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Correspondence to

Yong Min Kim, MD, PhD


Department of Otorhinolaryngology-Head and Neck Surgery, Research Institute for Medical Science, Chungnam National University School of Medicine, 282 Munhwa-ro, Jung-gu, Daejeon 35015, Korea.
Tel: +82-42-280-7696
Fax: +82-42-253-4059
E-mail: entkym@cnu.ac.kr


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ORCID iDs


Mi-Ra Choi 
<https://orcid.org/0000-0003-0734-8360>

Jun Xu 
<https://orcid.org/0000-0002-6080-118X>

Seulgi Lee 
<https://orcid.org/0000-0001-7323-2476>

Sun-Hee Yeon 
<https://orcid.org/0000-0003-3078-4419>

Soo-Kyoung Park 
<https://orcid.org/0000-0002-5163-536X>

Ki-Sang Rha 
<https://orcid.org/0000-0001-9981-057X>

Chloroquine Treatment Suppresses Mucosal Inflammation in a Mouse Model of Eosinophilic Chronic Rhinosinusitis

Mi-Ra Choi ^{1,2}, Jun Xu ^{1,3}, Seulgi Lee ¹, Sun-Hee Yeon ¹, Soo-Kyoung Park ¹,
Ki-Sang Rha ¹, Yong Min Kim ^{1,2*}

¹Department of Otorhinolaryngology-Head and Neck Surgery, Research Institute for Medical Science, Chungnam National University School of Medicine, Daejeon, Korea

²Department of Medical Science, Chungnam National University School of Medicine, Daejeon, Korea

³Department of Otorhinolaryngology-Head and Neck Surgery, State Key Laboratory of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, China

ABSTRACT

Purpose: The Toll-like receptor 9 (TLR9) signaling pathway is involved in the pathogenesis of chronic rhinosinusitis (CRS) with nasal polyposis. The aim of this study was to assess the therapeutic potential of the TLR9 pathway inhibitor chloroquine in CRS mice.

Methods: The expression of type I interferons (IFNs) in human CRS tissues was evaluated using quantitative polymerase chain reaction (qPCR), western blotting, and immunofluorescence. Mice were divided into 4 treatment groups: the control, nasal polyp (NP), chloroquine treatment (NP + Chlq), and dexamethasone treatment (NP + Dexa) groups. The effects of chloroquine on polyp formation and mucosal inflammation were examined by hematoxylin and eosin staining. The expression levels of type I IFN, B-cell activating factor (BAFF), TLR9, high mobility group box 1 (HMGB1), and proinflammatory cytokine expression levels were assessed using qPCR, western blot, or enzyme-linked immunosorbent assay.

Results: IFN- α and IFN- β mRNA levels were significantly higher in patients with eosinophilic NPs (EPs) than in healthy individuals or non-EP patients. The polyp score, epithelial thickness, mucosal thickness, and the number of eosinophils in nasal mucosa were significantly higher in the NP group compared with the control, NP + Chlq, and NP + Dexa groups. NP + Chlq or NP + Dexa significantly suppressed the induction of type I IFN and BAFF expression in the NP group; these treatments also significantly suppressed the induction of TLR9, HMGB1, interferon regulatory factors, interleukin (IL)-6, IL-1 β , tumor necrosis factor- α , and Th cytokine expression in the NP group. The secreted levels of anti-dsDNA Immunoglobulin G (IgG) were significantly higher in the NP group than in the control, NP + Chlq, and NP + Dexa groups. There were significant positive correlations between BAFF and mRNA levels of IFN- α/β /the protein levels of anti-dsDNA IgG.

Conclusions: Chloroquine may be used for the treatment of patients with eosinophilic CRS.

Keywords: Chloroquine; rhinitis; sinusitis; nasal polyps; Toll-like receptor 9; interferons; models, animal; therapeutics

Yong Min Kim <https://orcid.org/0000-0001-5414-8332>**Disclosure**

There are no financial or other issues that might lead to conflict of interest.

INTRODUCTION

Chronic rhinosinusitis (CRS) is a persistent mucosal inflammatory disease of the nose and paranasal sinuses; CRS with nasal polyposis (CRSwNP) is one of the 2 subtypes of CRS. CRS has been divided into 2 endotypes that are characterized by distinct pathophysiological mechanisms: eosinophilic CRS (E CRS) and non-E CRS.¹ The majority of patients with CRS can be successfully treated with proper pharmacological and/or surgical therapy; however, some patients with CRS, especially those with E CRS, do not respond to treatment.² For such patients, repetitive administration of systemic corticosteroids is performed to control the disease; however, disease symptoms often recur after completion of treatment.³ Recent advances in the understanding of CRS pathogenesis may yield novel therapeutic approaches.⁴⁻⁶

Previous studies have shown that Toll-like receptors (TLRs) 7 and 9 play pivotal roles in inflammatory diseases, such as inflammatory bowel disease, systemic lupus erythematosus, and psoriasis; specific TLR7 and TLR9 antagonists might be beneficial for controlling these diseases.⁷ Several studies have demonstrated that TLR9 deficiency or inhibition has effects similar to those of chloroquine treatment, suggesting that TLR9 is a potential therapeutic target of chloroquine.^{8,11} However, the exact mechanisms by which chloroquine and TLR9 inhibitors exert their effects remain unclear.

