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ORIGINAL ARTICLE



COX-2 induces T cell accumulation and IFN- γ production during the development of chromium allergy

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ABSTRACT

Chromium (Cr) is commonly added into various metal alloys to improve some mechanical properties such as corrosion resistance, strength, and workability. However, Cr is also known to be a metal allergen for some individuals. Metal allergy is a T cell-mediated disease with symptoms of inflammation and swelling that involve inflammatory cytokines and prostaglandins. Hence, suppressing these inflammation paths by using COX-2 inhibitor might be useful in treating Cr allergy. In this study, mice were used with Cr-induced allergy challenge model. The mice were injected with celecoxib once per day for 7 days one hour after the challenge. Footpad samples were stained with haematoxylin and eosin (H&E), and lymphocytes were isolated from popliteal lymph nodes for the flow cytometric analysis. The results show that both prostaglandin E₂ (PGE₂), a known mediator of inflammation, and cyclooxygenases (COX)-2 have important roles in the development of Cr allergy. Further, COX-2 inhibitor, celecoxib, was effective in relieving swelling and inflammation in Cr-allergic mice concordant with suppression of IFN- γ production by CD8⁺ T cells and T cell accumulation in the lymph nodes. Therefore, the inhibition of COX-2 may be a therapeutic target for Cr allergy, and additional molecules in the PGE₂ signalling pathway may also be an effective therapeutic target for the treatment of metal allergy.

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Introduction

Metal allergy is categorized as allergic contact dermatitis that is caused by metal sensitization [1,2]. In dentistry, many treatments utilize metals including dental restoration, crowns, orthodontics, implants, and dentures [3]. Nickel, cobalt, and chromium (Cr) are often utilized in alloys to increase properties including corrosion resistance, strength, and workability. In fact, Cr is frequently used in alloys found in coins, dental materials, and daily goods. Unfortunately, however, these metals are also known to cause allergic contact dermatitis [4–6].

Symptoms of metal allergy include inflammation, redness, fever, swelling, and pain. The onset of this T cell-mediated disease occurs approximately 24 hours after exposure to the causal metal [3,6–8]. In several reports, various cytokines have been shown to control the activation or inhibition of immune responses during metal allergy [5,9,10]. Of these, IFN- γ an inflammatory cytokine that enhances immune responses is produced by pathogenic T cells during metal allergy and is considered most important in this process [6,11,12]. In addition to cytokines, prostaglandins also play a key role in the generation of

inflammatory responses [8,13]. Prostaglandin E₂ (PGE₂) is the major lipid mediator in inflammation, which mediates its various cellular functions through binding to the PGE₂, EP1, EP2, EP3, and EP4 receptors [13,14]. Cyclooxygenase (COX)-1 and COX-2 are the enzymes responsible for the oxidation of arachidonic acid during the production of PGE₂; therefore, increases in PGE₂ during inflammation are mediated by COX-2 [14–17]. Although COX-2 expression has been observed in various inflammatory diseases [17–21], the role of COX-2 in metal allergy is unclear. Furthermore, how COX-2 and IFN- γ contribute to the onset of Cr allergy is unknown. Therefore, we investigated whether COX-2 is involved in Cr allergy and whether a COX-2 inhibitor might be a potential treatment for Cr allergy.

Material and methods

Ethics statement

All animals experimental procedures were conducted under approval from the Institutional Committee for the Use and Care of the Animal Laboratory of the Tohoku University, this experiment was approved by the Tohoku University

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 Supplemental data for this article can be accessed [here](#).

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Ethics Review Board (No. 2012AcA-069), and Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication of NIH 85-23, revision 1996). All experimental animals conducted under free pathogens conditions. Isoflurane (Merck & Co., Inc., Whitehouse Station, NJ, USA) were an anaesthetic drug in all operations. Mice were sacrificed with minimal suffering using cervical dislocation method to collect tissue samples.

Mice

Seven-week-old female mice type C57BL/6 purchased from CLEA Japan. The animals used in this study were approved by the Institutional Committee for the Use and Animal Care of the Tohoku University Laboratory.

