

Letter to the Editor

Therapeutic potential of D prostanoid receptor 1 signal enhancement in a murine model of food allergy

To the Editors:

Mast cells play a key role in the development of antigen-induced food allergies. Activated mast cells release several inflammatory mediators that induce local intestinal inflammation and lead to systemic allergy symptoms. This intestinal inflammation stimulates infiltration of mucosal mast cells (MMC) into the intestinal tract, resulting in expansion of intestinal mast cells. Furthermore, patients with food allergies possess greater numbers of intestinal mast cells than those without food allergies.¹ Experimental studies in murine models have shown that numbers of intestinal mast cells are positively correlated with the severity of the allergic response to food.² Therefore inhibiting intestinal mast cell expansion could be an effective solution for managing food allergy problems.

Although mast cells in patients with food allergies are known to release large amounts of prostaglandin D₂ (PGD₂), its role in the inflammation process caused by food allergens has been hitherto unclear. Using hematopoietic PGD₂ synthase-deficient mice, we demonstrated that mast cell-derived PGD₂ inhibits oral ovalbumin (OVA)-induced intestinal MMC expansion and the development of allergy symptoms.³ Although the role of D prostanoid receptor 1 (DP1) in allergic patients remains contentious, we found in an *in vitro* study that pharmacologic DP1 activation inhibited increased production of the mast cell chemoattractant stromal cell-derived factor 1 α (SDF-1 α).³ Based on these results, we hypothesize that pharmacologic stimulation of DP1 could provide therapeutic value for managing food allergies.

We began this study by first examining the preventive effect of enhanced DP1 signaling on the development of food allergies. Mice were injected intraperitoneally with either vehicle (2% dimethyl sulfoxide) or the selective DP1 agonist BW245C (100 μ g/kg) 3 times a day for 18 days (Fig 1, A). In vehicle-treated mice repeated OVA challenges gradually led to greater allergy symptom scores and incidences of diarrhea (Fig 1, B and C, and see Table E1 in this article's Online Repository at www.jacionline.org). Ten allergic episodes from OVA stimulation led to a 12- and 60-fold increase in MMC numbers in the jejunum and colon, respectively (Fig 1, D-F). Expression of MMC-specific mouse mast cell protease 1 (mMCPT-1) mRNA was also found to be greater in the small and large intestines (Fig 1, E and F) in vehicle-treated mice. Pretreatment with BW245C was found to prevent increases in allergy symptom scores and diarrhea incidence (Fig 1, B and C). Furthermore, BW245C treatment strongly inhibited the increase in MMC numbers to less than half in the jejunum and less than one tenth in the colon compared with vehicle treatment. This BW245C treatment significantly decreased intestinal mRNA levels of *mMcp1*-1 and decreased, although not significantly, serum levels of mMCPT-1 (Fig 1, D-G). BW245C treatment reduced the number of mast cell progenitors (MCps) in the jejunum by a small amount, but the reduction in the colon was significant (Fig 1, H). BW245C did not influence serum levels of OVA-specific IgE (Fig 1, I).

Pharmacologic blocking of DP1 signaling by the specific DP1 antagonist BWA868C (1 mg/kg) accelerated the increase in allergy symptom scores and diarrhea incidence (see Fig E1, A-C, in this article's Online Repository at www.jacionline.org). Furthermore, pretreatment with BWA868C also significantly increased intestinal MMC numbers and serum levels of mMCPT-1 (see Fig E1, D-F). BWA868C treatment tended to increase mRNA levels of *Sdf-1 α* but significantly increased mRNA levels of matrix metalloproteinase 9 in colon tissue compared with the vehicle (see Fig E1, G and H). These results showed the same tendency as those of the previous report.³ Therefore it is clear that enhancing DP1 signaling attenuates OVA-induced allergic reactions and intestinal mast cell expansion, whereas inhibition of DP1 signaling exacerbates these conditions.

