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Pyruvate enhances oral tolerance via GPR31

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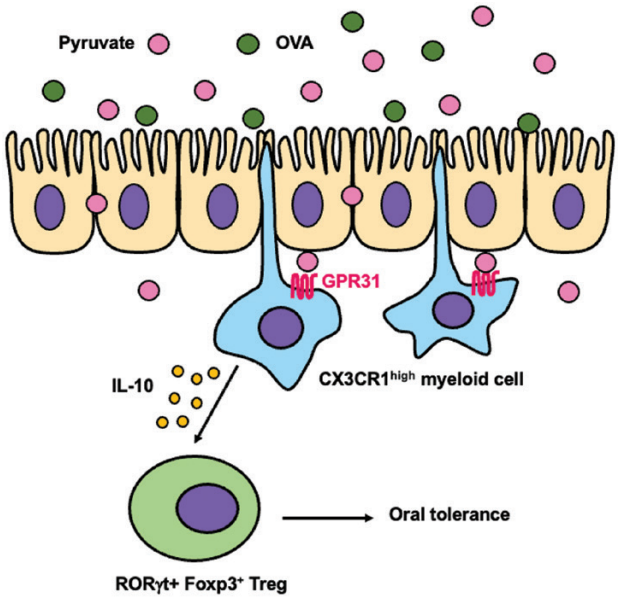
Abstract

CX3CR1^{high} myeloid cells in the small intestine mediate the induction of oral tolerance by driving regulatory T (Treg) cells. Bacterial metabolites, e.g. pyruvate and lactate, induce a dendrite extension of CX3CR1^{high} myeloid cells into the intestinal lumen via GPR31. However, it remains unclear whether the pyruvate–GPR31 axis is involved in the induction of oral tolerance. Here, we show that pyruvate enhances oral tolerance in a GPR31-dependent manner. In ovalbumin (OVA)-fed Gpr31-deficient mice, an OVA-induced delayed-type hypersensitivity response was substantially induced, demonstrating the defective induction of oral tolerance in Gpr31-deficient mice. The percentage of RORγt^{+} Treg cells in the small intestine was reduced in Gpr31-deficient mice. In pyruvate-treated wild-type mice, a low dose of OVA efficiently induced oral tolerance. IL-10 production from intestinal CX3CR1^{high} myeloid cells was increased by OVA ingestion in wild-type mice, but not in Gpr31-deficient mice. CX3CR1^{high} myeloid cell-specific IL-10-deficient mice showed a defective induction of oral tolerance to OVA and a decreased accumulation of OVA-specific Treg cells in the small intestine. These findings demonstrate that pyruvate enhances oral tolerance through a GPR31-dependent effect on intestinal CX3CR1^{high} myeloid cells.

Graphical abstract



Keywords: bacterial metabolites, IL-10, mucosal immunity, oral tolerance, Treg

Introduction

The suppressive state of immune responses against antigens that the host was exposed to in the gastrointestinal tract has been recognized for a long time and is known as oral tolerance (1–3). Oral tolerance is required for the prevention of immune disorders at the mucosal tissues, such as food allergy, celiac disease and inflammatory bowel disease. Disruption of oral tolerance is further implicated in the pathogenesis of systemic immune disorders. Accordingly, the mechanisms for the induction of oral tolerance have been extensively investigated.

Initially, the depletion of antigen-specific T cells in the gut-associated lymphoid tissues (GALTs) of mice orally administered the antigen was demonstrated to be responsible for the induction of oral tolerance (4). Subsequently, the central role of Foxp3$^+$ regulatory T (Treg) cells in mediating oral tolerance was shown in many studies (5–9). In addition to their development in the thymus, Treg cells are induced in the peripheral organs, particularly the intestine (10). A substantial proportion of intestinal Treg cells express RORγt and mediate oral tolerance and the suppression of colonic inflammation (11–13). The development of RORγt$^+$ Treg cells in the colon and small intestine is induced by microbiota and dietary antigens, respectively (12, 14, 15). Intestinal Treg cells develop in the GALTs, such as the mesenteric lymph nodes, through instruction by CD103$^+$ dendritic cells that migrate to there after receiving fed antigens via CX3CR1$^+$ myeloid cells in the lamina propria (16–18). Treg cells that differentiate in the GALTs are then recruited to the intestinal lamina propria to suppress the immune responses to dietary antigens and microbiota. Several mechanisms have been proposed to be responsible for the maintenance of intestinal Treg cells in the lamina propria. A recent study indicates that group 3 innate lymphoid cells (ILC3) support RORγt$^+$ Treg cells in the intestine through the production of IL-2 (7). In addition, IL-10 produced by intestinal macrophages has been implicated in the maintenance of intestinal Treg cells (19, 20). Although the induction of oral tolerance in IL-10-deficient mice is still controversial (21, 22), IL-10 was shown to enhance the oral tolerance (23). Among several subsets of intestinal macrophages, myeloid cells that highly express CX3CR1 have been shown to produce IL-10 and induce Treg cells. Indeed, intestinal CX3CR1^{high} myeloid cells were shown to mediate oral tolerance (8, 18, 24).

Intestinal CX3CR1^{high} myeloid cells residing just beneath the small intestinal epithelial layer possess the unique property of protruding their dendrites into the lumen for the direct uptake of luminal antigens (25, 26). Dendrite protrusion by intestinal CX3CR1^{high} myeloid cells has been shown to be mediated by bacterial metabolites, e.g. pyruvate and lactate, via GPR31 (27). However, it remains unclear whether the GPR31-mediated dendrite extension of CX3CR1^{high} myeloid cells is involved in the induction of oral tolerance. In this study, we investigated whether oral tolerance is induced in the absence of GPR31 and whether the GPR31 ligand pyruvate regulates oral tolerance.