Reagents

CrCl₂ with 99.99% purity was acquired from Sigma-Aldrich (St. Louis, MO, USA). Celecoxib (C₁₇H₁₄F₃N₃O₂S) with more than 98% purity was acquired from Tokyo Chemical Industry (Tokyo, Japan). Lipopolysaccharide (LPS) prepared from *Escherichia coli* O55:B5 acquired from Sigma (St Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS).

Induction of Cr allergy and measurement of allergic footpad swelling

Mice were inducing Cr allergy as describe in previous paper [12]. Sensitization was injected twice at an interval of 7 days *via* the intradermal (i.d.) route into the left and right groin of mice, 125 µl of indicated CrCl₂ with 10 µg/ml of LPS in PBS (250 µl per mice) [22]. At day 7, after the 2nd sensitization, mice were challenged by i.d. injection of 25 µl of indicated dose of CrCl₂ (without LPS) in sterile saline into both the left and right footpads. As a control, mice were injected with PBS and then challenged with indicated dose of CrCl₂ (without LPS). Footpad swelling was measured before and at 24–72 h after the challenge using a Peacock Dial Thickness Gauge (Ozaki MFG Co. Ltd, Tokyo, Japan) [23], and the difference in footpad thickness before and after challenge was calculated. Celecoxib treatment, one hour after challenge the mice were injected by intraperitoneally (i.p.) with celecoxib once per day from for 7 days. The dose of celecoxib was 13 mg/kg of weight in 250 µl of polyethylene glycol (PEG) 400/saline (2:1, v/v). PEG400/saline (2:1, v/v) was used in control mice were injected with i.p. instead of celecoxib.

Histological analysis

Mice were sacrificed at 24 h after the challenge. Footpad samples were embedded in Tissue-Tek O.C.T Compound and sectioned using a cryostat (10 µm). After slides were fixed with acetone, sections were stained with haematoxylin and eosin (H&E).

Flow cytometric analysis of cytokine production

The flow cytometric analysis procedure was arranged in the same procedure as previous paper to examine IFN-γ production [12].

Statistical analysis

Student's *t*-test was used in each group to analyze statistical significance ($\alpha = 0.05$) regarding changes in footpad swelling and IFN-γ production by using SPSS software version 21 (IBM Corp, New York, USA). Super-scripted-asterisks (*) and (**) indicate statistical significance at $p < .05$ and $p < .01$, respectively.

Results

Footpad swelling in Cr allergic mice

First, altered a published model of Cr allergy in mice to include challenge with 3 mM CrCl₂ (without LPS) because we observed that challenge with 10 mM CrCl₂ resulted in severe spontaneous swelling unrelated to the allergic response (see [Supplementary Figure S1](#)) [22,24]. To assess the induction of Cr allergy in this model, inflammatory responses were evaluated by footpad swelling measurement. Sensitization with CrCl₂ and LPS was done twice, and then challenge with CrCl₂ only was done once ([Figure 1\(a\)](#)), and footpad swelling was measured before and after challenge at the indicated times shown in [Figure 1\(a\)](#).

Although the peak in footpad swelling was observed at 24 h after challenge in both groups ([Figure 1\(b\)](#)), footpad swelling in the Cr-sensitized group was significantly enhanced compared with the PBS-sensitized group at this time. Moreover, this difference continued at 48 and 72 h after challenge. Further, Cr-sensitized mice were found to be slow in their recovery from footpad swelling compared with PBS-sensitized mice ([Figure 1\(b\)](#)).

COX-2 is upregulated during the development of chromium allergy

Next, to examine whether COX-2 is involved in Cr allergy, we used our modified model of Cr-allergic mice. Upregulation of COX-2 expression is responsible for increased release of PGE₂ under inflammatory conditions [15,25]. Thus, the amount of COX-2 mRNA in the spleens of Cr-sensitized mice was measured using quantitative RT-PCR. A significant increase of COX-2 mRNA was observed in the spleens of Cr-allergic mice compared with wild-type mice ([Figure 2\(a\)](#)).