Immunostaining has shown that in allergic mice intestinal epithelial cells strongly express DP1 receptors (Fig 1, J). Some immune cells also express this receptor (Fig 1, J). We assessed mRNA levels of several factors that promote mast cell expansion by stimulating proliferation and/or enhancing chemotaxis in mouse intestines (Fig 1, K and L).⁴ We found that OVA challenge increased mRNA levels of the major epithelium-derived pro-T_H2 cytokine *Il-33*, along with that of its downstream cytokine *Il-4*.^{5,6} Pretreatment with BW245C tended to inhibit the increase in levels of *Il-33*, whereas inhibition of *Il-4* in the jejunum was significantly stronger (Fig 1, K). Previous work suggests that there are large variations in epithelial responses to antigens between the small and large intestines.⁷ In our study neither OVA challenges nor DP1 receptor stimulation influenced expression levels of T_H2 cytokines in the colon (Fig 1, L). However, OVA challenges significantly increased mRNA and protein levels of the mast cell chemoattractant chemokine CCL2, which was inhibited by BW245C pretreatment (Fig 1, L and M) in the colon. We believe that enhancing DP1 receptor signaling can indirectly inhibit mast cell expansion by inhibiting the production of IL-4, CCL2, or both in the intestines. It is also possible that DP1 receptor stimulation directly modulates mast cell migration. However, *in vitro* treatment with BW245C did not influence stem cell factor-induced chemotaxis of bone marrow-derived mast cells (BMMCs; Fig 1, N).

Finally, we examined the therapeutic potential of DP1 signal enhancement. We began administering BW245C (100 μ g/kg) intraperitoneally after mice had diarrhea caused by 3 to 4 challenges with OVA (Fig 2, A). Although the vehicle-treated control mice exhibited increased allergy symptom scores and high diarrhea occurrence in response to the additional OVA challenges, mice treated with BW245C exhibited a gradual decrease in allergy symptom scores and diarrhea incidence (Fig 2, B and C). BW245C treatment also decreased levels of the urinary tetranor-prostaglandin D metabolite (te-PGDM; 8.2 ± 3.7 ng/mg creatinine), an index of food allergy,⁸ whereas in control mice te-PGDM levels remained high (14.9 ± 3.7 ng/mg creatinine). Furthermore, BW245C treatment also decreased MMC numbers and serum levels of mMCPT-1 (Fig 2, D-F).

Because activation of the Gs-coupled DP1 receptor is known to increase intracellular cAMP levels and previous reports indicate that an increase in intracellular cAMP levels inhibits mast cell