Methods

Mice

C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). *Gpr31b*$^{-/-}$ mice were generated as previously described (27). CD45.1 mice and *Cx3cr1*^{creER} knockin mice were purchased from the Jackson Laboratory. *Cx3cr1*^{gfp} knockin mice (28), OT-II mice (29) and *Il10*^{lox} mice (30) have been described previously. The mice were maintained under specific pathogen-free (SPF) conditions. Age- and sex-matched mice were used for experiments. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

Cell isolation

Cells in the small intestinal lamina propria were isolated as previously described (31) with minor modifications. In brief, small intestinal tissues were opened longitudinally and washed extensively with phosphate-buffered saline (PBS) after removal of the Peyer's patches. Small intestinal segments were incubated in PBS containing 10 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 30 min and then washed several times with PBS. These small intestinal segments were next digested with continuous stirring in RPMI 1640 with 10% fetal calf serum (FCS), 400 U ml^{-1} of collagenase D (Roche, Basel, Switzerland) and 100 μg ml^{-1} of DNase I (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 45 min. Low-density myeloid cells were enriched by the use of 17.5% Histodenz (Sigma-Aldrich) solution. Lymphocytes were enriched by the use of 40 and 80% Percoll (GE Healthcare, Chicago, IL, USA).

Cells from the colonic lamina propria were prepared as previously described (32) with minor modifications. In brief, the colons were opened longitudinally and washed with PBS to remove the feces. The colons were then shaken in Hanks' Balanced Salt Solution (HBSS) with 5 mM EDTA and incubated at 37°C for 20 min. After being washed in PBS, the tissues were cut into small pieces and incubated in RPMI 1640 containing 4% FCS, 1 mg ml^{-1} of collagenase D, 0.5 mg ml^{-1} of dispase (Thermo Fisher Scientific, Waltham, MA, USA) and 40 μg ml^{-1} of DNase I for 40 min at 37°C in a shaking water bath. Lymphocytes were enriched by the use of 40 and 80% Percoll.

Cells from spleens and mesenteric lymph nodes were prepared as previously described (32) with minor modifications. In brief, spleens and mesenteric lymph nodes were cut into small fragments that were then ground between glass slides, and the cells from these lysates were passed through 40-μm-pore-sized nylon meshes and suspended in RPMI 1640 containing 10% FCS. Splenocytes were treated with red blood cell (RBC) lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA) for 2 min before their suspension in RPMI 1640 containing 10% FCS.

Pyruvate administration

Sodium pyruvate (Sigma-Aldrich) was orally administered to mice via their drinking water at a concentration of 50 mM for the indicated periods. For the oral tolerance and adoptive transfer experiments, sodium pyruvate was administered 4 weeks before and throughout the experiments.

Induction and measurement of delayed-type hypersensitivity responses

Mice were tolerized through gavage with two doses of 50 mg of ovalbumin (OVA) (Grade III, Sigma-Aldrich) on 2

consecutive days. One week later, these mice were immunized via a subcutaneous injection with 300 μg of OVA (Grade VI, Sigma-Aldrich) in 200 μl of an emulsion of PBS and complete Freud's adjuvant (Sigma-Aldrich). Two weeks after immunization, these mice were challenged via a subcutaneous injection with 50 μg of OVA (Grade VI, Sigma-Aldrich) in 20 μl of PBS into the right ear pinna while 20 μl of PBS without OVA were injected into the left ear pinna for control purposes. Ear thicknesses were measured with a digimatic micrometer (Mitutoyo, Kawasaki, Japan) before and 48 h after injection. OVA-specific ear swelling was calculated as follows: $(\text{right ear thickness} - \text{left ear thickness})_{48h} - (\text{right ear thickness} - \text{left ear thickness})_{0h}$.

For OVA-specific IgG1 quantification, mice were further challenged through subcutaneous injection with 300 μg of OVA (Grade VI, Sigma-Aldrich) 1 week after ear thickness measurement. Three days later, serum was collected for use in an enzyme-linked immunosorbent assay (ELISA).

For the evaluation of IL-10 production by CX3CR1^{high} cells, mice were challenged via a subcutaneous injection with 300 μg of OVA (Grade VI, Sigma-Aldrich) at 2 weeks post-immunization. Three days later, CX3CR1^{high} cells were collected from the small intestinal lamina propria. These cells were cultured at a concentration of 10^5 cells/100 μl RPMI 1640/10% FCS in wells pre-coated with fibronectin (Corning, Corning, NY, USA). The supernatants were harvested 16 h later, and the concentration of IL-10 was measured by ELISA.

Enzyme-linked immunosorbent immunoassay

For OVA-specific IgG1 quantification, 96-well plates (Corning) were coated with 40 μg ml^{-1} OVA (Grade VI, Sigma-Aldrich) in PBS for 16 h at 4°C. The plates were then washed and blocked in 1% bovine serum albumin (BSA)/PBS for 2 h. After the plates were washed again, mouse serum, diluted 10^5–10^6 times in 1% BSA/PBS, and anti-OVA IgG1 (Cayman Chemical, Ann Arbor, MI, USA) at concentrations ranging from 0.781 to 100 ng ml^{-1} were added to the wells and incubated for 2 h at room temperature. OVA-specific IgG1 was detected by using a horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody (Jackson ImmunoResearch, West Grove, PA, USA). Finally, a substrate solution (R&D Systems, Minneapolis, MN, USA) and 2 N H_2SO_4 were added to the wells, and the plates were read at 450 nm with a spectrometer.

For IL-10 quantification, the concentration of IL-10 in the culture supernatant of CX3CR1^{high} cells was measured by using a Mouse IL-10 DuoSet ELISA (R&D Systems) in accordance with the manufacturer's protocol.

Flow cytometry and sorting

The antibodies used for flow cytometry and magnetic-activated cell sorting (Miltenyi Biotec, Auburn, CA, USA) are listed in Supplementary Table 1. Dead cells were stained with 7-AAD Viability Staining Solution (BioLegend, San Diego, CA, USA). Flow cytometric analysis was performed with a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FlowJo software (Tree Star, Ashland, OR, USA).

For the isolation of CX3CR1^{high} cells, cells from the small intestinal lamina propria were stained with 7-AAD and anti-CD45, anti-CD11b, anti-CD11c, anti-CD64 and anti-CX3CR1 antibodies. 7AAD$^-$CD45$^+$CD11b$^+$CD11c$^+$CD64$^+$CX3CR1$^+$ cells were isolated with a FACSAria flow cytometer (BD Biosciences).

For intracellular nuclear protein staining, the cells were stained with anti-Foxp3, anti-RORγt and anti-GATA3 antibodies after their fixation and permeabilization with Fixation/Permeabilization Solution (Invitrogen, Waltham, MA, USA).

