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A Deficiency in the Prostaglandin D$_2$ Receptor CRTH2 Exacerbates Adjuvant-Induced Joint Inflammation

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Although the cyclooxygenase metabolites PGs are known to be involved in the progression of arthritis, the role of PGD$_2$ remains unclear. In this study, we evaluated the contribution of signaling mediated through a PGD$_2$ receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), in the progression of adjuvant-induced joint inflammation. Injection of CFA into the ankle joint stimulated PGD$_2$ production and induced paw swelling in both CRTH2-naïve (WT) and CRTH2$^{-/-}$ mice. CRTH2$^{-/-}$ mice presented more severe arthritic manifestations than did WT mice. Through bone marrow transplantation experiments between WT and CRTH2$^{-/-}$ mice, we showed that CRTH2 deficiency in bone marrow–derived immune cells is involved in disease progression. Morphological studies showed that CRTH2 deficiency accelerated the infiltration of macrophages into the inflamed paw. Consistent with this finding, we observed that treatment with the macrophage inactivator GdCl$_4$ or the macrophage-depleting agent liposomal clodronate improved arthritis symptoms in CRTH2$^{-/-}$ mice. Adoptive transfer of CRTH2$^{-/-}$ macrophages exacerbated joint inflammation in WT mice. In addition, CRTH2 deficiency accelerated, whereas CRTH2 agonism inhibited, the expression of a macrophage-activating cytokine (GM-CSF) and a chemokine receptor (CXCR2) in CFA-treated peritoneal macrophages. Together, these observations demonstrate that PGD$_2$-CRTH2–signaling plays a protective role in joint inflammation by attenuating the infiltration of macrophages.

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Abbreviations used in this article: BM, bone marrow; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DK-PGD$_2$, 13,14-dihydro-15-keto-PGD$_2$; DP, D prostanoid; Gr-1, granulocyte receptor-1; Mf, BM-derived macrophage; RA, rheumatoid arthritis; WT, wild-type.

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Increasingly, studies are investigating the pathophysiological role of PGD$_2$. The administration of PGD$_2$ or a DP agonist has anti-inflammatory effects in rat bowel inflammation (18) and mouse atopic dermatitis (19). DP receptor agonists also inhibit eosinophil migration (20) and dendritic cell activation (21) in a mouse model of asthma. Additionally, we showed that DP deficiency promotes, mouse acute lung inflammation, tumor growth, and dermatitis by inhibiting vascular permeability, whereas DP agonists suppress acute lung inflammation (22–24). Together, these studies suggest that the PGD$_2$-DP pathway exerts an anti-inflammatory effect.

In contrast, there are several studies showing that proinflammatory responses also are mediated through CRTH2. In vitro studies show that CRTH2 agonism stimulates chemotactic activation of Th2 cells, basophils, eosinophils, and macrophages (25, 26). In line with these observations, treatment with a CRTH2 agonist aggravates atopic dermatitis, whereas CRTH2 deficiency improves it (27, 28). Concomitant CRTH2-mediated anti-inflammatory reactions were reported. CRTH2 deficiency promoted the infiltration of eosinophils into asthmatic mice (29), as well as the infiltration of neutrophils in septic mice (30). Thus, PGD$_2$ signaling can regulate inflammation differently, depending on the context of the disease.

PGD syntheses are expressed at high levels in arthritic joints, and PGD$_2$ production is detected in the serum and arthritic paws of model mice (31, 32). However, the pathophysiological roles of PGD$_2$ in RA remain unknown. In this study, we investigated the role of CRTH2-mediated signaling in arthritic inflammation using genetically modified mice. Our results demonstrate that CRTH2 signaling attenuates adjuvant-induced joint inflammation by inhibiting macrophage accumulation and activation.

