

RESEARCH ARTICLE

PGD₂/CRTH2 signaling promotes acquired immunity against bee venom by enhancing IgE production

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Abstract

IgE-dependent/independent activation of mast cell (MC) has been assumed to play a host defensive role against venom injection in skin. However, its detailed mechanisms remain unknown. We aimed to investigate the contribution of MC-derived prostaglandin D₂ (PGD₂)-mediated signaling in host defense against bee venom (BV). To achieve this, we utilized gene-deficient mice of a PGD₂ receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). We first confirmed that subcutaneous injection of BV produced PGD₂ equally in wild-type (WT) and CRTH2-deficient (*Crth2*^{-/-}) mice skins. The BV injection dropped body temperature and impaired kidney equally in both lines of mice. In WT mice, pre-injection of BV (3 weeks) significantly inhibited the hypothermia and kidney impairment caused by second BV injection. In contrast, this pre-injection was not effective for the second BV injection in *Crth2*^{-/-} mice. We also found that BV injections increased serum BV-specific IgE levels in WT mice, and its serum transfused mice improved the BV-induced hypothermia in naïve WT mice. In contrast, serum BV-specific IgE level was significantly lower in *Crth2*^{-/-} mice. FACS analysis showed the BV injection stimulate migration of dendritic cells (DCs) into regional lymph nodes in WT mice. In *Crth2*^{-/-} mice, its number was significantly smaller than that of WT mice. In conclusion, PGD₂/CRTH2 signaling plays defensive role against second BV injection. This signaling promotes BV-specific IgE production at least partially by promoting DCs migration into regional lymph node.

KEYWORDS

biological defense, IgE production, lipid mediator

1 | INTRODUCTION

Humans and other animals have risks being attacked by venomous animals, such as honeybee. Bee venom (BV)

contains various toxic substances including histamine, melittin, phospholipase A₂ (PLA₂), and mast cell degranulating peptide.^{1,2} These components cause skin inflammation and activate mast cells (MCs) directly. Activated

Abbreviations: BSA, bovine serum albumin; BV, bee venom; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; *Crth2*^{-/-}, CRTH2 deficient; DC, dendritic cell; DP, D prostanoid; DW, distilled water; H-PGDS, hematopoietic PGD synthase; LC, liquid chromatographic; LC-MS/MS, LC-tandem mass spectrometry; L-PGDS, lipocalin-type PGD synthase; MC, mast cell; PBS, phosphate-buffered saline; PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂; PLA₂, phospholipase A₂; RPMI, Roswell Park Memorial Institute medium; RT, room temperature; WT, wild type.

MCs further release prostaglandin D₂ (PGD₂), one of the lipid mediators.³ BV and mediators released from MCs sometimes cause systematic inflammation which leads to hypothermia and blood pressure drop. Activated MCs also release detoxication substances such as heparin and proteases which presumably bind to invaded venom to denature and detoxicate it.⁴ Previous study showed that MCs deficiency exacerbate the body temperature drop and decrease of survival rate against the first time BV injection suggesting host defensive role of MCs against BV.⁵

At the same time of defending against BV injection, antigen presenting cells capture BV antigens and promote the production of BV-specific IgE. The produced IgEs bind the FcεRI on the surface of MCs. This IgE production process is called “sensitization.” From the next BV injection after sensitization, BV components activate MCs in both the above direct and IgE-dependent indirect pathway by cross-linking of antigen and IgE. It is known that IgE-mediated MCs activation results in forming of larger and more heterogeneously shaped granule structures that undergo prolonged exteriorization.⁶ In this reaction, which is called allergic reaction, animals can defense against venom injection more strongly than first time venom injection. Previous study showed that pharmacological neutralization or gene deficiency of functional IgE exacerbate the reaction against BV injection.⁵ This supports the idea that IgE-mediated MCs activation; allergic reactions strengthen host defense against subsequent BV injection.

Upon activation, MCs synthesize and release abundant PGD₂ as well as various mediators.³ PGD₂ is a lipid mediator synthesized by cyclooxygenase and hematopoietic PGD synthases (H-PGDS) from cell membrane component, arachidonic acid. Produced PGD₂ exerts its biological functions through a receptor named chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2).⁷ PGD₂/CRTH2 signaling has been emerged as a pro-inflammatory factor in allergic reactions. Spik et al reported that PGD₂ exacerbates allergic skin inflammation by promoting eosinophil infiltration and IgE production.⁸ Other studies reported that activation of CRTH2 signaling exacerbates the chronic allergic inflammation of skin.⁹ These reports imply that PGD₂/CRTH2 signaling promotes the allergic reaction against subsequent antigen injection at the skin. However, the role of PGD₂/CRTH2 signaling in biological reaction against BV invasion remains unknown.

In the present study, we revealed that BV injection activates MCs to produce PGD₂ and that PGD₂/CRTH2 signaling plays a crucial role in host defense against second-time BV injection by promoting IgE production in vivo.