The instrument compensation was set in each experiment by using single-color, two-color or four-color stained samples.

Adoptive cell transfer

Naive OT-II T cells (CD3$^+$CD4$^+$CD25$^-$7AAD$^-$ cells) were purified from the spleens and mesenteric lymph nodes of OT-II transgenic mice (CD45.1 C57BL6/J strain) by using a combination of magnetic-activated cell sorting (Miltenyi Biotec) and flow cytometry performed on a FACSAria (BD Biosciences) with anti-cell surface marker antibodies. The purified naive OT-II T cells (4×10^6 cells) were intravenously transferred into recipient mice (CD45.2 C57BL6/J strain) on day 0. These mice received two doses of 50 mg of OVA through gavage on day 1 and day 2. The cells collected from the small intestinal lamina propria of the recipient mice at 12 days post-transfer were analyzed by flow cytometry.

OVA uptake experiment

The OVA injection surgery was performed as previously described (33) with minor modifications. Under combined anesthesia with medetomidine (0.3 mg kg^{-1}), midazolam (4 mg kg^{-1}) and butorphanol tartrate (5 mg kg^{-1}), the abdominal skin and peritoneal membrane were incised, and then the proximal small intestine was exteriorized. Next, 150 μg of OVA or Alexa Fluor 647-conjugated OVA (Invitrogen) in 200 μl of PBS were injected into the proximal small intestine. The intestine was reintroduced into the abdominal cavity, and the incision was closed with wound clips. Mice were maintained under anesthesia for 1 h before sacrifice.

One hour after OVA administration, the proximal half of the small intestine was collected, and the percentage of CX3CR1^{high} cells from the proximal small intestine containing Alexa Fluor 647-conjugated OVA was analyzed by using a FACSCanto II flow cytometer (BD Biosciences) with FlowJo software (Tree Star).

Generation of CX3CR1^{high} cell-specific IL-10-deficient mice

Cx3cr1$^{creER/+}$ *Il10*$^{flox/flox}$ mice, in which a deletion of *Il10* gene (exon1) in CX3CR1^{high} cells is induced by tamoxifen treatment, were generated by mating *Cx3cr1C*^{reER} knockin mice and *Il10*-flox mice. Tamoxifen treatment was performed as previously described (34). In brief, tamoxifen (Sigma-Aldrich) was given as a solution in corn oil (Nacalai Tesque, Kyoto, Japan) by intra-peritoneal injection or oral gavage. Mice received five doses of tamoxifen (2 mg per dose of tamoxifen for intra-peritoneal injection, 3 mg per dose of tamoxifen for oral gavage), with a separation of 24 h between doses. When indicated, mice were given an intra-peritoneal injection with one dose of 2 mg of tamoxifen per week.

To confirm the deletion of the *Il10* allele in CX3CR1^{high} cells, genomic DNA was isolated from CX3CR1^{high} cells by cell lysis with 50 mM NaOH at 98°C for 1 h and subjected to polymerase chain reaction (PCR) performed with two pairs of *Il10*-specific primers: 5′-CCAGCATAGAGAG CTTGCATTACA-3′ and 5′-GAGTCGGTTAGCAGTATGTTG TCCAG-3′ for detecting the *Il10*^{flox} or *Il10*^{wt} alleles (35) and 5′-CAGGATTTGACAGTGCTA GAGC-3′ and 5′-AAACCCAGC TCAAATCTCCTGC-3′ for detecting the *Il10* allele lacking the floxed fragment (34).

Statistical analysis

Differences between the control and experimental groups were evaluated by a two-tailed unpaired Student's *t*-test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism 9.1.2. Differences where the *P*-value was <0.05 were considered statically significant.

Results

Defective induction of oral tolerance in Gpr31-deficient mice

To analyze whether GPR31, which is expressed on CX3CR1^{high} myeloid cells in the small intestine, is involved in the induction of oral tolerance to fed antigens, we examined a delayed-type hypersensitivity (DTH) response to OVA in OVA-fed mice. Wild-type, *Cx3cr1*$^{gfp/gfp}$ and *Gpr31b*$^{-/-}$ mice were gavaged with two doses of 50 mg of OVA or PBS on days 0 and 1, immunized subcutaneously with OVA and complete Freund's adjuvant on day 8, and subcutaneously challenged with OVA in the ear pinna on day 22. The ear thickness was then measured 48 h after the OVA challenge (Fig. 1A and B). Prior oral administration of OVA caused a marked reduction in the amount of ear swelling in OVA-challenged wild-type mice, but, as previously reported, *Cx3cr1*$^{gfp/gfp}$ mice were defective in the induction of oral tolerance, and OVA-fed *Cx3cr1*$^{gfp/gfp}$ mice showed severe ear swelling after the OVA challenge. Like *Cx3cr1*$^{gfp/gfp}$ mice, *Gpr31b*$^{-/-}$ mice did not develop oral tolerance, and OVA-fed *Gpr31b*$^{-/-}$ mice had thickened ears after the OVA challenge. We also measured the concentration of OVA-specific IgG1 in the sera of mice 3 days after the second subcutaneous OVA challenge (Fig. 1A and C). The anti-OVA IgG1 concentration was significantly lower in the sera of OVA-fed wild-type mice compared to those wild-type mice that were not fed with OVA. In contrast, the serum concentration of anti-OVA IgG1 was comparable after the OVA challenge in both OVA-fed and -unfed *Gpr31b*$^{-/-}$ mice, thus indicating that OVA-fed *Gpr31b*$^{-/-}$ mice exhibit DTH responses to OVA. These findings demonstrate that *Gpr31b*$^{-/-}$ mice display a defective induction of oral tolerance to fed antigens.