Celecoxib is a COX-2-specific inhibitor, which has been shown to be effective for clinical suppression of inflammation [17,26,27]. Thus, to examine whether COX-2 plays a role in the development of Cr allergy, mice were treated with celecoxib at 1 h after Cr challenge (treatment schedules are shown in [Figure 2\(b\)](#)). Indeed, celecoxib treatment significantly decreased footpad swelling following Cr challenge of sensitized mice as compared with mock-sensitized (PBS)

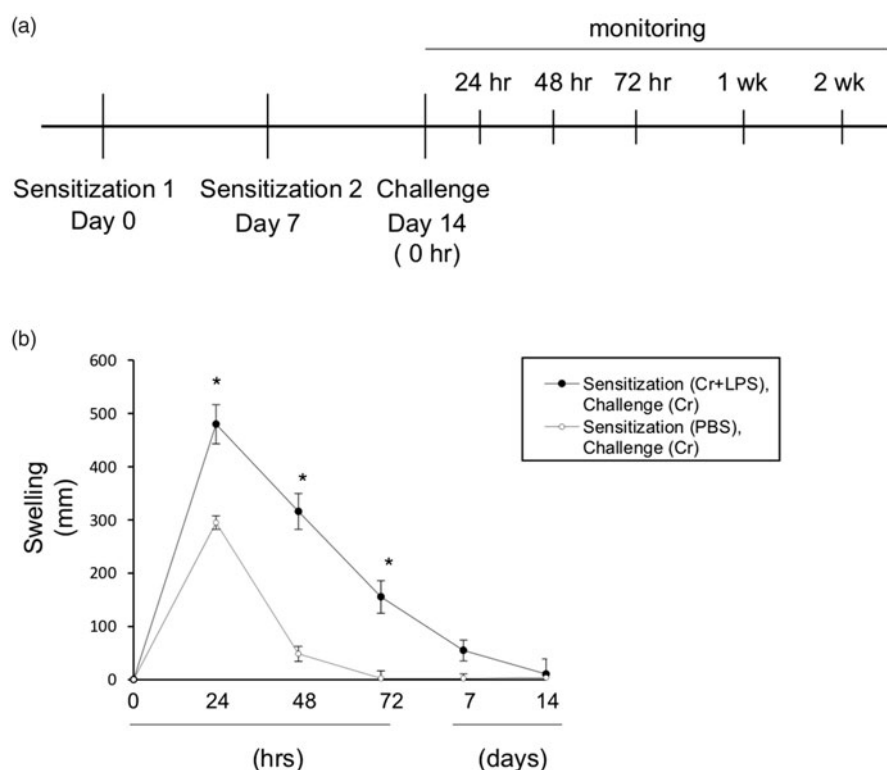


Figure 1. Induction of Cr allergy and measurement of allergic footpad swelling. (a) Induction schedule of Cr allergy in mice. 7-week-old female mice, type C57BL/6, were sensitized into the left and right groin by intradermal (i.d.) injection of CrCl₂ and LPS twice with an interval of 7 days. Seven days after the 2nd sensitization, the left and right footpads of mice were challenged by i.d. injection of CrCl₂ (without LPS) in PBS. As a control, mock sensitization of PBS was injected into the mice and the challenge used CrCl₂ (without LPS). (b) Footpad swelling was measured before and at 24–72 h after the challenge. The difference in footpad thickness before and after challenge was calculated. Cr-allergic mice represented in closed circles (●), while the control represented in open circles (○). Observations in both groups in footpad swelling 24 h after the challenge with Cr. Data are represented as the mean $\pm$ SD ($n = 5$). Unpaired Student's *t*-test was used in statistical significance (* $p < .05$). Two independent experiments were performed. Representative data are shown.

mice (Figure 2(c)). These results indicate that COX-2 is involved in the development of Cr allergy, and celecoxib should be considered as a potential treatment for the reduction of swelling during Cr allergy.

In histological analyses found that Cr-allergy groups showed thickening of the epidermal layer, partial separation of epidermal keratinocytes creating spongiotic dermatitis, and liquefaction degeneration (Figure 2(d), compare left and middle panels). Further, treatment with celecoxib prevented this thickening of the epidermal layer and reduced the inflammation of the dermal layer in the foot of treated mice compared with those left untreated prior to challenge (Figure 2(d), compare middle and right panels). These data suggest that inhibition of COX-2 attenuates inflammation during Cr allergy.