TABLE 1 Experimental condition of BV injection

BV dose	Frequency	Experiments
80 μg	One time	Measuring PGD ₂ and PGE ₂
		Analyzing DCs migration
	Two times every 2 weeks	Measuring serum IgE
	Four times every 2 weeks	Making BV-sensitized serum
80 or 160 μg	One time	For sensitization
80 or 560, 640 μg	One time	Measuring body temperature

2 | MATERIALS AND METHODS

2.1 | Mice and BV injection

All experiments and animal care were conducted in conformity with the institutional guidelines of The University of Tokyo. CRTH2-deficient C57BL/6J and Balb/c female mice (*Crth2*^{-/-}) and the age-matched (8-12 weeks old) wild-type female mice (WT) were bred and used as previously described.¹⁰

We injected BV solution in 200 μL of saline subcutaneously into shaved back skin at four sites 50 μL of each (up and down, left and right). We showed elaborated condition of BV injection in Table 1. Since bioactivities of animal venoms including BV have difference in lot, we pooled multiple lots of BV together and used it for each experiment. We measured rectal temperature using BAT-12 Microprobe Thermometer and RET-3 Rectal Probe for Mice (Physitemp, Clifton, NJ, USA). We calculated survival rate after euthanasia treatment by observing water and food intake and immobility time.

2.2 | H&E staining

We dissected kidney tissue and fixed it in 4% paraformaldehyde for overnight at 4°C and embedded it in paraffin. We stained the 4 μm thick sections with hematoxylin and eosin based on conventional methods.

2.3 | PGD₂ and PGE₂ measurement by LC-MS/MS

After 30 minutes BV injection, we dissected the skins of the injection sites and immediately frozen them in liquid nitrogen. We homogenized skins in 1 mL of 100% ethanol.

After centrifugation, we diluted 100 μL of supernatants with 850 μL of 0.1% formic acid water, then we added PGD₂-d₄ (100 ng/mL) and PGE₂-d₄ (50 ng/mL) as internal standard. We applied solutions to Oasis HLB 1 cc Vac Cartridge (Waters, Milford, MA, USA), and washed cartridges with 1 mL of ultrapure water and 1 mL of n-hexane. We eluted the analyte from the cartridge with 1 mL of methanol. We dried the analyte and reconstituted residue in 100 μL of methanol.

We introduced sample solutions into an LCMS-8060 system (Shimadzu, Kyoto-city, Kyoto, Japan) equipped with an electrospray interface. We conducted the liquid chromatographic (LC) separation with the InertSustain C8 column (GL Sciences, Torrance, CA, USA) using a mobile phase consisting of 0.05% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). The gradient is as following: initial (0-1 minute) at 70:30 (solvent A: solvent B), 1-6 minutes at 20:80, 6-6.1 minutes at 5:95, and 8.51 minutes at 90:10. We operated LC-tandem mass spectrometry (LC-MS/MS) in the negative ion modes. For analysis, the monitored transition for PGD₂ was m/z 351.30 $\rightarrow$ 271.25, for PGD₂-d₄ was m/z 355.30 $\rightarrow$ 275.25, for PGE₂ was m/z 351.30 $\rightarrow$ 271.20, and for PGE₂-d₄ was m/z 355.30 $\rightarrow$ 275.20. Elution time of PGD₂, PGD₂-d₄, PGE₂, and PGE₂-d₄ was 3.756 minutes, 3.750, 3.635, and 3.639 minutes, respectively. The amount of PGD₂ and PGE₂ was divided by tissue weight.

2.4 | IgE measurement by EIA

For the detection of BV-specific serum IgE, we coated 96-well plate (Nunc MaxiSorp flat-bottom, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with 200 $\mu\text{g/mL}$ of whole BV for overnight at 4°C, followed by blocking with 1% bovine serum albumin (BSA) in PBS for 2 hours at RT. We diluted the analyte in 1% BSA in PBS for fivefold dilution and incubated for overnight at 4°C. We detected IgEs using biotinylated detection antibodies (Biotin anti-mouse IgE, 2 mg/mL in 1% BSA in PBS) for 1 hour at RT, followed by incubation with avidin-HRP for 30 minutes at RT. After each incubation step, we conducted four washing steps using PBS containing 0.05% tween-20. Then, we added 1-Step Ultra TMB-ELISA Substrate Solution and reacted for 40 minutes at RT with light blinding. After TMB reaction, we added stop solution (2N H₂SO₄) to the well and measured the absorbance at 450 nm by plate reader.

2.5 | Flow cytometry assay for dendritic cells

After triturating lymph nodes, we centrifuged (5 minutes at 3000 g, RT) and suspended the pellet in 10% FBS in RPMI. For removing erythrocyte, we added RBC lysis buffer (NH₄Cl

0.15 M, KHCO₃ 0.01 M, and EDTA 0.5 M, pH 7.2) and left to stand for 5 minutes and washed with PBS by centrifugation. After removing the supernatant, we added assay buffer (0.5% BSA, 2 mM EDTA in PBS with filtration) to make 100 μL 10×10^6 cells/mL solution. For counting cell number, we used automatic cell counter (NanoEntek, Waltham, MA, USA).

For labeling cell surface antigens, we incubated their specific antibodies with cell solutions for 30 minutes on ice with light blinding. The used antibodies listed below: Alexa Fluor 488 anti-mouse CD80 antibody (4 μL), PE/Cy5 anti-mouse CD86 antibody (1.25 μL), Alexa Fluor 647 anti-mouse CD11c antibody (0.5 μL), and PE anti-mouse I-A/I-E antibody (1.25 μL). For color compensation, we used spleen cell as same method above. We washed excessive antibodies with PBS by centrifugation for 2 times (10 minutes at 700 g, 4°C). We suspended the pellet with assay buffer and passed this analyte through nylon mesh sheet. We used BD Accuri C6 Flow Cytometer (BD Biosciences, East Rutherford, NJ, USA) for analysis. After color compensation we defined total dendritic cells (DCs) as CD11c and MHC II (I-A/I-E) double-positive cells and activated DCs as CD80 and CD86 double-positive cells. We calculated the number of DCs by the number of living cells $\times$ percentage of surface marker double-positive DCs.