Reduced percentage of intestinal RORγt^{+} Treg cells in Gpr31-deficient mice

Foxp3^{+} Treg cells in the small intestine, many of which express RORγt, have been shown to mediate oral tolerance (5–9, 13). Therefore, we analyzed the percentages of Foxp3^{+} Treg cells and RORγt^{+}Foxp3^{+} Treg cells in the lamina propria of the small intestine in *Gpr31b*$^{-/-}$ mice (Fig. 2A–C). The percentage of Foxp3^{+} cells among the CD4^{+} T cells in the small intestine of *Gpr31b*$^{-/-}$ mice was comparable to that in wild-type mice. However, the percentage of RORγt^{+}Foxp3^{+} cells among this cell population was markedly lower than that in wild-type mice. The percentages of RORγt^{+}Foxp3^{+} cells among the CD4^{+} T cells in other tissues, such as the colon, mesenteric lymph nodes, peripheral lymph nodes and the spleen, were not lower in *Gpr31b*$^{-/-}$ mice than in wild-type mice (Supplementary Fig. 1). These findings indicate that the percentage of RORγt^{+}Foxp3^{+} Treg cells was selectively reduced in the small intestine of *Gpr31b*$^{-/-}$ mice.

Next, we assessed the percentage of dietary antigen-specific RORγt^{+}Foxp3^{+} Treg cells in the small intestine by using OVA-specific OT-II T-cell receptor transgenic (CD45.1^{+} OT-II) mice (Fig. 2D and E). Naive CD4^{+} T cells were isolated from the spleen and mesenteric lymph nodes of CD45.1^{+} OT-II mice and were adoptively transferred into congenic CD45.2^{+} wild-type or *Gpr31b*$^{-/-}$ mice. OVA was then orally administered on days 1 and 2 after the adoptive transfer. At 10 days after the OVA administration, the percentage of CD45.1^{+}RORγt^{+}Foxp3^{+} Treg cells in the small intestine was substantially lower in the *Gpr31b*$^{-/-}$ recipient mice compared with the wild-type recipient mice. Together, these findings indicate that GPR31 is required for the maintenance of dietary antigen-specific RORγt^{+}Foxp3^{+} Treg cells in the small intestine of mice.

Efficient induction of oral tolerance by pyruvate

GPR31 has been shown to be essential for the pyruvate-dependent dendrite extension of CX3CR1^{high} myeloid cells into the small intestinal lumen (27). Therefore, in the next experiment, we analyzed whether oral treatment with pyruvate enhances the oral tolerance. The OVA-induced DTH response was assessed in wild-type and *Gpr31b*$^{-/-}$ mice that had been orally treated with pyruvate for 4 weeks or left untreated before being fed a low dose (10 mg per day for 2 days) of OVA. Oral administration of a low dose of OVA did not reduce the amount of ear swelling after OVA challenge in wild-type mice that were not treated with pyruvate, indicating that the low dose of OVA did not induce oral tolerance (Fig. 3A), whereas in wild-type mice that were orally treated with pyruvate, the subsequent oral administration of a low dose of OVA effectively induced oral tolerance. In contrast, in pyruvate-treated *Gpr31b*$^{-/-}$ mice, a subcutaneous OVA challenge induced ear swelling in both groups of mice, i.e. those with or without oral administration of a low dose of OVA. These findings indicate that pyruvate enhances oral tolerance via GPR31.

We next analyzed whether oral pyruvate treatment increases dietary antigen-specific RORγt^{+}Foxp3^{+} Treg cells (Fig. 3B). Naive CD4^{+} T cells isolated from the spleen and mesenteric lymph nodes of CD45.1^{+} OT-II mice were adoptively transferred to congenic CD45.2^{+} wild-type or *Gpr31b*$^{-/-}$ mice. These mice were left untreated or orally treated with pyruvate from 4 weeks before the adoptive transfer until the end of the experiment, and OVA was orally administered on days 1 and 2 after the transfer. At 10 days after the OVA administration, the percentage of CD45.1^{+}RORγt^{+}Foxp3^{+} Treg cells in the small intestine was analyzed. Pyruvate-treated wild-type mice exhibited a higher percentage of CD45.1^{+}RORγt^{+}Foxp3^{+} Treg