COX-2 inhibition suppresses IFN- γ production by T cells in Cr allergic mice

Since IFN- γ is believed to be the most important cytokine in metal allergy [2,5,6,11,14,28,29], examined of IFN- γ production from T cells in Cr-allergic mice treated with or without celecoxib. Mice were sacrificed at 24 h after the challenge and lymphocytes were isolated from draining lymph nodes followed by co-staining with anti-IFN- γ mAb, anti-CD4 mAb, and anti-CD8 mAbs for the detection of IFN- γ in T cells. Flow cytometric analysis revealed that

while CD4⁺ T cells do not produce IFN- γ in Cr-allergic mice (Figure 3), and 31.3 $\pm$ 1.73% of CD8⁺ T cells in lymph nodes of untreated Cr-allergic mice expressed IFN- γ (Figure 3). In contrast, treatment of mice with celecoxib resulted in only 19.1 $\pm$ 3.82% of CD8⁺ T cells expressing IFN- γ following Cr challenge (Figure 3). Thus, we found that CD8⁺ T cells, but not CD4⁺ T cells, are the main source of IFN- γ in the draining lymph nodes of Cr-allergic mice.

Furthermore, to examine whether T cell accumulation is affected by COX-2 in Cr-allergic mice, cell numbers in draining lymph nodes from Cr-allergic mice treated with or without celecoxib were measured. The total lymphocytes in draining lymph nodes from celecoxib-treated Cr-allergic mice was reduced (Figure 4(a)). Moreover, not only CD8⁺ T cells, but also CD4⁺ T cells, were decreased following celecoxib treatment *in vivo* (Figure 4(b)). Of note, approximately 1.1 million IFN- γ ⁺ CD8⁺ T cells were present in lymph nodes of Cr-allergic mice (Figure 4(c)), while only 0.3 million IFN- γ ⁺ CD8⁺ T cells were present in lymph nodes of Cr-allergic mice treated with celecoxib (Figure 4(c)). However, the frequency of CD4⁺ and CD8⁺ T cells in the lymph nodes was not significantly affected, indicating that both populations were equally decreased (Figure 4(d)). Further, flow cytometric analysis revealed a significant decrease in the frequency of IFN- γ expressing CD8⁺ T cells in the draining lymph nodes of mice treated with celecoxib