2.6 | Reagents

Isoflurane, ethanol, paraformaldehyde, xylene, n-hexane, methanol, formic acid, ethyl acetate, acetic acid, acetonitrile, hematoxylin, eosin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), EDTA (NIPPON GENE, Chiyoda-ku, Tokyo, Japan), Roswell Park Memorial Institute medium (RPMI)-1640, fetal bovine serum (Nichirei Biosciences Inc, Chuou-ku, Tokyo, Japan), BV from *Apis mellifera* (Latoxan, Portes lés, Valence, France), Otsuka Normal Saline (Otsuka Pharmaceutical Factory, Naruto-city, Tokushima, Japan), Triton X-100 (MP biomedical, Santa Ana CA, USA), Multi Mount 480 (MATSUNAMI, Kishiwada, Osaka, Japan), bovine serum albumin (BSA) (PROSPEC, Ness-Ziona, Israel), Tween-20 detergent (Calbiochem, San Diego, CA, USA), 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA, USA), 0.4% trypan blue stain (NanoEntek, Waltham, MA, USA), Avidin-HRP, Alexa Fluor 488 anti-mouse CD80 antibody, PE/Cy5 anti-mouse CD86 antibody, Alexa Fluor 647 anti-mouse CD11c antibody, PE anti-mouse I-A/I-E antibody, Biotin anti-mouse IgE (BioLegend, San Diego, CA, USA), PGD₂-d₄, PGE₂-d₄, PGD₂, and PGE₂ (Cayman, Ann Arbor, MI, USA).

2.7 | Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed with Prism 3.0. Two-group comparisons were

analyzed by Student's *t* test. Multiple-group comparisons of body temperature were analyzed by two-way ANOVA and other experiments by one-way ANOVA with Tukey's post-test each.

3 | RESULTS

3.1 | BV injection stimulates PGD₂ production in mouse skin

We first measured the concentration of PGD₂ as well as a major pro-inflammatory lipid mediator PGE₂ in mice skins. Before the BV injection (naïve), PGD₂ and PGE₂ were scarcely detected in WT and *Crth2*^{-/-} mice skins (Figure 1A,B). The 80 µg of BV injection significantly increased PGD₂ production in WT mouse skin (221.02 ± 37.52 ng/g). Gene deficiency of CRTH2 did not influence the PGD₂ production (216.08 ± 50.22 ng/g).

The BV injection also produced PGE₂ in mouse skin. There was no difference in its productions between mouse lines (WT: 119.22 ± 5.78 ng/g, *Crth2*^{-/-}: 119.21 ± 216.08 ng/g) suggesting these mice represent similar inflammation against BV injection at their skins.

3.2 | CRTH2 signaling does not influence the damage of first BV injection

BV injection causes biological damages including body temperature drop.¹¹ In WT mice, 80 µg BV injection immediately dropped body temperature (5 minutes) and its drop was gradually recovered to comparable level as before BV injection (15 minutes to 24 hours, Figure 2A). A total of 640 µg BV injection also dropped body temperature immediately (5 minutes), but it was gradually decreased until 90 minutes (Figure 2B). After that, body temperature declined again sharply in 90 to 300 minutes (maximum; -9.9 ± 0.5°C, 300 minutes). After the 640 µg BV injection, 6 of 10 (60%) mice died. We calculated survival rate after euthanasia treatment by observing water and food intake and immobility

time. The gene deficiency of CRTH2 did not influence the 80 and 640 µg BV-induced body temperature drop (maximum; -11.0 ± 0.4°C, 640 µg BV).

High dosage of BV is known to impair kidney in rat.¹² One week after the 640 µg BV injection, we observed that the eosin-stained substances, presumably debris of damaged renal structure by BV components, were stuck in the cavity of renal tubule and glomerulus in WT mice as shown in Figure 2C. In *Crth2*^{-/-} mice, we also observed such impairment in kidney like WT mice. These observations showed that first BV injection caused similar biological reactions regardless of CRTH2 deficiency.

In the following experiment, we used 640 µg BV for high-dose challenge conferring some lethality.

3.3 | Activation of CRTH2 signaling attenuates the damage of second BV injection

We pre-injected 80 or 160 µg BV to mice back skin for sensitization. Three weeks after that, we injected 640 µg BV again (second BV injection). As shown in Figure 3A and Supporting Information Figure S1, in WT mice, the second BV injections dropped body temperature until 90 minutes after the injection like the first 640 µg BV injection. From 90 to 360 minutes, the second BV injections did not sharply drop the body temperature and the deepest body temperature drop was significantly attenuated compared to the first 640 µg BV injection (first; -9.3 ± 1.5°C, second after 80 µg BV sensitization; -5.1 ± 1.3°C, second after 160 µg BV sensitization; -5.0 ± 1.3°C). Since there was no difference in the effects of 80 or 160 µg BV injections, we used 80 µg BV for sensitization in following experiments.

These attenuations showed that sensitization establish the acquired immune response. In *Crth2*^{-/-} mice, second BV injection also dropped the body temperature as well as WT mice until 90 minutes after the injection. However, after that, the body temperature declined sharply again and the severe points of the body temperature drop were significantly exacerbated in *Crth2*^{-/-} mice compared to WT mice.