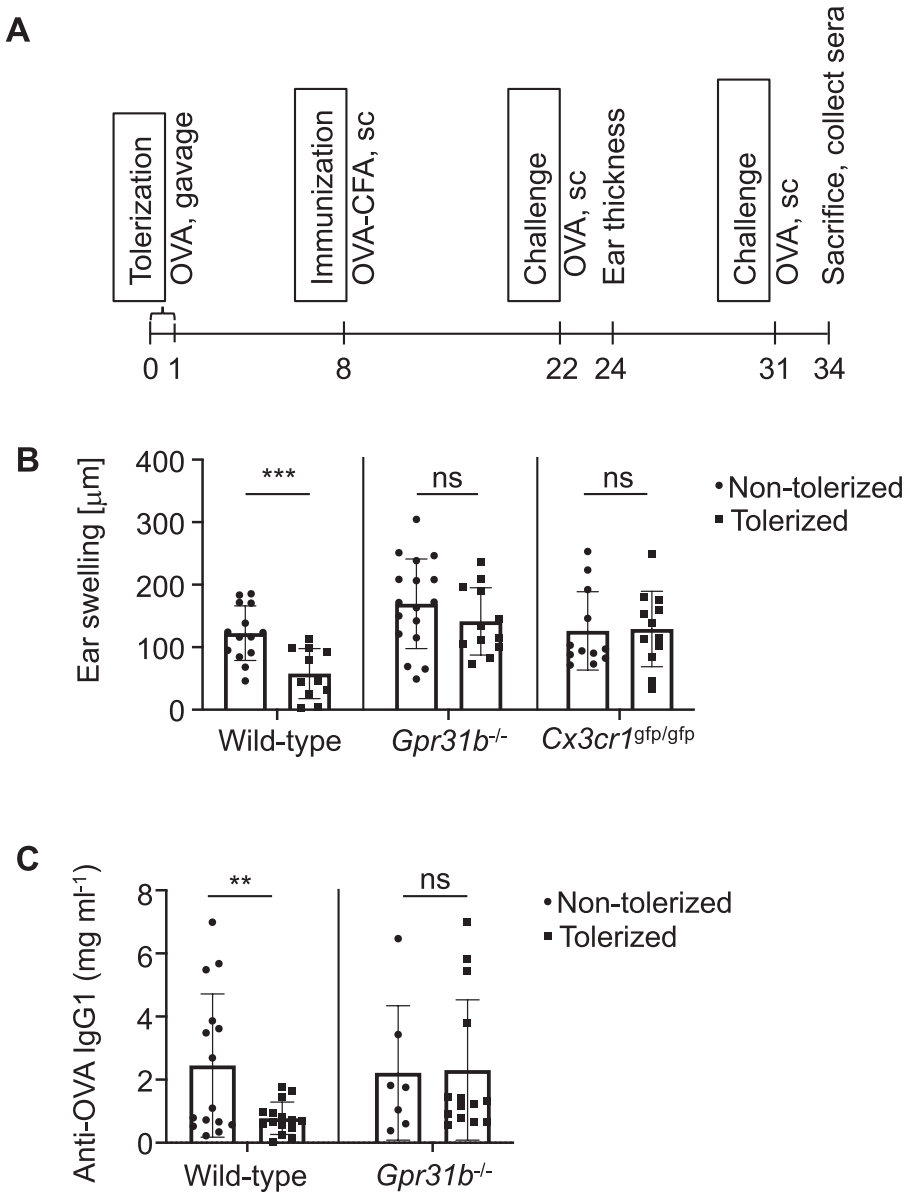


Fig. 1. GPR31 is required for oral tolerance to dietary antigen. (A) Experimental protocol for the evaluation of oral tolerance to OVA in a DTH model. CFA: complete Freund's adjuvant, sc: subcutaneous injection. (B) Oral tolerance in wild-type, *Gpr31b*$^{-/-}$ and *Cx3cr1*$^{gfp/gfp}$ mice was determined by measuring the amount of ear swelling in a DTH model. Data are pooled from three independent experiments. (C) Serum concentration of anti-OVA IgG1 in wild-type and *Gpr31b*$^{-/-}$ mice with or without tolerization. Results are presented as the mean ± SD. A two-tailed Student's *t*-test was performed to determine statistically significant differences between groups. $^{**}P < 0.01$, $^{***}P < 0.001$, ns: not significant.

cells in the small intestine compared with untreated wild-type mice, whereas the percentage of these cells was not altered by pyruvate treatment in *Gpr31b*$^{-/-}$ mice. Together, these findings indicate that pyruvate increases the percentage of intestinal RORγt^{+}Foxp3^{+} Treg cells and enhances oral tolerance in a GPR31-dependent manner.

IL-10 produced from intestinal CX3CR1^{high} myeloid cells mediates oral tolerance

Pyruvate has been shown to induce dendrite extension from CX3CR1^{high} myeloid cells in the small intestine (27). Because intestinal CX3CR1^{high} myeloid cells have been shown to directly uptake luminal antigens by extending their dendrites into the lumen (18, 25, 26, 33), we analyzed whether pyruvate enhances the uptake of luminal antigens by CX3CR1^{high} myeloid cells. Alexa Fluor 647-labeled OVA was injected into the gut lumen of mice that were left untreated or orally treated with pyruvate for 3 weeks. The percentage of CX3CR1^{high} myeloid cells containing dye-labeled OVA in the small intestine 1 h after the OVA injection was analyzed by flow cytometry (Supplementary Fig. 2A–C). Pyruvate treatment did not increase the fluorescence intensity of CX3CR1^{high} myeloid cells. In addition, similar to the previous result showing the normal uptake of injected OVA by CX3CR1$^{gfp/gfp}$ myeloid cells (33), OVA uptake was not impaired in the CX3CR1^{high} myeloid