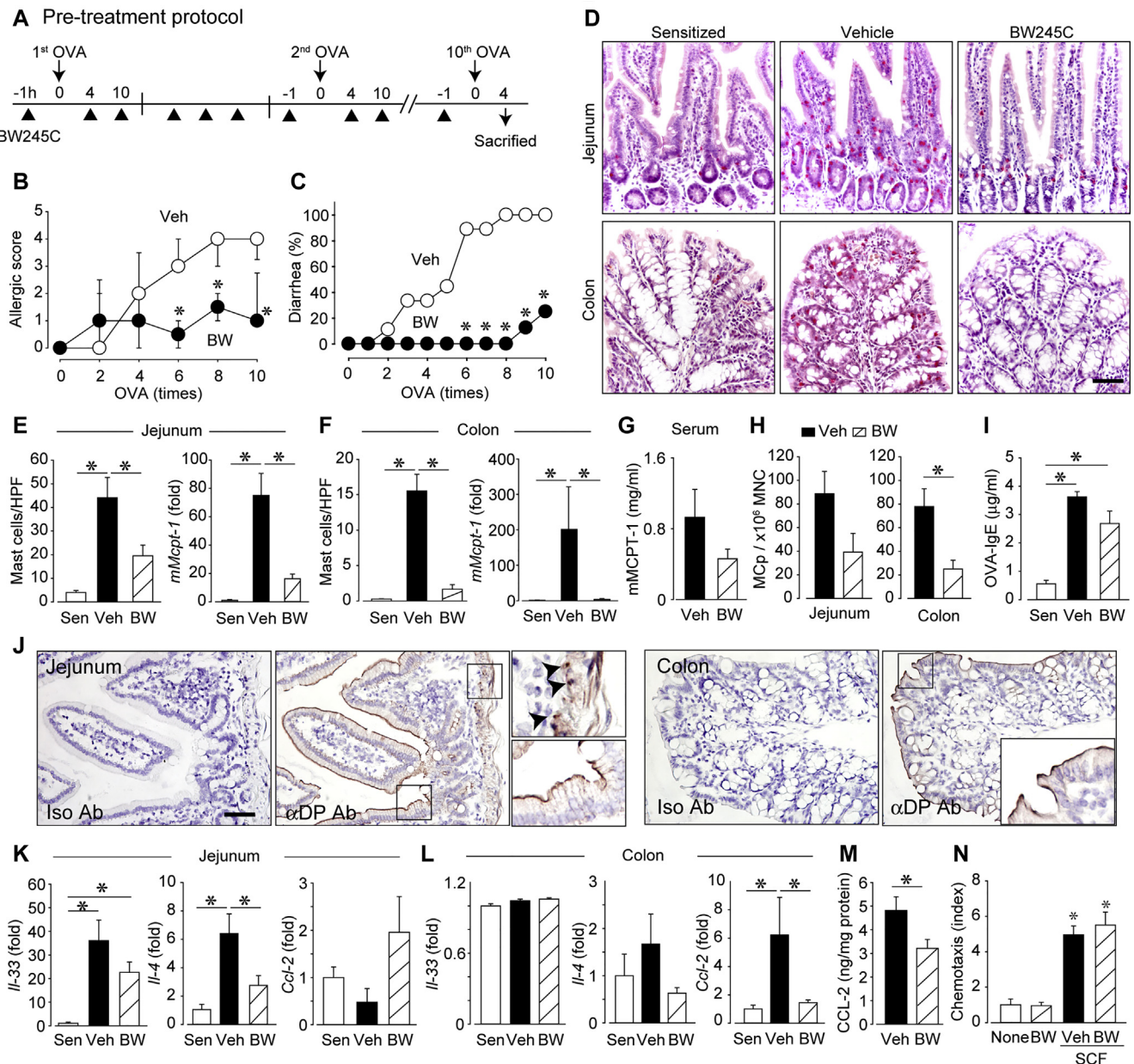


FIG 1. DP1 signal enhancement prevents development of food allergy. **A**, Experimental protocol. BW245C (BW; 100 μg/kg) was intraperitoneally administrated into mice 3 times a day. **B** and **C**, Allergy symptom scores (Fig 1, B) and diarrhea incidence (Fig 1, C). Symptom scores are shown as medians and interquartile ranges (25th-75th). **D**, Representative pictures of chloroacetate esterase-stained intestine. Scale bar = 50 μm. **E** and **F**, Numbers of mast cells per high-power field (HPF; original magnification ×400) and mRNA expression of mMcpt-1 in the jejunum (Fig 1, E) and colon (Fig 1, F). **G** and **I**, Serum levels of mMCPT-1 (Fig 1, G) and OVA-specific IgE (Fig 1, I). **H**, Number of MCPs in vehicle- or BW245C-treated jejunum and colon. **J**, Representative pictures of immunostaining of DP1 in the 10th OVA-challenged intestine. Arrowheads indicate DP1 receptor-positive immune cells. Scale bar = 50 μm. **K** and **L**, Relative mRNA expression of *Il33*, *Il4*, and *Ccl2* in the jejunum (Fig 1, K) and colon (Fig 1, L). **M**, Protein levels of CCL-2 in colon tissues. **N**, Effect of BW (30 nmol/L) in stem cell factor (10 ng/mL)-induced BMMC chemotaxis. BW245C was administered 10 minutes before stem cell factor stimulation. Data were obtained in 2 to 3 independent experiments (n = 5-9) and are represented as means ± SEMs. **P* < .05. Veh, Vehicle.

degranulation,⁹ it is possible that the anti-inflammatory effects of BW245C are mediated through inhibition of mast cell degranulation. As hypothesized, numbers of degranulated mast cells were significantly reduced in the intestines of BW245C-treated mice (Fig 2, D and G). Our *in vitro* experiments have also shown that