Materials and Methods

Reagents

The following reagents were used: IFA and heat-killed Mycobacterium tuberculosis strain H37RA (Becton Dickinson, San Diego, CA); paraformaldehyde, Triton X-100, and GD$_3$C (WAKO, Osaka, Japan); liposomal clodronate (FormuMax Scientific, Palo Alto, CA); 13,14-dihydro-15-keto-PGD$_2$ (Cayman Chemicals, Ann Arbor, MI); M-CSF (ProSpec, East Brunswick, NJ); DAPI, BSA, HBSS, and RPMI 1640 (Sigma-Aldrich, St. Louis, MO); random RT primers, ReverTra Ace, and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan); FITC-labeled Y chromosome probes (Chromosome Science Labo, Hokkaido, Japan); anti–granulocyte receptor-1 (anti–Gr-1), anti-CD4, and anti-CD8 Ab (BioLegend, San Diego, CA); anti-CD68 Ab (AbD Serotec, Oxford, U.K.); anti-F4/80 Ab (BMA Biomedicals, Augst, Switzerland); and anti-rat IgG conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR).

Mice

All experiments and animal care were conducted in conformity with the institutional guidelines of The University of Tokyo. CRTH2$^{-/-}$ mice were generated, backcrossed for 10 generations to C57BL/6J mice, and bred as previously described (28). Control wild-type (WT) mice of the same generation were used.

Stimulation of adjuvant-induced joint inflammation

The adjuvant-induced joint inflammation model is known to exhibit features similar to human RA (33). WT and CRTH2$^{-/-}$ mice, 8–12 wk old, were injected s.c. at two sites around the left ankle joint with 150 µg CFA. The volume of the hind paw was measured using a digital plethysmometer (LE7500; Panlab, Barcelona, Spain). Results are expressed as the difference ($\Delta$ ml) in paw volume before and after administration of CFA.

A series of bone marrow (BM) transplantation experiments was performed. To ablate the BM, recipient mice (4–5 wk old) were irradiated (9 Gy). BM cells (2 × 10$^6$) were isolated from the femurs and tibiae of donor mice (8–12 wk old) and injected into the tail vein of recipient mice. The subsequent experiments were conducted on the mice 6 wk posttransplantation.

Morphological analysis

Hind paws were fixed in 10% neutral-buffered formalin for 4 d, decalcified in 30% formic acid for 24 h, and embedded in paraffin. Four-micrometer-thick sections of the ankles were stained with H&E or toluidine blue.

Immunofluorescence was performed on frozen sections (4 µm). The primary Abs used were anti–CD68 (1:1,000), anti-F4/80 (1:500), anti–Gr-1 (1:500), anti-CD4 (1:500), and anti-CD8 (1:500). The images were captured using an Eclipse E800 fluorescence microscope (Nikon, Tokyo, Japan). The number of CD68$, F4/80$, Gr-1$, CD4$, and CD8$^+$ cells was counted in 10 randomly selected fields.

Measurement of PGD$_2$ and PGE$_2$

The content of PGD$_2$ and PGE$_2$ in the paws was measured as previously described (23). Briefly, excised paws were quickly frozen in liquid nitrogen and homogenized in ethanol containing 0.02% HCl, and the samples were separated by HPLC. The quantification was performed using an LCMS-8030 Triple Quadropole Mass Spectrometer (Shimadzu, Kyoto, Japan).

Isolation of peritoneal macrophages

Mice were injected i.p. with 150 µg CFA and sacrificed after 72 h. Peritoneal exudate cells were collected by washing with 5 ml HBSS. The cells were incubated in RPMI 1640 for 2 h, and the adherent cells (peritoneal macrophages) were collected. In some experiments, the CRTH2 agonist D$_3$-PGD$_2$ (10 µg/kg) was injected i.p. in mice 2 h prior to CFA treatment and twice daily thereafter.

RT-PCR

Total RNA was extracted from intact hind paws or peritoneal exudate macrophages. The first-strand synthesis of cDNA was performed using a random 9-mer primer and ReverTra Acc. For RT PCR analyses, cDNA was amplified using THUNDERBIRD SYBR qPCR Mix and an ABI Prism 7500 (Applied Biosystems, Foster City, CA). The expression levels were determined by the ΔCt method, using 18S rRNA as an endogenous control gene. The primer sequences used are shown in Table I.

Inactivation of macrophages

To decrease macrophage number and activity in an in vivo situation (34), GDc$_3$1 (100 mg/kg) was administered i.p. 1 d prior to CFA treatment and, subsequently, on alternating days. The macrophage-depletion agent liposomal clodronate (100 µl/head) also was administered i.p. 1 d prior to, and every 3 d following, CFA injection.