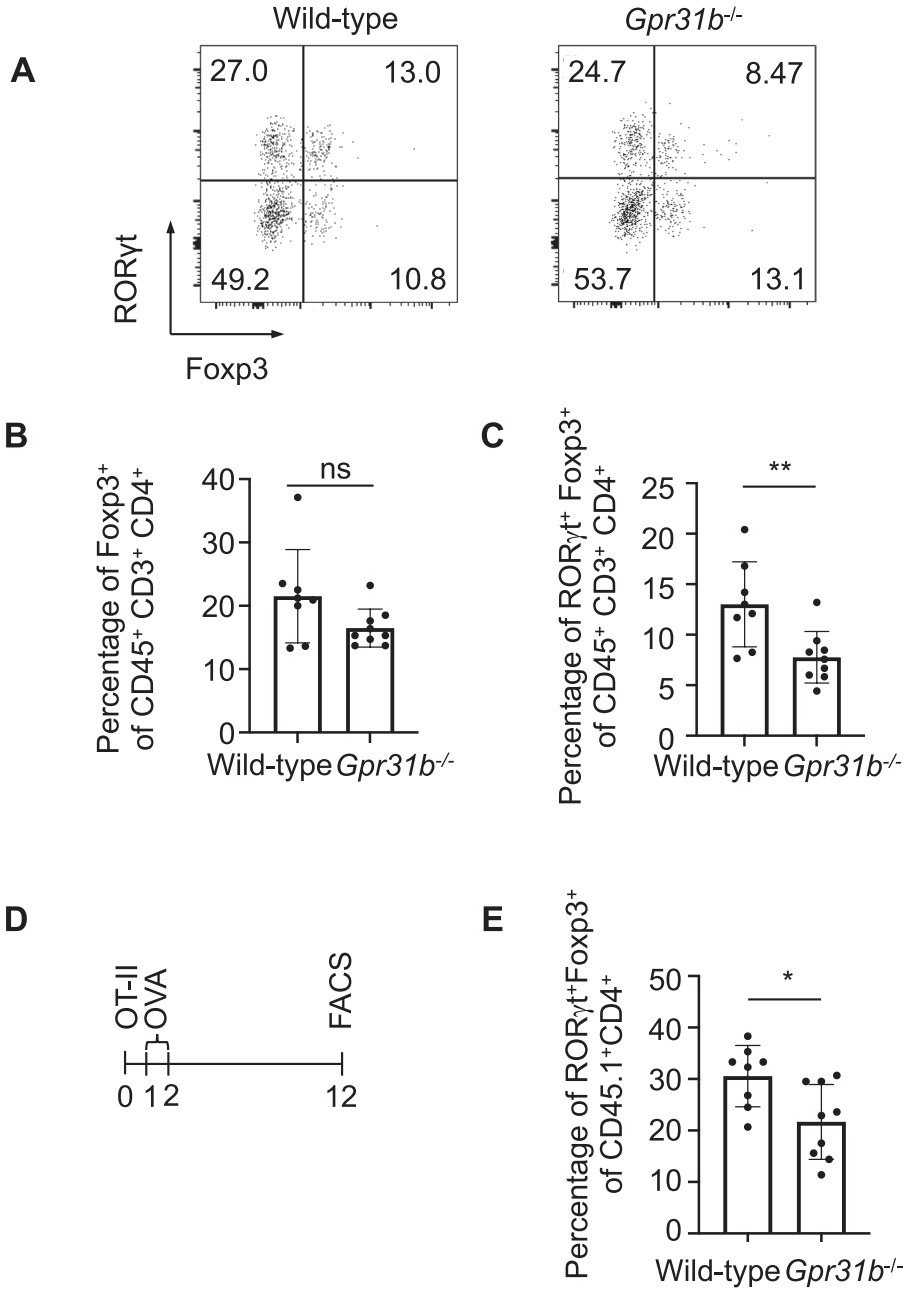


Fig. 2. The frequency and accumulation of RORγt⁺ Treg cells is reduced in the small intestinal lamina propria of Gpr31-deficient mice. (A) Representative flow cytometric plots of CD4⁺ T cells, stained with anti-RORγt antibody and anti-Foxp3 antibody, from the small intestinal lamina propria of wild-type or *Gpr31b*$^{-/-}$ mice. The numbers of the plots indicate the percentages of cells in each compartment. (B and C) The percentages of Foxp3⁺ Treg cells among CD4⁺ T cells (B) and of RORγt⁺Foxp3⁺ Treg cells among CD4⁺ T cells (C) in the small intestinal lamina propria of wild-type mice or *Gpr31b*$^{-/-}$ mice are shown. Data are pooled from three independent experiments. (D and E) Naive CD4⁺ T cells from OT-II TCR transgenic mice (CD45.1) were transferred to wild-type and *Gpr31b*$^{-/-}$ mice (CD45.2). CD45.1⁺CD4⁺ T cells in the small intestinal lamina propria of the recipient mice were analyzed by flow cytometry after gavage with OVA. An overview of the experimental protocol is shown in (D). The percentages of RORγt⁺Foxp3⁺ Treg cells among the CD45.1⁺CD4⁺ T cells (E) are shown. Data are pooled from five independent experiments. Results are presented as the mean ± SD. A two-tailed Student's *t*-test was performed to determine statistically significant differences between groups. **P* < 0.05, ***P* < 0.01, ns: not significant.

cells of *Gpr31b*$^{-/-}$ mice (Supplementary Fig. 2D). Thus, the pyruvate–GRP31 axis does not influence the OVA uptake by CX3CR1^{high} myeloid cells in the small intestine of mice.

Because intestinal CX3CR1^{high} myeloid cell-derived IL-10 is required for the maintenance of intestinal Treg cells (8, 19, 20, 24), we analyzed the expression of IL-10 by intestinal CX3CR1^{high} myeloid cells. CX3CR1^{high} myeloid cells were isolated from the small intestine of OVA-gavaged wild-type and *Gpr31b*$^{-/-}$ mice and cultured for 16 h. The IL-10 concentration in the culture supernatants was then analyzed (Fig. 4A).

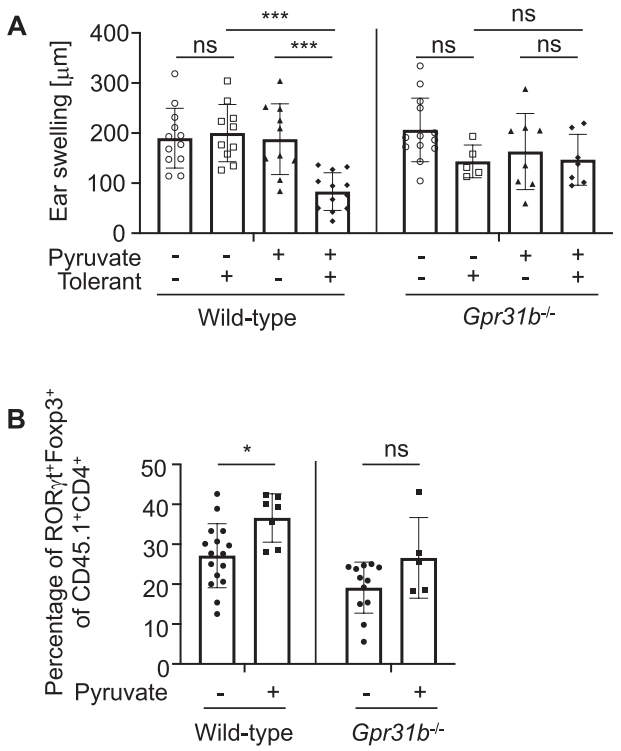


Fig. 3. Pyruvate enhances oral tolerance in a GPR31-dependent manner. Mice were treated with 50 mM pyruvate through drinking water from 4 weeks before the start of tolerization until the end of the experiment. (A) Oral tolerance in wild-type and G*pr31b*$^{-/-}$ mice with or without pyruvate treatment was determined by ear swelling in a DTH model. Data are pooled from two independent experiments. Results are presented as the mean ± SD. A one-way ANOVA followed by Tukey's multiple comparisons was performed to determine statistically significant differences between groups. ***$P < 0.001$, ns: not significant. (B) Naive CD4$^+$ T cells from OT-II TCR transgenic mice (CD45.1) were transferred to wild-type and G*pr31b*$^{-/-}$ mice with or without pyruvate treatment. The percentages of RORγt$^+$ Foxp3$^+$ Treg cells among the CD45.1$^+$ CD4$^+$ T cells in the small intestinal lamina propria of the recipient mice are shown. Data are pooled from five independent experiments. A two-tailed Student's *t*-test was performed to determine statistically significant differences between groups. Results are presented as the mean ± SD. *$P < 0.05$, ns: not significant.

The IL-10 production by CX3CR1^{high} myeloid cells from OVA-gavaged wild-type mice was higher than that in CX3CR1^{high} myeloid cells from untreated wild-type mice. In contrast, the level of IL-10 production was not higher in the CX3CR1^{high} myeloid cells from OVA-gavaged *Gpr31b*$^{-/-}$ mice than in the same cells from untreated *Gpr31b*$^{-/-}$ mice. These findings indicate that IL-10 is secreted from intestinal CX3CR1^{high} myeloid cells in a GPR31-dependent manner.