BW245C inhibits antigen-induced degranulation of BMDCs in a dose-dependent manner, which was similar to that exhibited by the intracellular cAMP increaser forskolin (Fig 2, H). BWA868C did not change the degranulation, which suggests that the mast cell-derived PGD₂ did not inhibit its own

A Post-treatment protocol

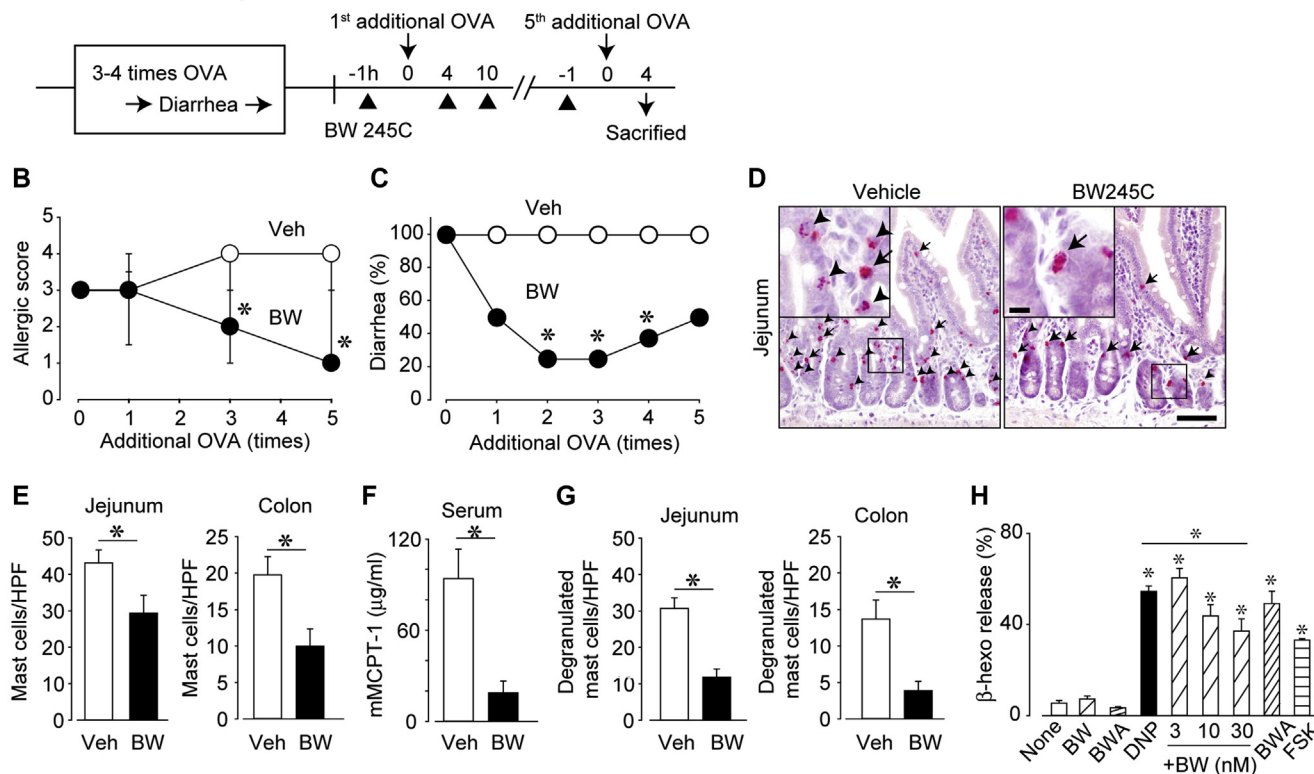


FIG 2. DP1 signal enhancement improves development of food allergy. **A**, Experimental protocol. Intraperitoneal administration of BW245C (100 μ g/kg administered 3 times a day) started after mice had diarrhea caused by 3 to 4 OVA challenges. **B** and **C**, Allergy symptom score (Fig 2, B) and diarrhea incidence (Fig 2, C). Symptom scores are shown as medians and interquartile ranges (25th-75th). **D**, Representative pictures of the fifth additional OVA-challenged jejunum. Arrows or arrowheads indicate nondegranulated or degranulated mast cells, respectively. Scale bar = 50 μ m (inset = 10 μ m). **E** and **G**, Numbers of total (Fig 2, E) and degranulated (Fig 2, G) mast cells in the jejunum or colon. **F**, Serum levels of mMCPT-1. **H**, Effect of BW245C (BW; 3-30 nmol/L), the DP1 antagonist BWA868C (BWA; 1 μ mol/L), or forskolin (FSK; 30 μ mol/L) on dinitrophenyl (DNP)-BSA (100 ng/mL)-induced degranulation of DNP-IgE (100 ng/mL)-sensitized BMMCs. Data were obtained in 2 to 3 independent experiments (n = 5-8) and are represented as means $\pm$ SEMs. **P* < .05.

degranulation (Fig 2, H). Therefore we believe that DP1 receptor activation exerts a therapeutic effect on food allergen-induced intestinal inflammation by inhibiting both the infiltration and degranulation of mast cells.

In conclusion, we demonstrated that enhancing DP1 receptor signaling can exert a preventive and therapeutic effect on development of food allergies by inhibiting infiltration and degranulation of mast cells in the intestines. Currently, medications used to treat food allergies, such as adrenaline and antihistamines, are only effective in providing symptomatic relief. However, our findings might help in development of a new therapeutic strategy that can suppress the onset of food allergies.

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METHODS

Mice

All experiments were approved by the University of Tokyo's Institutional Animal Care and Use Committee. Five-week-old female BALB/c mice were purchased from CLEA Japan (Meguro-ku, Japan).