Adoptive transfer of macrophages

To prepare BM-derived macrophages (Mfs), BM cells from male WT or CRTH2$^{-/-}$ mice were cultured in RPMI 1640 supplemented with 10% FBS and 20 mg/ml M-CSF for 7 d. Mfs (1 × 10$^6$) from each mouse group were transferred into female WT hosts by i.v. injection immediately before and every 3 d after CFA treatment. The cryosections were labeled with the FITC-labeled Y chromosome probes (Chromosome Science), following the manufacturer’s protocol, and the number of FITC$^+$ cells was counted in randomly selected fields.

Statistics

Data are expressed as mean ± SEM. Unpaired Student t test was used to compare data between two groups. One-way ANOVA followed by a Tukey or Dunnett test or two-way ANOVA followed by the Bonferroni post hoc test were used to compare multiple groups. A p value < 0.05 was considered significant.

Results

CRTH2 deficiency exacerbates arthritis

As shown in Fig. 1A and 1B, nontreated WT and CRTH2$^{-/-}$ mice had the same initial paw size, and the injection of CFA (150 µg) induced paw swelling. Their paw swelling peaked on day 11 and lasted until day 15 (paw volume on day 15, WT mice: 0.15 ± 0.013 ml; CRTH2$^{-/-}$ mice: 0.29 ± 0.023 ml; n = 5 for each). CRTH2$^{-/-}$ mice showed a progressive development of paw swelling throughout the test period. By day 11 postinjection, the pad volume of CRTH2$^{-/-}$ mice was ~2-fold greater than that of WT mice.

No morphological differences were observed between WT and CRTH2$^{-/-}$ hind paws prior to CFA stimulation (Fig. 1C, 1D, 1E, 1F).
The data are presented as the ratio of expression relative to untreated paws. A log value of the mean mRNA levels of CFA-treated WT paws is shown in Supplemental Fig. 1B, and the quantifications are shown in Fig. 2C or Supplemental Fig. 2A, and the quantifications are shown in Fig. 2D or Supplemental Fig. 2B.) or the neutrophil marker Gr-1. Few cells expressed the T cell markers CD4 and CD8. We found that the number of CD68+ macrophages in CFA-treated mice was comparable between the mouse strains. These observations suggest that a genetic CRTH2 deficiency in hematopoietic cells aggravates adjuvant-induced joint inflammation.

CRTH2 deficiency in hematopoietic lineage cells aggravates arthritis

To investigate the contribution of CRTH2 signaling in hematopoietic and nonhematopoietic lineage cells during arthritis progression, BM chimeric mice were generated and analyzed. WT hosts with transplanted CRTH2-deficient BM (WT + CRTH2+/−BMM) exhibited a CFA-induced increase in paw volume equal to that of nonchimeric CRTH2−/− mice (CRTH2+/− + CFA) (Fig. 2A). Conversely, chimeric CRTH2+/− mice possessing WT BM (CRTH2+/− + WTBM) displayed a reduced paw volume in response to CFA injection compared with that of nonchimeric CRTH2−/− mice. These results suggest that a genetic CRTH2 deficiency in hematopoietic cells aggravates adjuvant-induced joint inflammation.

CRTH2 deficiency accelerates infiltration of macrophages in inflamed paws

We performed a histological analysis on paws on the third day post-CFA injection, a time point when the manifestations of arthritis are first observed. H&E staining of paw cross-sections revealed that the majority of infiltrating cells exhibited multilobulated or notched nuclei, which are typical of neutrophils or monocytes/macrophages, respectively (Fig. 2B, insets). Consistent with these observations, immunostaining showed that many cells expressed the macrophage marker CD68 or iNOS. Future studies of CD68+ macrophages in CRTH2−/− paws were ∼2.4-fold greater than that in WT paws. Notably, the numbers of Gr-1+ neutrophils and CD4+CD8+ T cells were comparable between the mouse strains. These observations suggest that the majority of infiltrating cells exhibited multilobulated or notched nuclei, which are typical of neutrophils or monocytes/macrophages, respectively (Fig. 2B, insets). Consistent with these observations, immunostaining showed that many cells expressed the macrophage marker CD68 or iNOS. Future studies of CD68+ macrophages in CRTH2−/− paws were ∼2.4-fold greater than that in WT paws. Notably, the numbers of Gr-1+ neutrophils and CD4+CD8+ T cells were comparable between the mouse strains. These observations...
suggest that CRTH2 deficiency accelerates the infiltration of macrophages into CFA-injected paws.