To analyze the role of IL-10 secreted from CX3CR1^{high} myeloid cells, we crossed *Cx3cr1*$^{CreER/+}$ mice with *Il10*$^{flox/flox}$ mice. The repeated injection of tamoxifen successfully deleted the floxed allele of the *Il10* gene in intestinal CX3CR1^{high} myeloid cells (Supplementary Fig. 3). Using these CX3CR1$^+$ cell-specific IL-10 knockout (*Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$) mice, we analyzed the effect of CX3CR1^{high} myeloid cell-derived IL-10 on the induction of oral tolerance (Fig. 4B and C). *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice without tamoxifen treatment developed oral tolerance after OVA gavage. In contrast, oral tolerance was not induced in tamoxifen-treated *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice. The possibility of a non-specific effect by tamoxifen was ruled out by the finding that tamoxifen-treated *Il10*$^{flox/flox}$ mice developed oral tolerance (Supplementary Fig. 4).

Because intestinal macrophage-derived IL-10 is required for the maintenance of intestinal Treg cells, we analyzed the effect of IL-10 deficiency in CX3CR1^{high} myeloid cells on intestinal RORγt$^+$Foxp3$^+$ Treg cells (Fig. 4D and E). Naive CD4$^+$ T cells of CD45.1$^+$ OT-II mice were adoptively transferred into CD45.2$^+$ *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice with or without tamoxifen treatment. At 10 days after the OVA administration, the percentage of CD45.1$^+$RORγt$^+$Foxp3$^+$ Treg cells in the small intestine was analyzed. In tamoxifen-treated *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice, the percentage of CD45.1$^+$RORγt$^+$Foxp3$^+$ Treg cells in the small intestine was significantly lower than that in mice without tamoxifen treatment. These findings indicate that IL-10 derived from intestinal CX3CR1^{high} myeloid cells is involved in the maintenance of intestinal RORγt$^+$Foxp3$^+$ Treg cells.

Discussion

In the present study, we showed that oral pyruvate treatment enhances oral tolerance in a GPR31-dependent manner. In pyruvate-treated mice, being fed a low dose of OVA effectively induced oral tolerance to OVA. Pyruvate did not have this effect in *Gpr31b*$^{-/-}$ mice. IL-10 production from CX3CR1^{high} myeloid cells and the percentage of RORγt$^+$Foxp3$^+$ Treg cells in the small intestine were also lower in *Gpr31b*$^{-/-}$ mice. Thus, pyruvate acts on small intestinal CX3CR1^{high} myeloid cells via GPR31 and mediates the maintenance of intestinal Treg cells through the production of IL-10, thereby contributing to the effective induction of oral tolerance.

Several previous studies showed that probiotic *Bifidobacterium* and *Lactobacillus*, which produce the GPR31 ligands lactate and pyruvate, induce oral tolerance (36–38). This effect of probiotic bacteria might be elicited through the GPR31-dependent action of pyruvate (and lactate) on CX3CR1^{high} myeloid cells in the small intestine.

In CD45.2$^+$ mice transferred with CD45.1$^+$ OT-II naive CD4$^+$ T cells, CD45.1$^+$RORγt$^+$Foxp3$^+$ Treg cells were observed after OVA administration. Because adoptively transferred Foxp3$^+$ Treg cells in the intestine were not present without OVA administration (8), OVA-specific Treg cells were induced by OVA administration. In addition, the induction of OVA-specific Foxp3$^+$ Treg cells in the intestine was impaired in mice lacking MHC class II in CX3CR1$^+$ cells (24), indicating the requirement of OVA presentation by MHC class II on CX3CR1^{high} myeloid cells. Together with the fact that pyruvate did not enhance the uptake of OVA by CX3CR1^{high} myeloid cells, we propose the following mechanism of the pyruvate-dependent enhancement of oral tolerance: CX3CR1^{high} myeloid cells, which capture luminal antigens and thereby induce Treg cells independently of the GPR31 signaling, respond to pyruvate and produce increased amounts of IL-10 via the GPR31 signaling. IL-10 enhances the maintenance of luminal antigen-specific RORγt$^+$ Treg cells that mediate oral tolerance.

An elevated level of IL-10 production by intestinal CX3CR1^{high} myeloid cells during the induction of oral tolerance