Reagents

The following reagents were obtained from the indicated suppliers: naphthol AS-D chloroacetate and monoclonal anti-dinitrophenyl antibody produced in mice (clone SPE-7), OVA, and DNase I were from Sigma-Aldrich (St Louis, Mo); formamide, N,N-dimethylformamide, sodium nitrate, stem cell factor, IL-3, and collagenase I were from Wako (Tokyo, Japan); BSA and 2,4-dinitrophenylated were from Calbiochem (La Jolla, Calif); BW245C, BWA868C, and rabbit anti-DP polyclonal antibody were from Cayman Chemical (Ann Arbor, Mich); and new fuchsin was from MP Biomedicals (Santa Ana, Calif).

Food allergy model

Five-week-old mice were sensitized by means of intraperitoneal injection with 50 μ g of OVA and 1 mg of aluminum potassium sulfate in saline. Two weeks later, 50 μ g of OVA was intraperitoneally injected to boost. Two weeks after the last sensitization, 10 mg of OVA was orally administered after 4 hours of fasting every other day. Allergy symptoms were observed during a 45-minute period after each challenge. Watery feces are defined as diarrhea, and the other allergy symptoms were scored as shown in Table E1, as previously described.^{E1,E2} In some experiments the DP1 receptor agonist BW245C (100 μ g/kg in 100 μ L of saline with 2% dimethyl sulfoxide) was intraperitoneally injected 1 hour before and 4 and 10 hours after each OVA challenge or the DP1 receptor antagonist BWA868C (1 mg/kg in 100 μ L of saline with 2% dimethyl sulfoxide) was intraperitoneally injected 1 hour before and 10 hours after each OVA challenge.

Morphologic studies

Dissected tissues were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Four-micrometer sections were stained with chloroacetate esterase, as previously described.^{E3} Chloroacetate esterase-positive total mast cells and degranulated mast cells were counted in 20 to 25 randomly selected fields ($\times 400$ magnification) per section. For immunohistochemistry, deparaffinized tissue sections were incubated with methanol containing 0.3% hydrogen peroxidase for 30 minutes at room temperature (RT) and then treated with 50 mmol/L Tris buffer containing 0.1% trypsin and 0.1% calcium chloride for 15 minutes at 37°C. After incubation with PBS containing 0.1% Triton X-100 and 5% normal goat serum for 30 minutes at RT, rabbit anti-DP1 receptor antibody (1:200) was administered to sections at 4°C overnight.