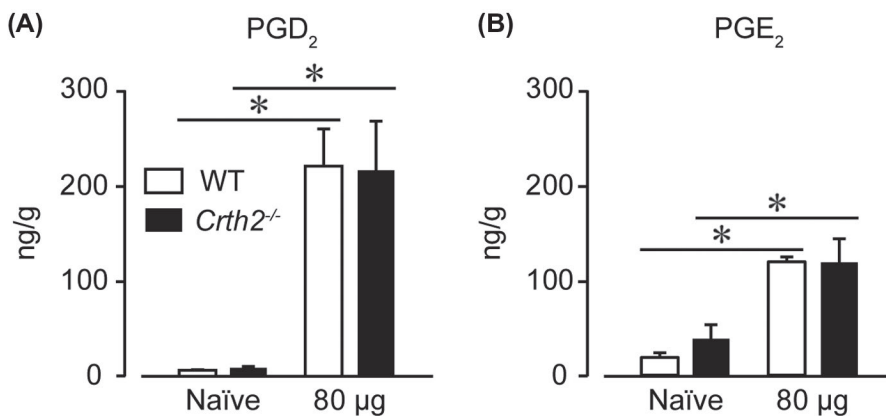


FIGURE 1 BV injection stimulates PGD₂ production in mouse skin. The concentrations of PGD₂ (A) and PGE₂ (B) in the naïve or 80 µg BV-injected (30 minutes after) skins of WT (white) and *Crth2*^{-/-} (black) mice. *n* = 4 each (in one group). **P* < .05

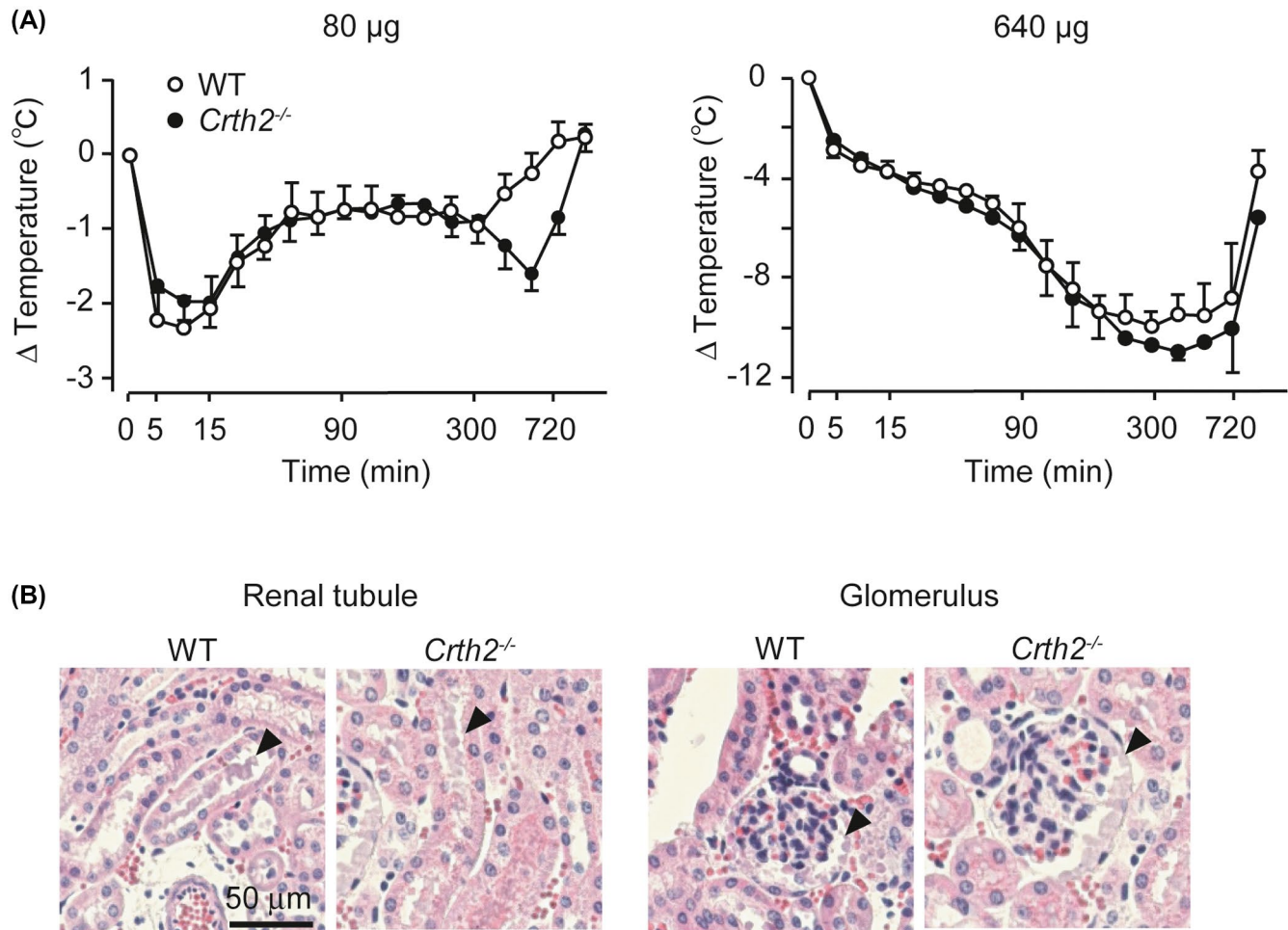


FIGURE 2 CRTH2 signaling does not influence the damage of first BV injection. Body temperatures drop after the first subcutaneous injection of 80 (left) and 640 μg (right) BV injection in WT (white) and *Crth2*^{-/-} (black) mice (A). $n = 8$ (80 μg , from a total of four separate groups), $n = 10$ (640 μg , from a total of four separate groups) each. The typical H&E staining pictures of renal tubule and glomerulus 1 week after the 640 μg BV injection in WT and *Crth2*^{-/-} mice (B). Arrowheads show depositions of eosinophilic substances

As Figure 3B showed, 80 and 640 μg BV injection caused depositions of eosinophilic substances in renal tubule and glomerulus of WT mice kidneys. Interestingly, the depositions in renal tubule and glomerulus were scarcely detected in WT mice suggesting that sensitization supported mice to gain resistance to BV. In *Crth2*^{-/-} mice, the depositions were detected as well as first 640 μg BV injected mice. CRTH2 signaling may promote detoxicating BV components and tissue protection by enhancing sensitization.