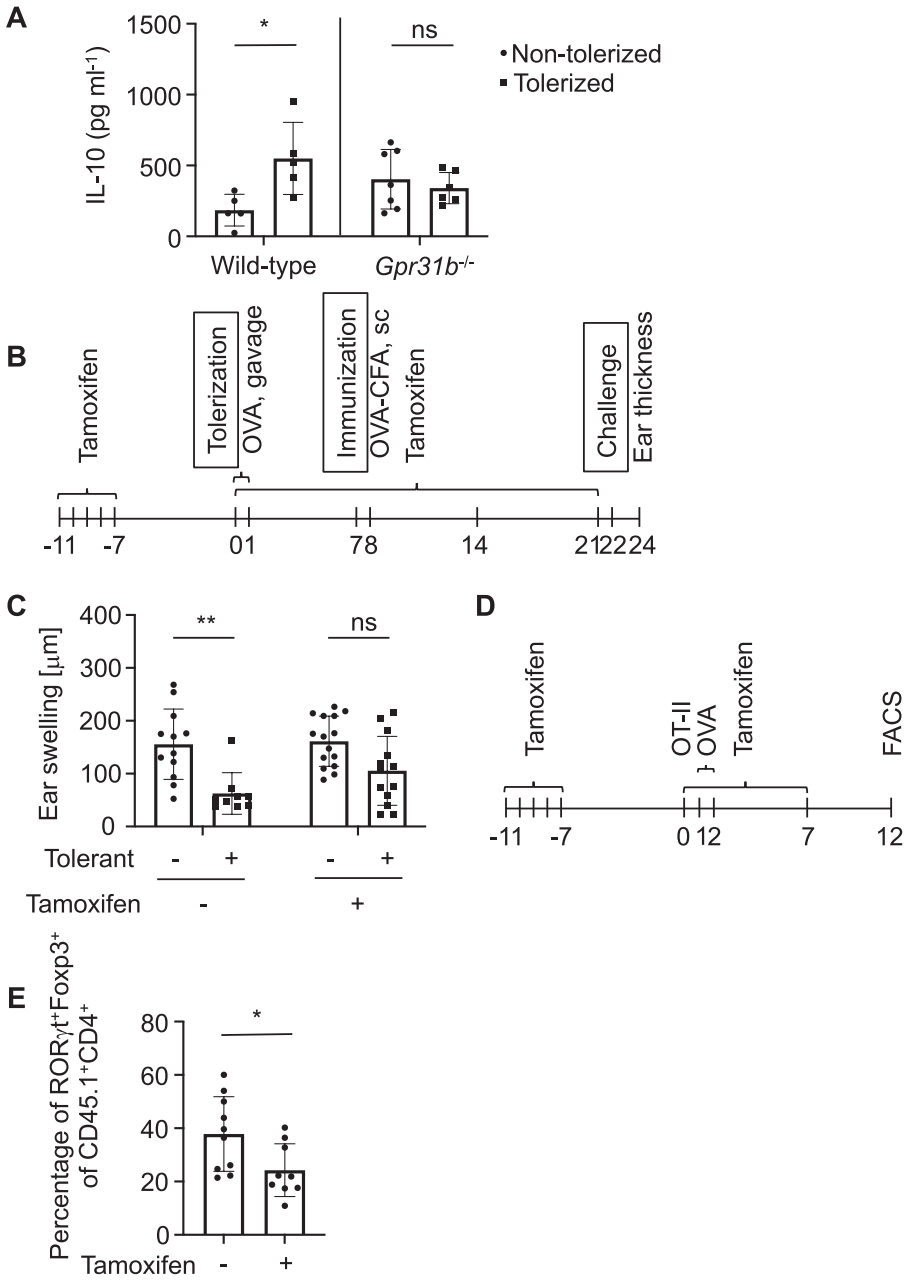


Fig. 4. GPR31-dependent IL-10 production of CX3CR1^{high} cells is required for oral tolerance. (A) The concentration of IL-10 in the culture supernatant of CX3CR1^{high} cells, isolated from the small intestinal lamina propria of wild-type or G*pr31b*$^{-/-}$ mice 3 days after OVA challenge via subcutaneous injection in a DTH model, was measured by ELISA. Results are presented as the mean ± SD. A two-tailed Student's *t*-test was performed to determine statistically significant differences between groups. *$P < 0.05$, ns: not significant. (B) Experimental protocol for our DTH model in *Cx3cr1*$^{creER/+}$*Il10*$^{flox/flox}$ mice. (C) Oral tolerance in *Cx3cr1*$^{creER/+}$*Il10*$^{flox/flox}$ mice with or without tamoxifen treatment was determined by measuring the amount of ear swelling in a DTH model. Data are pooled from five independent experiments. Results are presented as the mean ± SD. A two-way ANOVA followed by Tukey's multiple comparisons was performed to determine statistically significant differences between groups. **$P < 0.01$, ns: not significant. (D and E) Naive CD4$^+$ T cells from OT-II TCR transgenic mice (CD45.1) were transferred to *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice (CD45.2) with or without tamoxifen treatment. CD45.1$^+$ CD4$^+$ T cells in the small intestinal lamina propria of the recipient mice were analyzed by flow cytometry after gavage with OVA. An overview of the experimental protocol is shown in (D). The percentages of RORγt$^+$Foxp3$^+$ cells among the CD45.1$^+$CD4$^+$ T cells (E) from the small intestinal lamina propria of the recipient *Cx3cr1*$^{CreER/+}$ *Il10*$^{flox/flox}$ mice are shown. Data are pooled from three independent experiments. Results are presented as the mean ± SD. A two-tailed Student's *t*-test was performed to determine statistically significant differences between groups. *$P < 0.05$.

to OVA was not observed in *Gpr31b*$^{-/-}$ mice. However, in the steady state, the level of mRNA expression of *Il10* in intestinal CX3CR1^{high} myeloid cells was comparable between wild-type and *Gpr31b*$^{-/-}$ mice (our unpublished results). In addition, IL-10 production by intestinal CX3CR1^{high} myeloid cells was not increased by pyruvate treatment alone (our unpublished

results). Therefore, pyruvate is expected to act on intestinal CX3CR1^{high} myeloid cells via GPR31 and modulate their cell activity to enhance IL-10 production.

Although we expected pyruvate treatment to up-regulate the uptake of luminal antigens because pyruvate has been shown to mediate the protrusion of dendrites by intestinal CX3CR1^{high} myeloid cells into the intestinal lumen (27), in our experimental setting, we did not observe any pyruvate/GPR31-dependent enhancement of antigen capture (Supplementary Fig. 2). However, the antigen capture by CX3CR1^{high} myeloid cells in this experimental setting was only subtle, so discriminating the difference between pyruvate-treated or -untreated groups would be difficult. A previous study showed the apparent CX3CR1-dependent uptake of luminal antigens by intestinal CX3CR1^{high} myeloid cells when using a different experimental procedure with the oral administration of high amounts of OVA (18). We also tried this method but still could not see a difference between the wild-type and *Gpr31b*$^{-/-}$ mice (our unpublished results). Thus, more careful experiments will be required to conclude that the pyruvate–GPR31 axis is not involved in the enhancement of luminal antigen uptake.

Food allergy, the incidence of which has been increasing over recent years, is caused by the disturbance of oral tolerance and is correlated with dysbiosis (39–41). It is possible that the concentrations of the GPR31 ligand pyruvate and lactate are below normal levels in the dysbiotic intestinal lumen of patients with food allergy. Therefore, given that oral administration of pyruvate enhances oral tolerance, pyruvate could be a good candidate for a clinical treatment for food allergy, acting through the effective induction of oral tolerance. To investigate the potential clinical application of pyruvate for the induction of oral tolerance, the human counterpart of mouse CX3CR1^{high} myeloid cells in the small intestine should be identified, and their pyruvate response should be clarified in the future.

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Author contributions: Q.L. performed most of the experiments, analyzed the data and wrote the manuscript; N.M., H.K. and Y.B. assisted with experiments; T.K., E.U. and R.O. supervised the study; and K.T. planned and directed the overall project and wrote the manuscript.

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