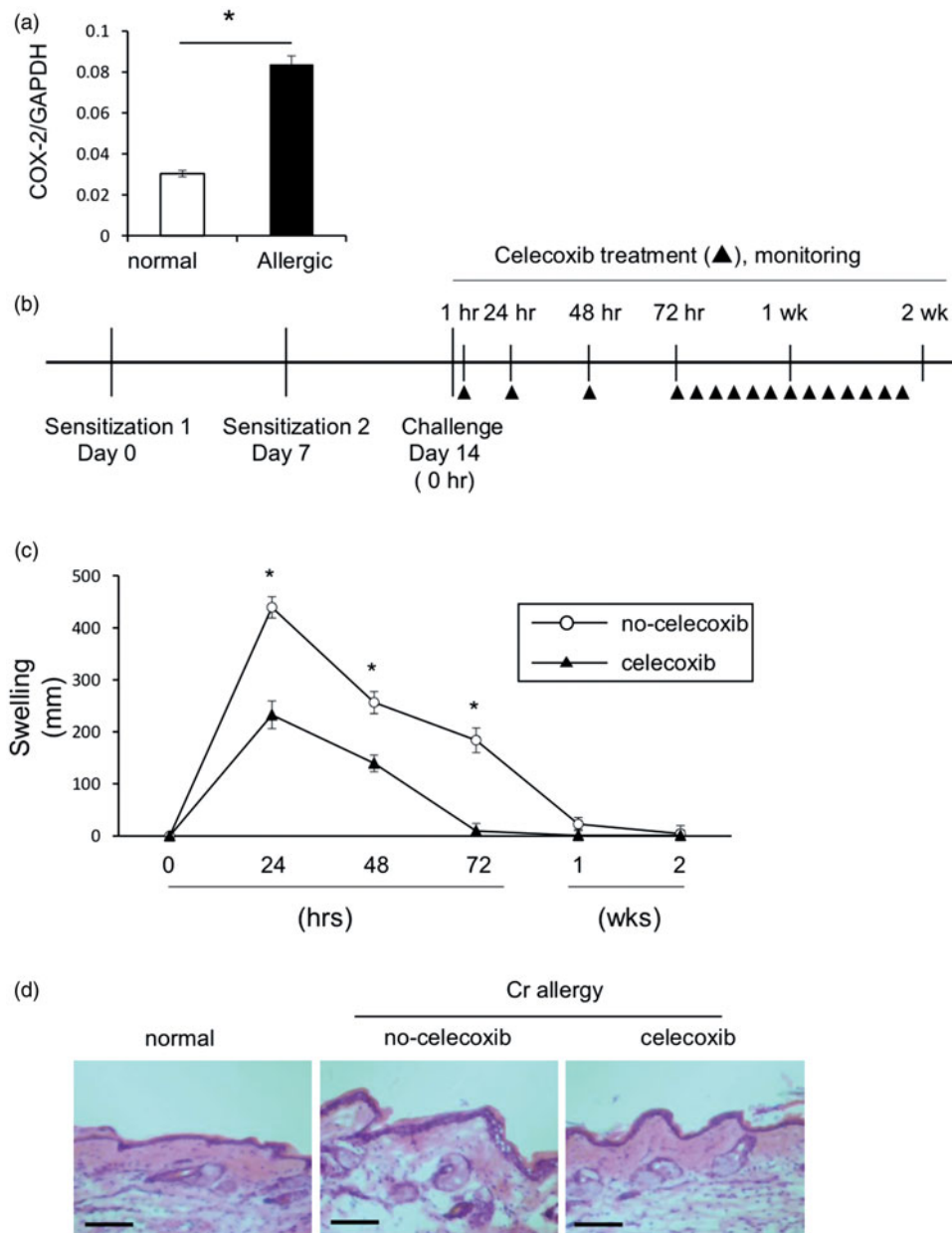


Figure 2. Effect of celecoxib in Cr-allergic mice. (a) Expression of the COX-2 transcript in the spleen. Spleens were isolated at 24 h before and after Cr challenge, and COX-2 mRNA expression was analyzed by quantitative RT-PCR. Shown on the graph are relative values normalized to the expression of GAPDH. The open column is normal foot tissue from wild-type mice and the closed column is allergic foot tissue from Cr-allergic mice. Bars indicate $\pm$ S.D. ($n=3$, per group). (b) Administration schedule of celecoxib in Cr-allergic mice. Cr allergy was induced in mice as indicated in Figure 1(a). Celecoxib was injected intraperitoneally at a daily interval starting 1 h after challenge (shown by arrowheads in this Figure). (c) Footpad swelling was measured before and up until 72 h after the challenge, and the difference in footpad thickness before and after challenge was calculated. Open circles (○) represent Cr-allergic mice without administration of celecoxib, closed triangles (▲) represent Cr-allergic mice administered celecoxib and closed circles (●) represent challenge only (unsensitized) mice. In all groups, footpad swelling peaked at 24 h after Cr challenge. Data are shown as the mean $\pm$ SD ($n=7$). Statistical significance was tested using unpaired Student's *t*-test (* $p < .05$). (d) Representative pictures of feet from wild-type or allergic mice treated with or without celecoxib at 24 h after challenge. The tissue sections were stained with haematoxylin and eosin (H&E). Scale bars indicate 50 μ m. Two independent experiments were performed. Representative data are shown.

compared with untreated Cr-allergic mice (Figure 4(e)). These data indicate that in the draining lymph nodes of Cr-allergic mice, the number of IFN- γ expressing CD8 $^{+}$ T cells is dramatically reduced by treatment with celecoxib, while CD4 $^{+}$ T cells did not express IFN- γ during chromium allergy.

Therefore, our findings suggest that COX-2 induced signalling promotes the accumulation of lymphocytes and the production of IFN- γ by CD8 $^{+}$ T cells, which leads to the development of Cr allergy.