**CRTH2 deficiency in macrophages aggravates arthritis**

Next, we investigated the contribution of macrophages to the development of adjuvant-induced joint inflammation in CRTH2−/− mice. As shown in Fig. 3A and 3B, treatment with the macrophage inactivator GdCl3 (100 mg/kg, 1 d before and alternating days post-CFA treatment) significantly reduced the CFA-induced paw swelling typically seen in CRTH2−/− mice to a level that was comparable to that in WT mice. Consistently, GdCl3 treatment also decreased the elevated mRNA levels of IL-6 and IL-1β that we observed in CRTH2−/− inflamed paws (Fig. 3C). Treatment with the macrophage-depleting agent liposomal clodronate (100 µl/head, 1 d before and every 3 d after CFA treatment) improved the paw swelling normally observed in CRTH2−/−, but not WT, mice (Fig. 3D). These results indicate that the infiltrating macrophages exacerbate joint inflammation in CRTH2−/− mice. As shown in Supplemental Fig. 3, GdCl3 treatment decreased the volume of the swollen paws induced by higher concentrations of CFA (500 µg) in WT mice. Therefore, the 150-µg dose of CFA might not have been sufficient to observe a response to macrophage inactivation/depletion in WT mice.

We next examined whether CRTH2−/− macrophages aggravated joint inflammation. Mfs isolated from male CRTH2−/− or WT mice were injected into female WT mice, and tissue infiltration of Y chromosome+ macrophages was assessed. On day 11, Y chromosome+ Mfs were detected in WT inflamed paws (Fig. 3E). The number of infiltrating CRTH2−/− Mfs was 2.0-fold greater than that of WT Mfs (Fig. 3F). The adoptive transfer of CRTH2−/− Mfs to WT hosts induced greater paw swelling than that of WT Mfs (Fig. 3G). These results further support the hypothesis that CRTH2 deficiency in macrophages exacerbates the manifestations of joint inflammation.

**CRTH2 deficiency enhances the accumulation and activation of macrophages**

We next attempted to clarify the mechanism by which CRTH2−/− macrophages aggravate adjuvant-induced joint inflammation. Three days after the i.p. injection of CFA (150 µg), peritoneal macrophages were isolated and characterized. Similar to the data obtained in arthritic ankles, CRTH2 deficiency increased the infiltration of macrophages into the peritoneal cavity in response to CFA injection (Fig. 4A). There are two subsets of macrophages. M1 macrophages produce the proinflammatory cytokines inducible NO synthase, IL-12, and IFN-γ, whereas M2 macrophages synthesize arginase-1, an enzyme that inhibits NO synthase activity and expresses a scavenger receptor (CD206). As shown in Supplemental Fig. 4, CRTH2 deficiency did not change the mRNA expression of these cytokines and cell surface Ag. However, CRTH2 deficiency increased the mRNA expression of a macro-

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**FIGURE 2.** The deficiency of CRTH2 enhances macrophage infiltration. (A) Time-dependent changes in paw volume (n = 9–10). *p < 0.05, versus WT + WTBM mice, p < 0.05, versus CRTH2−/− + WTBM mice on day 11. (B) Representative H&E staining of ankle sections (day 3). Scale bar, 20 µm. (C) Representative images of CD68+ cells in ankle sections. Scale bar, 20 µm. (D) Number of CD68+, Gr-1+, CD4+, and CD8+ cells in the ankle sections (n = 4 for each). *p < 0.05 versus CFA-treated WT paws, †p < 0.05 versus nontreated paws.