These results showed that activation of CRTH2 signaling attenuates the damage of second 640 μg BV injection by sensitization against BV.

3.4 | PGD₂/CRTH2 signaling promotes the production of BV-specific IgE

To investigate the effect of sensitization against the second BV injection, we transferred BV sensitized-serum into naïve WT mice before BV injection. Figure 4A showed that

in saline-transferred group, 560 μg BV injection dropped body temperature (maximum; $-11.8 \pm 1.7^{\circ}\text{C}$) as well as first 640 μg BV injected WT mice. We took serum from WT mice which were injected with 80 μg BV in every 2 weeks for four times and transferred into naïve WT mice. Interestingly, the transferring of sensitized serum attenuated the maximal drop of body temperature induced by 560 μg BV injection ($-9.9 \pm 1.3^{\circ}\text{C}$) slightly but not significantly. This implied that sensitization may play defensive role against BV injection partially via some serum components.

Previous study showed that presence of BV-specific IgE attenuates body temperature drop induced by BV.⁵ As shown in Figure 4B, we rarely detected BV-specific IgE in both WT and *Crth2*^{-/-} mice before the BV injection (indicated as 0 week in Figure 4B). The BV injections (80 μg , 2 times, every 2 weeks) gradually increased the serum IgE level in WT mice. In contrast, the level of BV-specific IgE in *Crth2*^{-/-} mice was significantly lower than that of WT mice. We found the difference of the serum IgE level in pre-BV injected WT and *Crth2*^{-/-} mice. This may be due to nonspecific binding of