Discussion

In this study, we demonstrate the involvement of both PGE $_2$ and COX-2 in the development of Cr allergy. It has been reported that COX-2 expression is generally very low in normal tissues and increases immediately during acute inflammation prior to the recruitment of leukocytes and the infiltration of immune cells [8,29,30]. COX-2 is the enzyme responsible for oxidation of arachidonic acid during the production of PGE $_2$, and thus, the increase in PGE $_2$ during

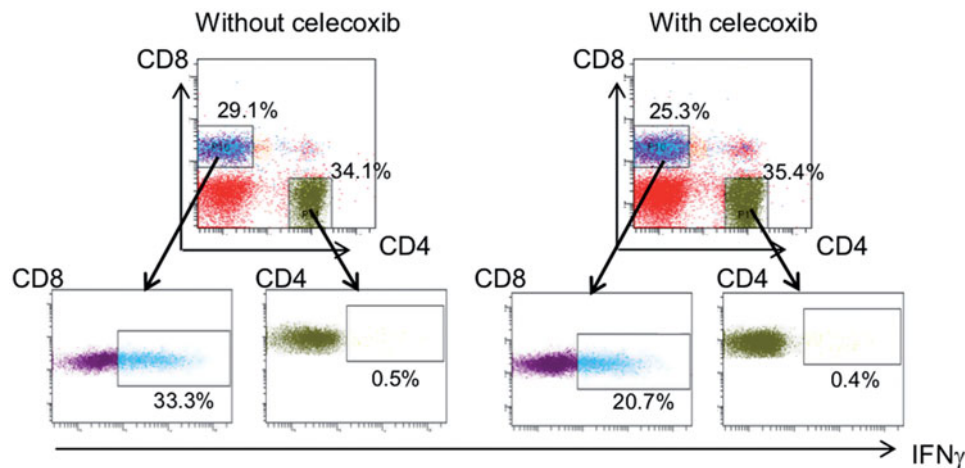


Figure 3. Effect of celecoxib on IFN- γ production by CD4⁺ and CD8⁺ T cells in the draining lymph nodes of Cr-allergic mice. Induction of Cr allergy in mice and celecoxib administration were as described in Figure 2(b). Popliteal lymph nodes cells were isolated at 24 h after Cr challenge, and cytokine production was analyzed by flow cytometry. Dot plots show (top) CD4⁺ and CD8⁺ T cells and (bottom) IFN- γ expressing cells in the popliteal lymph nodes of mice treated with celecoxib or left untreated. Three independent experiments were performed. Representative data are shown.