Biotinylated goat anti-rabbit antibody (1:500) was incubated for 2 hours at RT. After incubation with avidin-biotin complex (VECTASTAIN; Vector Laboratories, Burlingame, Calif) for 30 minutes at RT, sections were colored by means of incubation with 50 mmol/L Tris buffer containing 200 μ g/mL diaminobenzidine and 0.03% hydrogen peroxidase. Images were captured with an OPTIPHOT-2 microscope and DXM-1200C digital camera (Nikon, Shinagawa, Japan).

MCp assessment

Appearance ratios of MCps in intestinal tissues were determined by using a limiting dilution assay. Intestinal tissues were digested in 40 mL of RPMI-1640 containing 1 mg/mL collagenase I (Wako), 50 U/mL DNase I (Roche, Mannheim, Germany), and 10% FBS at 37°C for 30 minutes. Liberated cells were centrifuged over a 2-step 44% (density, 1.050 g/mL)/60% (density, 1.084 g/mL) Percoll gradient at 400g for 20 minutes at RT. Mononuclear cells at the gradient interface were collected and used for 4 serial 2-fold dilutions with RPMI-1640 containing 10% FBS, 50 μ mol/L 2-mercaptoethanol, and 10 ng/mL IL-3. Four different concentrations (10,000-1,250 per well) of mononuclear cell dilutions were added to 24 wells of a standard 96-well plate (Nunc). After a 10-day cultivation, wells containing mast cell colonies were counted. The appearance ratio of MCps was calculated by using L-Calc software (STEMCELL Technologies, Vancouver, British Columbia, Canada). The ratio is expressed as the number of MCps per 10^6 mononuclear cells.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed with SYBR Green Master Mix (Invitrogen, Carlsbad, Calif) and specific primers. Total RNA was isolated with TRIzol reagent and reverse transcribed to cDNA by using ReverTra Ace (Toyobo, Osaka, Japan). The real-time PCR reaction was performed with an AriaMx (Agilent Technologies, Santa Clara, Calif). Quantification was performed by using $\Delta\Delta$ cycle threshold methods. The used primers are as follows: *Ccl2* forward 5'-TGCCCTAAGGTCTTCAGCAC-3' and *Ccl2* reverse 5'-AAGGCATCACAGTCCGAGTC-3'; *Il4* forward 5'-ATGCCTG GATTCATCGATAAG-3' and *Il4* reverse 5'-GCTCAGTACTACGAG TAATCC-3'; *Il33* forward 5'-CATGCCAACGACAAGGACTA-3' and *Il33* reverse 5'-GCTCTCATCTTTCTCTCTCCA-3'; *Mcpt1* forward 5'-ACGGA CAGAGGTTCTGAGGA-3' and *Mcpt1* reverse 5'-GCTCACATCAT GAGCTCAA-3'; *Sdf1a* forward 5'-CTGCATCAGTGACGGTAAACC-3' and *Sdf1a* reverse 5'-CAGCCGTGCAACAATCTGAAG-3'; *Mmp9* forward 5'-CATTTTCGACGACGACGAGT-3' and *Mmp9* reverse 5'-AGTGGTG CAGGCAGAGTAGG-3'; and *18S* rRNA forward 5'-GACTCAACACGG GAAACCTCAC-3' and *18S* rRNA reverse 5'-CACCCACGAATCGA GAAAG-3'.

BMMC isolation

Bone marrow cells were collected from mouse femurs and cultured in RPMI-1640 containing 10% FBS, 50 μ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10 ng/mL IL-3 for up to 6 weeks at 37°C in a 5% CO₂ atmosphere.

Chemotaxis assay

The chemotactic response of BMMCs was examined with an 8- μ m pore size insert chamber (BD Biosciences, San Jose, Calif). Four-week-old BMMCs (1×10^6 cells/mL) in 400 μ L of RPMI-1640 containing 25 mmol/L HEPES and 0.1% BSA were added to the upper insert chamber. BW245C (3-30 nmol/L) was added to both the upper and lower chambers 10 minutes before stem cell factor (100 ng/mL) was added to the lower chamber. After 5 hours of incubation, migrated cells in the lower chamber were counted by means of flow cytometry (Accuri C6; BD Biosciences).