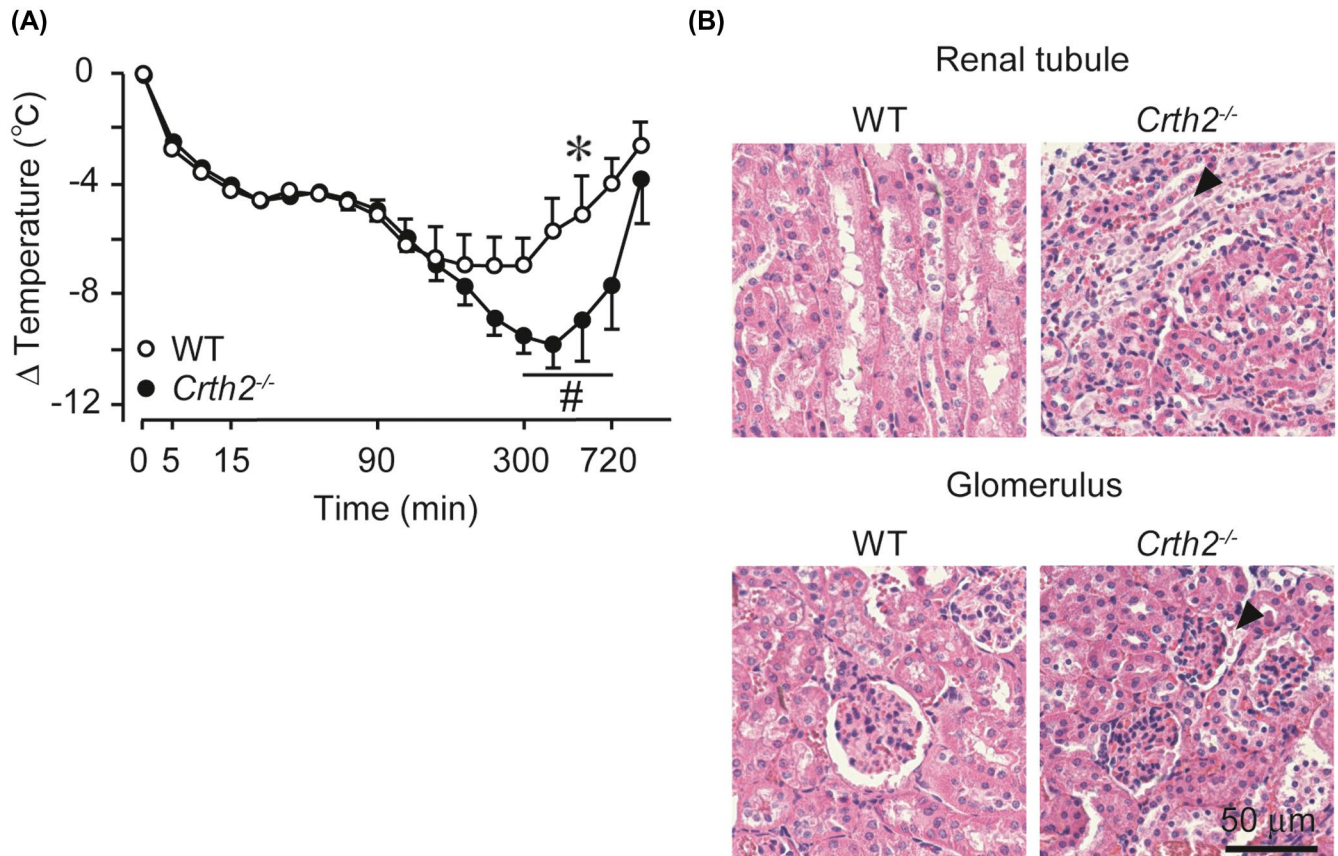


FIGURE 3 CRTH2 signaling attenuates the damage of second BV injection. Body temperature drop after the second subcutaneous injection of 640 μg BV, respectively, into WT (white) and *Crth2*^{-/-} (black) mice (A). $n = 8$ each (from a total of four separate groups). *, # $P < .05$. The typical H&E staining pictures of renal tubule and glomerulus after 1 week of second BV injection in WT and *Crth2*^{-/-} mice, respectively (B). Arrowheads show depositions of eosinophilic substances

serum components to BV. However, the exact reason of that difference remained to unveil.

Since immune responses vary with mouse strain, we also examined the serum IgE level in WT and *Crth2*^{-/-} Balb/c mice (3 weeks after 80 μg BV injection). Consistent with the data obtained from C57BL/6J mice lines, the serum level of BV-specific IgE was lower in *Crth2*^{-/-} mice than WT mice of Balb/c (Supporting Information Figure S2).

These observations suggest that PGD₂/CRTH2 signaling promotes the production of BV-specific IgE antibody.

3.5 | PGD₂/CRTH2 signaling promotes the migration of DCs to lymph nodes

Upon venom injection, skin-resident DCs capture antigens and migrate into regional lymph nodes to promote IgE production. In naïve mice, the numbers of CD11c- and MHC II-positive DCs in both axillary and inguinal lymph nodes were comparable between WT and *Crth2*^{-/-} mice (Figure 5A). After the 24 hours 80 μg BV injection, the number of CD11c- and MHC II-positive cells in regional lymph nodes tend to increase in WT mice. In *Crth2*^{-/-} mice, while the number of CD11c- and

MHC II-positive cells also tend to increase in regional lymph nodes, the number of them were lower than those of WT mice.

In naïve mice, the number of CD80 and CD86, the marker of activated DCs, expressing DCs in regional lymph nodes were comparable between WT and *Crth2*^{-/-} mice (Figure 5B). After the 24 hours 80 μg of BV injection, the number of CD80- and CD86-positive cells in CD11c- and MHC II-positive cells in WT mice significantly increased both in axillary ($7.7 \pm 0.4 \times 10^4$ cells) and inguinal lymph nodes ($6.3 \pm 0.6 \times 10^4$ cells) compared to those of naïve WT mice. The BV injection also migrated those cells into *Crth2*^{-/-} mice lymph nodes. However, their numbers were significantly lower in axillary ($4.0 \pm 0.6 \times 10^4$ cells) and inguinal ($3.9 \pm 0.4 \times 10^4$ cells) lymph nodes than WT mice.

These results indicated that PGD₂/CRTH2 signaling promotes the migration of DCs to regional lymph nodes.

4 | DISCUSSION

In the present study, we demonstrated that PGD₂/CRTH2 signaling plays a host defensive role against venom invasion using genetically modified mice. Upon BV injection into

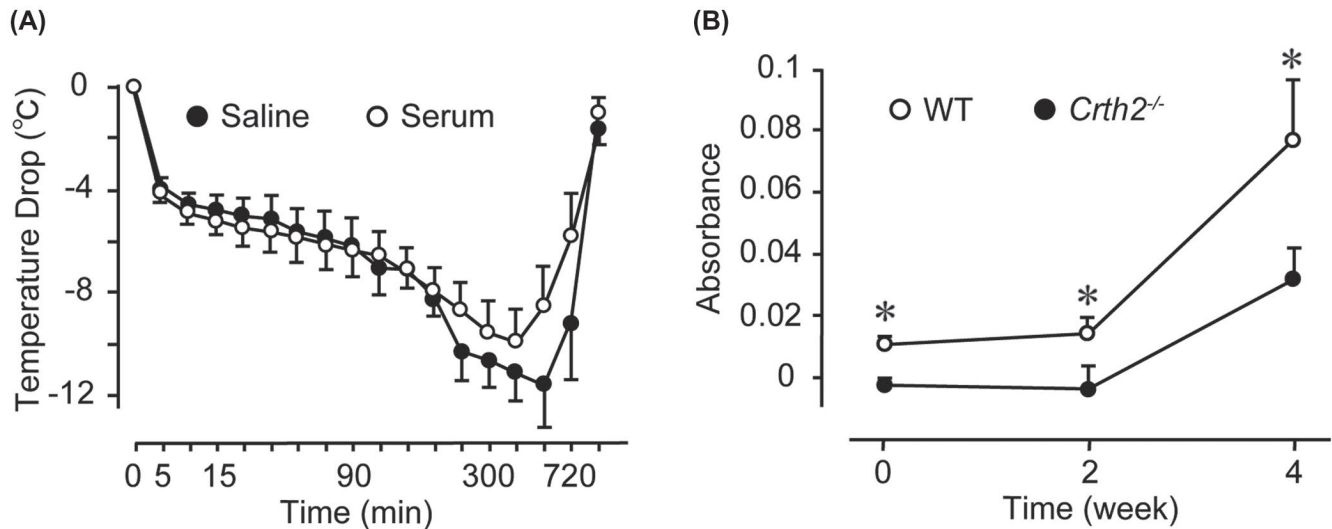


FIGURE 4 CRTH2 signaling promotes the production of BV-specific IgE. Body temperature drop after the subcutaneous injection of 560 μ g BV, respectively, into saline (black, $n = 11$, from a total of three separate groups) and serum (white, $n = 13$, from a total of three separate groups) transferred mice (A). Transferred serum was taken from WT mice after 2 weeks of the injection of 80 μ g BV every 2 weeks for four times. The titer of BV-specific IgE antibody expressed by absorbance measured in pre-BV injection (0 week), 2 weeks after the first 80 μ g BV injection (2 weeks) and 2 weeks after the second 80 μ g BV injection (4 weeks) in serum of WT (white) and *Crth2*^{-/-} (black) mice (B). $n = 5$ each (from a total of three separate groups). * $P < .05$

