immune system, especially, seems to be directly affected by the digestion and absorption of dietary materials. Intestinal tissues are primary sites for infection by many pathogenic microorganisms, and commensal bacteria are abundant. Thus, the intestinal immune system has to create harmonious immunological condition, and the disruption of the intestinal immune homeostasis leads to the development of allergic, inflammatory, and infectious diseases [1,2].

Dietary lipids seem to be the dietary materials most involved in the regulation of intestinal immune responses after the conversion into lipid mediators [3]. Among various lipid mediators, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that regulates cell trafficking and activation [4,5]. S1P is abundantly present in the blood and lymph, which is originated from the cell membranes from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells [6]. It is degraded by S1P lyase in the lymphoid tissues [7]. This metabolic pathway establishes an S1P gradient between the blood/lymph and lymphoid tissues and mediates cell trafficking.

The S1P gradient is recognized by cells expressing S1P receptors, and these cells migrate toward high concentrations of S1P. Of the five types of S1P receptor, type-1 S1P receptors (S1P1) are preferentially expressed by lymphocytes, and they determine lymphocyte emigration from and retention in the lymphoid tissues [8]. S1P1 is highly expressed in naive lymphocytes, including single-positive thymocytes expressing either CD4 or CD8, and expression is decreased upon lymphocyte activation. S1P1 expression recovers once the activated lymphocytes are fully differentiated and this recovery leads to their emigration from the lymphoid tissues into the blood circulation [4,5]. Studies indicate that the trafficking of macrophages, dendritic cells, and natural killer cells is mediated by S1P2, S1P3, and S1P5, respectively [9–11].

Recent studies have revealed additional functions of S1P in immune regulation that are independent of cell trafficking [4]. For example, differentiation of T cells is regulated by S1P1-mediated signaling [12–14]. It has also been demonstrated that a S1P2-mediated pathway is involved in the activation of mast cells [15] and macrophages [16], and that S1P3 are involved in dendritic cell endocytosis [10]. These findings together suggested that the S1P plays critical role in the activation and differentiation of immunocompetent cells involved in the both innate and acquired phases of immune responses in addition to their function of cell trafficking.

These biological and immunological functions show that S1P is involved in the maintenance of immunosurveillance as well as the development of immune diseases. In this review, we discuss the relationship between dietary materials (e.g., lipids, vitamin, and colorant) and S1P metabolism and describe the immunological functions of S1P, such as regulation of immunoglobulin A (IgA) production and intraepithelial T-lymphocyte trafficking, and its role in the development of intestinal immune diseases such as food allergy and intestinal inflammation.

2. Relationship Between S1P and Dietary Lipids

Several lines of evidence demonstrate that intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues [17]. There is no evidence of intestinal uptake of sphingolipids from