Measurement of urinary te-PGDM

Urinary te-PGDM levels were measured by using liquid chromatography-tandem mass spectrometry, as described previously.^{E4} Briefly, mouse urine (0.4 mL), which was collected for 24 hours in metabolic cages, was diluted up to 1 mL with acidified distilled water (pH 3-4) containing 5 ng of d6-te-PGDM. Samples were loaded on an OASIS 1-mL cartridge (Waters, Milford, Mass) and washed with distilled water and hexane and then eluted with 1 mL of ethyl acetate. After drying under a vacuum, the analyte was dissolved in 100 μ L of 10% acetonitrile and injected for liquid chromatography-tandem mass spectrometry. te-PGDM or d6-te-PGDM was identified and quantified by monitoring transition ions (m/z 327 $\rightarrow$ 309 or 333 $\rightarrow$ 315, respectively). Urinary creatinine concentrations were measured with a kit (Wako), and urinary te-PGDM levels were expressed as nanograms per milligram of creatinine.

Degranulation assay

Six-week-old BMMCs (5×10^4) were sensitized with dinitrophenyl-IgE (100 ng/mL) for 24 hours and then stimulated with dinitrophenyl-BSA (10 ng/mL) for 30 minutes at 37°C. In some experiments BW245C (3-30 nmol/L) or BWA868C (1 μ mol/L) was preincubated for 10 minutes or forskolin was preincubated for 30 minutes. A *p*-nitrophenyl N-acetyl- β -D-glucosaminide solution (3.5 mg/mL, 100 μ L) was added to the supernatant (50 μ L) of the BMMCs. After 90 minutes of incubation at 37°C, glycine solutions (400 mmol/L, 50 μ L) were added, and absorbance was measured at 405 nm.

ELISA

All ELISA procedures were performed according to the manufacturer's instructions. For OVA-specific IgE (BioLegend, San Diego, Calif) and mMCPT-1 (eBioscience, San Diego, Calif) measurement, sera were collected

from mice and diluted at 25- and 50,000-fold, respectively. For CCL2 (BioLegend), intestinal tissues were homogenized in PBS, and 10-fold diluted supernatants were used.

Data representation and statistical analysis

Allergic scores are shown as medians and interquartile ranges (25th-75th), but other data are shown as means $\pm$ SEMs. The statistical difference was determined by using the Student *t* test for 2-group comparisons, by using 1-way ANOVA with Tukey or Dunnett tests for multiple-group comparisons, by using the Mann–Whitney *U* test for allergic scores, and by using the Fisher exact test for diarrhea incidence.