**FIGURE 3.** CRTH2 deficiency in macrophages exacerbates adjuvant-induced joint inflammation. GdCl3 (100 mg/kg) was administered i.p. 1 d prior to CFA treatment and on alternating days thereafter. (A) Representative images of CFA-induced paw swelling on day 11. Scale bar, 1 cm. (B) Time-dependent changes in paw volume (n = 5–6). *p < 0.05 versus saline-treated WT paws, †p < 0.05, versus saline-treated CRTH2−/− paws on day 11. (C) mRNA expression levels of proinflammatory cytokines in arthritic paws (day 5, n = 5–9). A log value of the mean mRNA levels of saline-treated WT paws is regarded as 1.0. The data are presented as the ratio of expression relative to that in saline-treated WT paws. *p < 0.05 versus saline-treated WT paws, †p < 0.05 versus saline-treated WT paws, ‡p < 0.05 versus saline-treated CRTH2−/− paws. (D) Changes in paw volume on day 11 after CFA immunization (n = 5/group). Liposomal clodronate (100 µl/head) was administered i.p. 1 d before and every 3 d after CFA injection. *p < 0.05 versus saline-treated WT paws, †p < 0.05 versus saline-treated CRTH2−/− paws. (E) Changes in paw volume on day 11 after CFA immunization (n = 5–6). (F) Representative images of Y chromosome+ cells in the ankle sections. Scale bar, 20 µm. (G) Number of Y chromosome+ cells in the ankle sections (n = 6 for each). *p < 0.05 versus WT + WT Mfs, (G) Changes in paw volume on day 11 after CFA immunization (n = 4–5). Mfs (1.5 × 10⁶) from each group of mice were transferred into WT hosts by i.v. injection immediately before and every 3 d after CFA treatment. *p < 0.05 versus WT + WT Mfs.
mice (Fig. 4C).

pression levels of GM-CSF and CXCR2 by more than half in WT before and twice daily after CFA treatment) decreased the ex-

macrophages. * presented as the ratio of expression relative to that in WT or vehicle-treated levels of WT or vehicle-treated macrophages is regarded as 1.0. The data are

an endogenous PGD2-CRTH2 pathway that acts in an antiarthritic manner. PG-mediated signaling appears to modulate the progression of joint inflammation multilaterally, with proinflammatory or anti-
inflammatory effects, depending on the context.

The infiltration of macrophages into inflamed paws reportedly exacerbates RA progression by producing a set of proinflammatory/ proarachitic cytokines, including TNF-α, IL-6, and IL-1β (35). Indeed, the severity of RA positively correlates with the number of infiltrating macrophages in inflamed joints (2). Depletion of macrophages using drugs or gene recombination improves murine arthritis (3, 4). Therefore, researchers have explored ways to modulate macrophage activation to treat RA. In this study, we showed that the PGD2-CRTH2 pathway is a novel inhibitory signaling of the macrophage response in the adjuvant-induced joint inflammation model.

Adjuvant-induced joint inflammation exhibits features similar to human RA, such as infiltration of innate immune cells into the joint and articular cartilage and bone destruction (33). However, because we used a relatively low dosage of CFA (150 µg), the resulting manifestation was relatively weak in WT mice. This model can be used as an experimental model of incipient human RA. In addition, CFA-induced inflammation does not include an adoptive autoimmune response that is characterized by T cell infiltration, which is also crucial for RA progression. Thus, we could not clarify the contribution of CRTH2 signaling to the autoimmune response. Previous studies separately indicated the implications of D2 or macrophage in serum-induced mouse autoimmune arthritis (3, 4, 31). The PGD2-CRTH2 pathway may be common in various types of joint immune responses, and this signaling pathway may be exploited as a potential therapeutic target for the treatment of RA in the future. Further investigations are needed to clarify this point.

In vitro studies showed that CRTH2 agonism (DK-PGD2, 10 nM–1 µM) elicits the migration of immune cells, including macrophages, Th2 cells, eosinophils, and basophils (25, 26). Intradermal administration of DK-PGD2 (3 µg/site) in mice also promotes neutrophil accumulation (36). In contrast to these reports, our data showed that the concentration of PGD2 in inflamed WT paws was relatively lower (~35 ng/g) (Fig. 1F) and that CRTH2 deficiency accelerated CFA-induced macrophage accumulation in vivo (Figs. 2D, 3F, 4A). Furthermore, our group reported previously that CRTH2 deficiency or CRTH2 agonism (DK-PGD2, 100 µg/kg) does not influence LPS-induced neutrophil infiltration into the lung (22). This inconsistency in the chemotactic action of CRTH2 signaling on immune cells may be attributed to differences in the site of PGD2 production or CRTH2 stimulation and/or the strength and duration of signaling, the target tissue, and the type of stimulant.