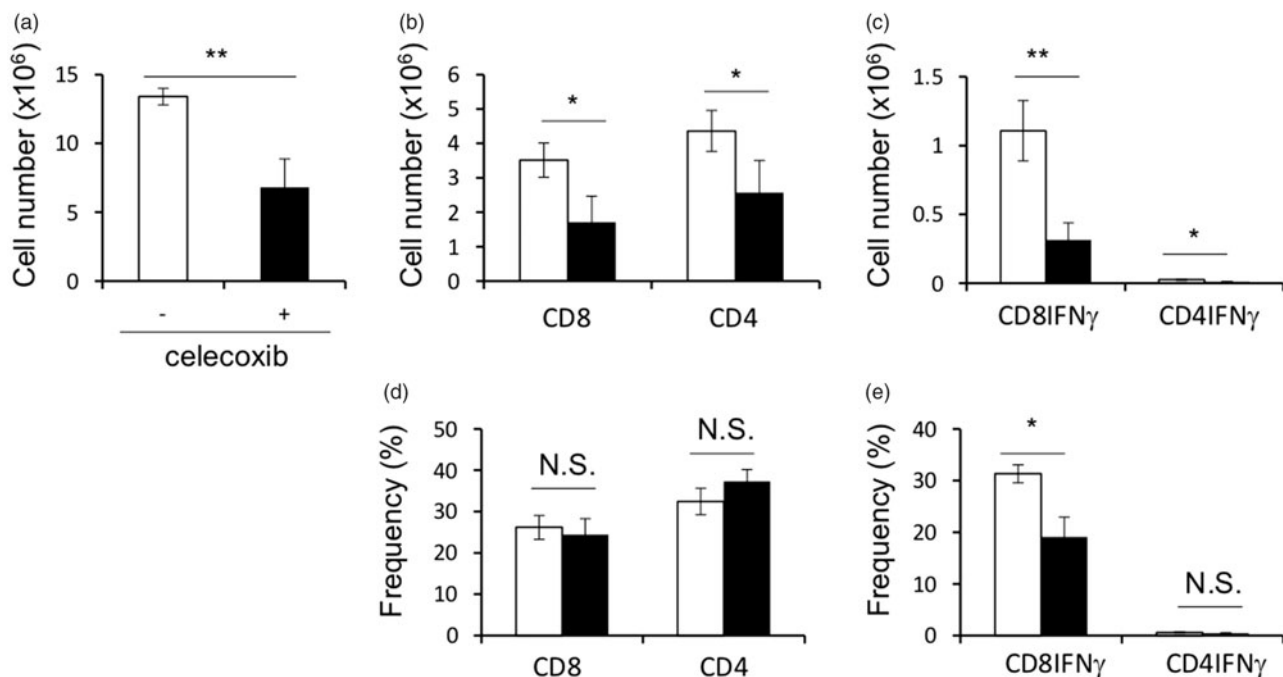


Figure 4. Quantitative analysis of IFN- γ expressing cells in the draining lymph nodes of Cr-allergic mice treated with or without celecoxib. Statistical analysis of IFN- γ production in CD8⁺ and CD4⁺ T cells based on the flow cytometric analysis as shown in Figure 3. Error bars indicate $\pm$ SD ($n = 3$, per group). Statistical significance was tested using unpaired Student's t -test (* $p < .05$). The percentage was calculated by setting total lymphocytes as 100%. Three independent experiments were performed. Representative data are shown.

inflammation is mediated by COX-2 [15]. Moreover, PGE₂ is involved in the processes that lead to the classic signs of inflammation including redness, swelling, and pain [8,31]. As shown in Figure 1(b) and Figure 2(a), COX-2 expression is dramatically upregulated concordant with footpad swelling in Cr-allergic mice. These results indicate that, as expected, COX-2 contributes to inflammation in Cr allergy.

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX-1 and COX-2 and possess analgesic, anti-inflammatory, and antipyretic properties making them effective in relieving pain, fever, and inflammation [5,8,27,32–34]. In this study showed that celecoxib, a COX-2-specific inhibitor

that suppresses PGE₂, is effective for relieving swelling and inflammation in Cr-allergic mice (Figure 2(c,d)). Likewise, COX-2 has also been shown to be involved in nickel elution and a COX-2 inhibitor reduced nickel elution from a wire implanted subcutaneously into mice [35]. Thus, these results strongly suggest that during metal allergy COX-2 contributes to inflammation and that a COX-2 inhibitor is effective at reducing symptoms of metal allergy.

IFN- γ is a major inflammatory cytokine known to be produced by T cells during various metal allergies [5,11,22]. As shown in Figure 3, we confirmed that IFN- γ is produced by T cells in Cr-allergic mice. Interestingly, celecoxib was

found to suppress not only footpad swelling but also IFN- γ production by T cells. There are two possible roles that celecoxib might play in IFN- γ production. First, as described above, COX-2 is known to be a key player in PGE₂ production and PGE₂ signalling through its receptors might result in IFN- γ production. To this end, we found that evidence of expression of the PGE₂ receptors EP1, EP2, EP3, EP4 on T cells in Cr-allergic mice could be found. Second, COX-2 expression may directly result in the production of IFN- γ ; however, further experiments will be required to explore the cooperative signalling cascades of PGE₂ and IFN- γ .

In addition, the number of T cells, and specifically CD8⁺ T cells, was greatly decreased in the draining lymph nodes and spleens of celecoxib-treated Cr-allergic mice (Figure 4(a)). This suggests that PGE₂ promotes not only the activation of CD8⁺ T cells leading to IFN- γ but also lymphocyte accumulation (Figure 4).

Therefore, celecoxib is thought to act as a comprehensive inhibitor for allergic events. Our findings suggest that this COX-2 inhibitor may be a powerful treatment for Cr allergy and in addition, that other PGE₂ signalling molecules may be effective therapeutic targets for the treatment of metal allergy.

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Authors contributions

R. M. S. performed experiments; K. I. designed and performed experiments, analyzed data, and prepared the manuscript. Y. S., M. I., K. S., K. U., N. H., T. N., N. H., and U. K. provided technical support and data discussion.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary Figure 1