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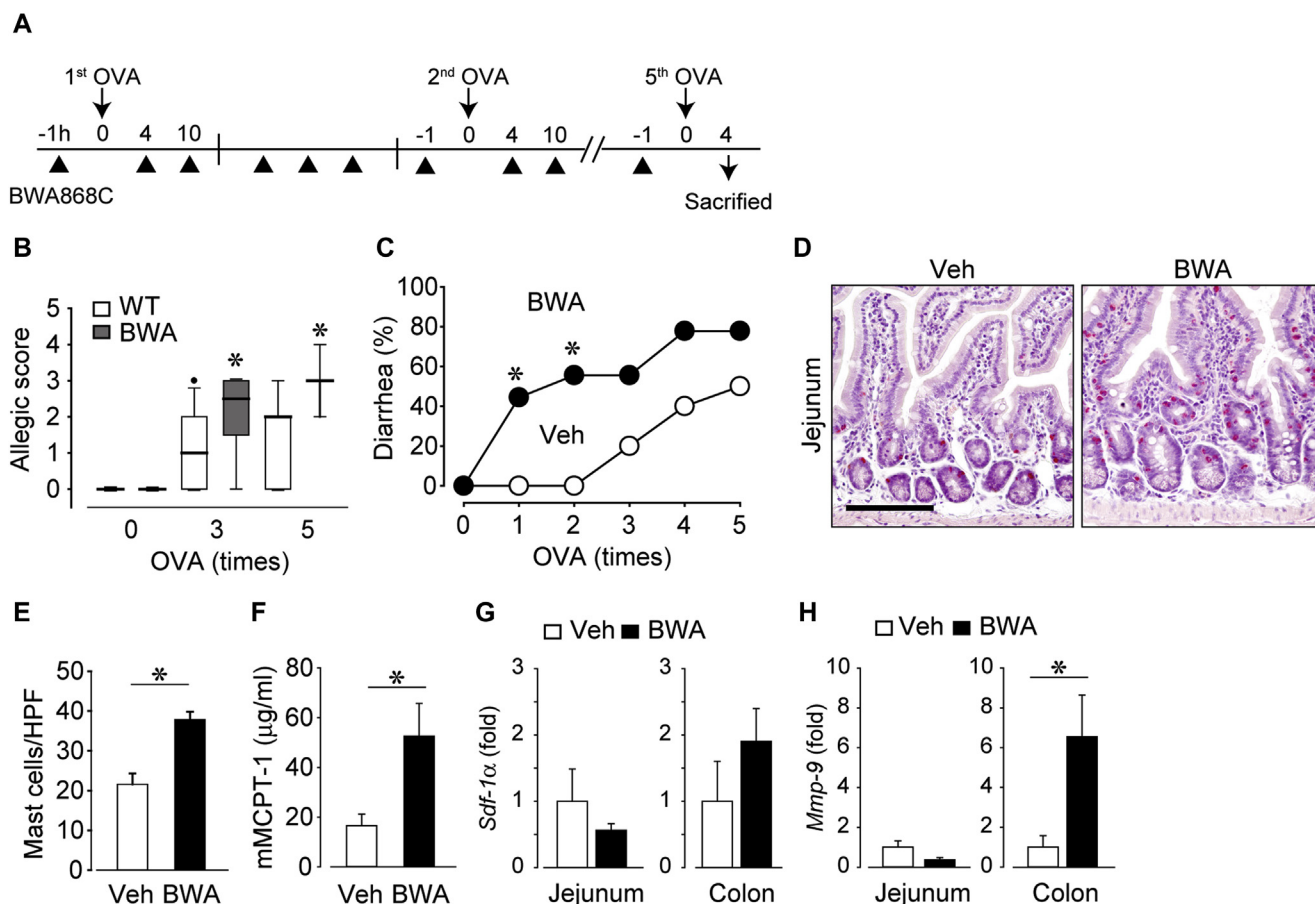


FIG E1. DP1 blockade promotes development of food allergy. **A**, Experimental protocol. Vehicle (2% dimethyl sulfoxide) or BWA868C (1 mg/kg) was intraperitoneally administered into mice 10 minutes before each OVA challenge. **B** and **C**, Allergy symptom score (Fig E1, *B*) and diarrhea incidence (Fig E1, *C*). Symptom scores are represented as medians and interquartile ranges (25th-75th). **D**, Representative pictures of the fifth OVA-challenged jejunum. Scale bar = 100 μm. **E** and **F**, Numbers of mast cells (Fig E1, *E*) and serum levels of mMCPT-1 (Fig E1, *F*) in the fifth OVA-challenged jejunum. **G** and **H**, mRNA expression of *Sdf-1α* (Fig E1, *G*) and matrix metalloproteinase 9 (*Mmp-9*; Fig E1, *H*) in vehicle- or BWA868C-treated jejunum and colon. Data are represented as fold increase to vehicle-treated intestines. **P* < .05. Data are represented as means ± SEMs. All experiments were performed as at least 2 independent experiments.

TABLE E1. Allergy symptom score

Score	Symptoms
0	No sign
1	Scratching nose and mouth less than 10 times in 15 minutes
2	Puffiness around the eyes and mouth, scratching nose and mouth more than 10 times in 15 minutes
3	Wheezing and labored respiration, cyanosis around the mouth and tail, diarrhea and difficulty in walking normally
4	No activity
5	Death