As shown in Fig. 2D, neutrophil infiltration is comparable in WT and CRTH2−/− paws, whereas macrophage infiltration is accelerated in CRTH2−/− paws. A previous study showed that a variety of chemokines for neutrophils, such as CXCL1, CXCL2, CXCL5, CCL2, and CCL7, were detected in murine serum–induced arthritic joints (37). These chemokines other than PGD2 may more dominantly modulate neutrophil infiltration into the inflamed paw.

Based on our data, we conclude that CRTH2 deficiency enhances, and CRTH2 agonism inhibits, the expression of the macrophage-stimulating factors GM-CSF and CXCR2, which further promotes the manifestations of arthritis (Fig. 4B, 4C) (37, 38). CRTH2 is coupled to Gi-type G proteins. Previous studies reported that CRTH2 agonism activates PI3K, which then suppresses IL-2–induced apoptosis in Th2 cells (39). Other studies suggested that the constitutive activation of PI3K, by creating a deficiency in the PTEN gene, inhibits the expression of proinflammatory cytokines, including TNF-α and IL-6, in macrophages (40). Although we did not fully elucidate the detailed mechanism of macrophage depression by CRTH2 signaling, our data suggest that it might act by inhibiting the expression of GM-CSF and CXCR2 via PI3K activation.

Although a previous study showed that chondrocytes and synovial cells in murine arthritic paws express PGD synthases (31), we did not identify the functional source of PGD2 during the progression of joint inflammation in our study. In addition, there is a possibility that PGD2 may exhibit pathophysiologic responses that act exclusively on DP when CRTH2 is absent. DP activation is reported to have anti-inflammatory effects in various pathological conditions, including asthma, dermatitis, and lung inflammation, as well as in tumors (21–24). Human osteoclasts express DP and CRTH2, and stimulations of both receptors inhibited osteoclast differentiation (41). Although we did not clarify the contribution of DP-mediated signaling in this study, it might also improve manifestations of arthritis. Further investigation is necessary to reveal the pathophysiological implications of PGD2 signaling.
In conclusion, we demonstrate that PGD2-CRTH2 signaling improves joint inflammation by inhibiting macrophage activation and accumulation. Our findings provide new insights into the protective effect and therapeutic potential of exogenous activation of CRTH2 signaling in treating arthritis.

Disclosures

The authors have no financial conflicts of interest.

References


CRTH2 deficiency aggravates joint inflammation. (A and B) Images of representative H&E and toluidine blue staining of ankle sections (day 11). White arrowheads indicate the infiltration of inflammatory cells. Arrows indicate bone erosion. Black arrowheads indicate cartilage destruction. ti, tibia; ta, talus; c, calcaneus. Scale bars: 200 μm (H&E) and 100 μm (toluidine blue).
Supplemental FIGURE 2.

The deficiency of CRTH2 accelerates macrophage infiltration. (A) Representative images of F4/80-positive cells in the ankle sections. (B) The number of F4/80-positive cells in the ankle sections (n = 4 for each). Scale bar: 10 µm. *, P < 0.05 indicates a significant difference in the number of positive cells relative to CFA-treated WT paws; and †, P < 0.05 relative to non-treated paws.
Supplemental FIGURE 3.

Treatment with GdCl$_3$ improves the swelling of WT paws induced by high concentrations of CFA. Changes in paw volume on day 11 after CFA immunization (n = 7 per group). *, $P < 0.05$ indicates a significant difference in paw volume relative to paws treated with 150 µg CFA; and †, $P < 0.05$ relative to 500 µg CFA-treated paws.
CRTH2 deficiency did not affect the phenotype of macrophage subsets. mRNA expression levels of M1 or M2 macrophage makers in CFA-elicited macrophages (n = 5–8). The data are represented as the ratio of expression relative to that of the WT.