skin, PGD₂ is produced at the local site of injection, which promotes the IgE production via CRTH2 signaling. The produced IgE is associated with host immune responses that are accompanied by attenuated hypothermia induced by the second BV injection.

Marichal et al previously reported that the deficiency of IgE-dependent MCs activation exacerbated body temperature drop and decreased survival rate induced by BV injection. They also excluded the involvement of IgG in this phenomenon. Upon activation, MCs release various mediators including histamine, proteases, and heparin. These mediators cause strong inflammation including body temperature and blood pressure drop. At the same time, heparin neutralize venom.⁴ These biological reactions by MCs-derived mediators may act as defense against venom invasion. We here suggested that MC-derived PGD₂ contributes to the development of acquired immunity, and that it can enhance defense against a second BV invasion. Thus, PGD₂ can be another crucial factor responsible for biological defense by MCs against venom invasion.

Of interest, there were two phases of body temperature drop, the first phase was observed until 90 minutes after the injection, and the second phase was after 90 minutes in the 640 μ g BV-injected mice. We expected that the first temperature drop may be due to inflammatory reactions to mediators released from MCs, while the second temperature drop may be attributable to organopathy after the venom spread throughout the body. Our data showed that PGD₂/CRTH2 signaling attenuates only the second phase of body temperature drop. IgE, whose production is promoted by PGD₂/CRTH2 signaling, may enhance detoxification effects of MCs

to prevent organopathy. In contrast, the sensitization with BV did not influence the first phase drop in body temperature. Since we utilized relatively large amount of BV (640 μ g) to stimulate mice after the sensitization, this amount of BV may strongly and directly; IgE-independently degranulated MCs which lead to drop body temperature to similar extents in both lines of mice.

In the acquired immunity, antigen-specific IgEs bind and mature MCs. Previous study showed that IgE-binding; sensitized MCs react against antigen injection more powerfully than unsensitized MCs.⁶ A possible mechanism of attenuating the body temperature drop by PGD₂/CRTH2 signaling is that mature IgE-binding MCs store more detoxifying substances and release them in response against the second BV injection. Further investigation is required to reveal the detailed mechanism.

Upon inflammatory stimulation, phospholipase A₂ (PLA₂) releases arachidonic acid from cell membrane which is metabolized into prostaglandin H₂ by cyclooxygenase.¹³ H-PGDS in MCs further metabolizes prostaglandin H₂ into PGD₂. BV contains PLA₂ which can directly activate MCs to produce PGD₂. BV also contain mastoparan which is reported to stimulate PLA₂ activity.¹⁴ Furthermore, BV releases MC degranulating peptide^{1,2} which activates GTP-binding protein in MCs and increases intracellular calcium concentration.¹⁵ These BV components stimulate MCs to produce PGD₂ by activating cyclooxygenase and H-PGDS.

To produce antigen-specific IgE, the process of antigen presentation is indispensable. Antigen presenting cells such as DCs capture antigen and migrate in lymph nodes to transport

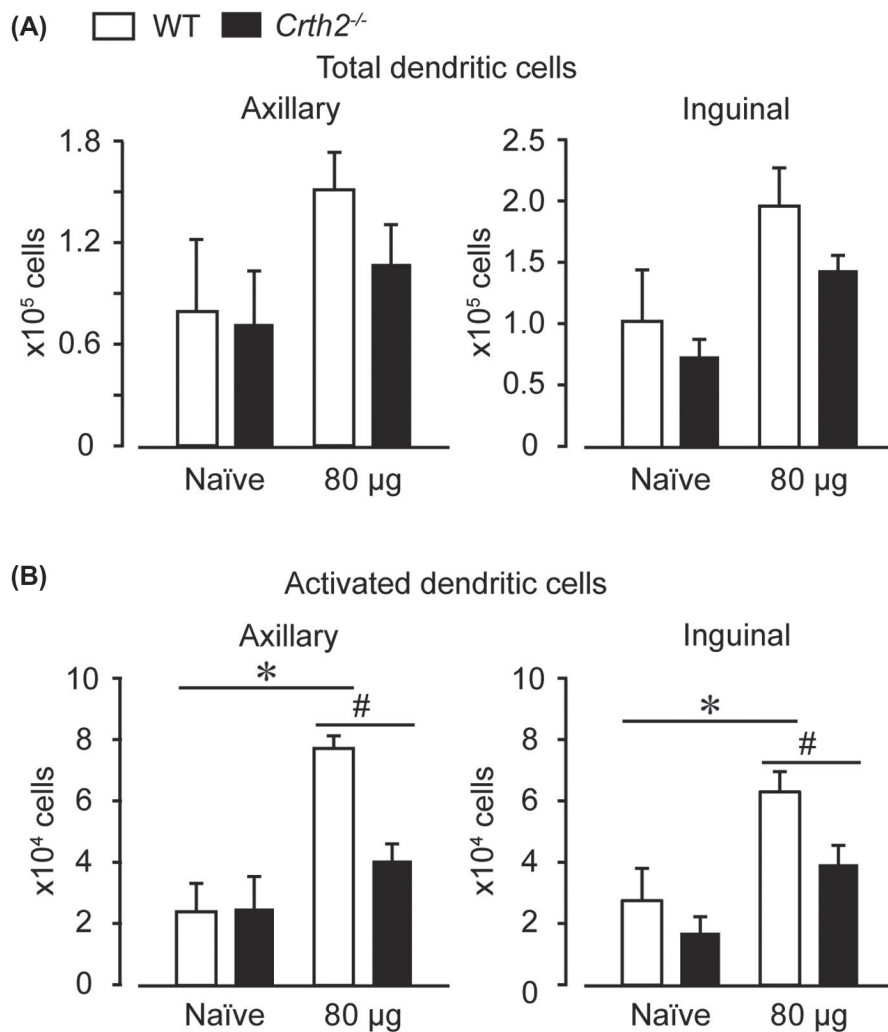


FIGURE 5 PGD₂/CRTH2 signaling promotes the migration of dendritic cells (DCs) to lymph nodes. The number of CD11c and MHC II expressing cells in axillary (right) and inguinal (left) lymph nodes (A) and the number of CD80 and CD86 expressing cells in CD11c and MHC II expressing cells in axillary (right) and inguinal (left) lymph nodes (B) of WT and *Crth2*^{-/-} mice before (naïve) and 24 hours after 80 µg BV injection. n = 5 each (from a total of three separate groups). *, # *P* < .05

the antigen information to naïve T cells. In this study, we revealed that activation of PGD₂/CRTH2 signaling promotes the migration of DCs into regional lymph nodes. Previous study reported that cultured DCs migrate in response to the specific CRTH2 agonist (DK-PGD₂) by chemotaxis assay.¹⁶ And, the other study reported that CRTH2 expression on DC is detected mildly by flow cytometry analysis.¹⁷ Thus, the migration of DCs in regional lymph nodes by activated PGD₂/CRTH2 signaling is at least partially responsible for the promotion in IgE production.

After the antigen presentation, the differentiation into Th2 cells from naïve T cells and interaction with B cells are proceeded. Since CRTH2 expression is also detected on Th2 cells,¹⁸ there is a possibility that PGD₂/CRTH2 signaling also participates in following steps of BV-specific IgE production. We need to further investigate the role of CRTH2 expressed on Th2 cells.

In our present study, we used 80 µg/head BV for sensitization and 640 µg/head BV for challenging in mice. Bees contain about more than 300 µg dry weight venom in their venom sacks and they pour about 50-140 µg dry weight venom into animals in one stinging.^{19,20} Therefore, we used nearly 1- to

10-sting BV for sensitization and challenge according to the previous study.⁵ Since these BV doses are likely to happen in the nature, our experimental condition may be reasonable to see biological actions against BV.

In conclusion, PGD₂/CRTH2 signaling promotes BV-specific IgE antibody production by promoting the migration of DCs into lymph nodes. And, its mechanism plays the role in host defensive reaction against second BV injection. This is the first report of elucidating that PGD₂ produced at the local skin site of BV injection contributes to the development of acquired immunity against subsequent venom injection in vivo.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

M. Kida, T. Nakamura, and T. Murata, designed the research and wrote the manuscript; M. Kida, T. Nakamura, and Y. Fujiwara performed the experiments and analyzed the data; M. Nakamura designed the research and provided experimental tools.

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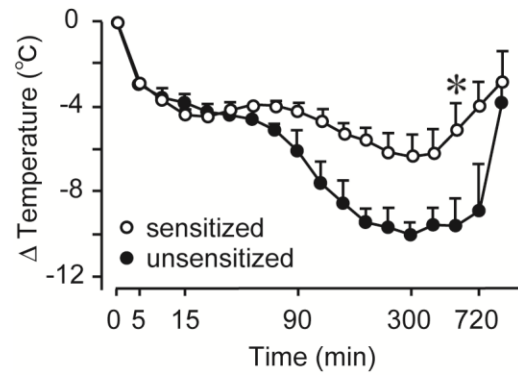
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SUPPORTING INFORMATION

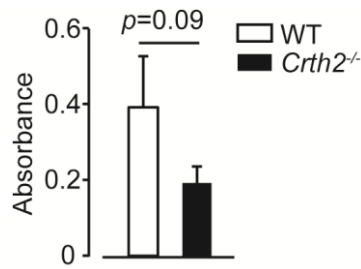
Additional Supporting Information may be found online in the Supporting Information section.

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Supplemental Fig. 1. CRTH2 signaling attenuates the damage of second BV injection.

Change in 24 hours body temperature after the first (black, unsensitized) and second subcutaneous injection of 640 μ g BV into WT mice sensitized with 160 μ g BV (white, sensitized). n=10 (from a total of 4 separate groups) and n=6 (from a total of 3 separate groups) each. The first BV injection data is duplicate data from Fig. 2B. * $p < 0.05$.



Supplemental Fig. 2. CRTH2 signaling promotes the production of BV specific IgE in Balb/c mice.

The titer of BV specific IgE antibody expressed by absorbance in serum of WT (white) and *Crth2*^{-/-} (black) Balb/c mice after 3 weeks of 80 µg BV injection. n=7 (WT) and n=8 (*Crth2*^{-/-}) (from a total of 3 separate groups).