Interactions between TLRs and their ligands during an infection initiate a signaling cascade that induces, among other things, the activation of nuclear factor- κ B (NF- κ B), interferon regulatory factor-3 (IRF3), IRF5, and IRF7 transcription factors. This activation results in the production of several proinflammatory cytokines and the activation of dendritic cells and B cells as well as the induction of adaptive immunity against pathogens.⁷ The administration of CpG DNA has been shown to induce high levels of interferon- α (IFN- α) in mice, resulting in IFN- α -dependent activation of T cells, B cells, and natural killer cells¹²; it also induced the production of other inflammatory cytokines such as interleukin-6 (IL-6), IFN- γ , IL-12, and tumor necrosis factor- α (TNF- α).^{7,12}

In our former study, we have demonstrated that CpG A-induced fibroblast activation and cytokine production were mediated via TLR9 stimulation in nasal polyp (NP)-derived fibroblasts, and that disrupting this process with an inhibitor targeting TLR9 or its downstream signaling pathways could represent a novel approach to CRSwNP therapy.¹³ We have previously reported an important role of the TLR9 signaling pathway and the subsequent induction of type I IFN immune responses in the NPs of patients with CRSwNP.¹⁴ In addition, we have demonstrated that exposure to TLR9 agonists increases *in vitro* expression of type I IFNs, which then increase the production of B-cell activating factor (BAFF) production; these events could be inhibited by the well-tolerated TLR9 inhibitor chloroquine.¹⁴ In the present study, we sought to elucidate the therapeutic potential of chloroquine in a mouse model of CRS, a clinically relevant model of E CRS.

MATERIALS AND METHODS**Patients**

Sinonasal tissues from healthy donors (n = 16) and NPs from patients with CRSwNP (n = 28) were harvested during endoscopic sinus surgery. Control tissues (uncinate tissue) were

obtained from patients without any sinus diseases during rhinologic surgeries such as skull base surgery, dacryocystostomy, or endoscopic orbital decompression.

The diagnosis of CRSwNP was made in accordance with the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines.¹ NPs were sub-classified into 2 groups, eosinophilic NPs (EPs) and non-eosinophilic NPs (N-EPs), based on the results of hematoxylin and eosin staining. NPs with an eosinophil count > 10% of the total inflammatory cells per high power field were classified as EPs; all other NPs were classified as N-EPs.¹⁵⁻¹⁷ Atopic status was evaluated by the skin prick test or by screening for serum specific immunoglobulin (Ig) E antibodies to common aeroallergens using the multiple allergen simultaneous test. A diagnosis of asthma was made by an allergist based on spirometry (change in forced expiratory volume in 1 second of 12% or more following administration of a short-acting β -agonist) or the methacholine challenge test.

Patients who used oral or nasal corticosteroids or other medications (*e.g.*, antibiotics or anti-leukotrienes) within 4 weeks before sample collection and patients with recent upper respiratory tract infections were excluded from the study. Patients undergoing revision sinus surgery were also excluded. Characteristics of included patients are shown in **Supplementary Table S1**. All patients and healthy donors provided written informed consent to be enrolled in the study. The study was approved by the Institutional Review Board of Chungnam National University Hospital (approval No. 2018-12-025).

Development of a CRS mouse model

Four-week-old female BALB/c mice were purchased from Orient-Bio Laboratory (Seongnam, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of Chungnam National University and were performed in accordance with the institutional guidelines (approval No. CNU-01113). Seventy-two mice were divided into 4 groups, as shown in **Fig. 1A**: control mice (n = 18), NP mice receiving phosphate-buffered saline (PBS) (NP; n = 20), NP mice receiving chloroquine treatment (NP + Chlq; n = 18), and NP mice receiving dexamethasone (NP + Dexa; n = 16).

The CRS mouse model was developed as previously described.^{17,18} In brief, mice were sensitized to ovalbumin on days 1 and 5 by an intraperitoneal injection of 25 mg of ovalbumin (Sigma-Aldrich, St. Louis, MO, USA) in 20 μ L of PBS solution containing 2 mg of aluminum hydroxide (alum; Sigma-Aldrich). Mice were then inoculated with 20 μ L of 3% ovalbumin solution through the nostrils into the nasal cavities daily from days 21 to 26, and then 3 times per week from weeks 4 to 12. Subsequently, mice were challenged with 10 ng of staphylococcal enterotoxins B (SEB; List Biological Laboratories Inc., Campbell, CA, USA) once per week from weeks 8 to 12, then sacrificed in week 13. The control group received PBS instead of ovalbumin solution, SEB, and drugs (chloroquine or dexamethasone). In the NP, NP + Chlq, and NP + Dexa groups, 20 μ L of PBS, 50 mg/kg of chloroquine, and 1 mg/kg dexamethasone, respectively, were administered intraperitoneally from weeks 8 to 12 (**Supplementary Fig. S1A**).

To determine the appropriate chloroquine treatment dose, mice (7 weeks old, 18–20 g) were divided into 4 groups (3 mice per group); the mice received 5 total doses of intraperitoneal injections with PBS (control) or one of 3 different doses of chloroquine (10, 50, or 100 mg/kg), between days 0 and 4. The body weights of the mice were measured every other day for 10 days. One day after the first injection, 2 mice died in the high-dose (100 mg/kg) group

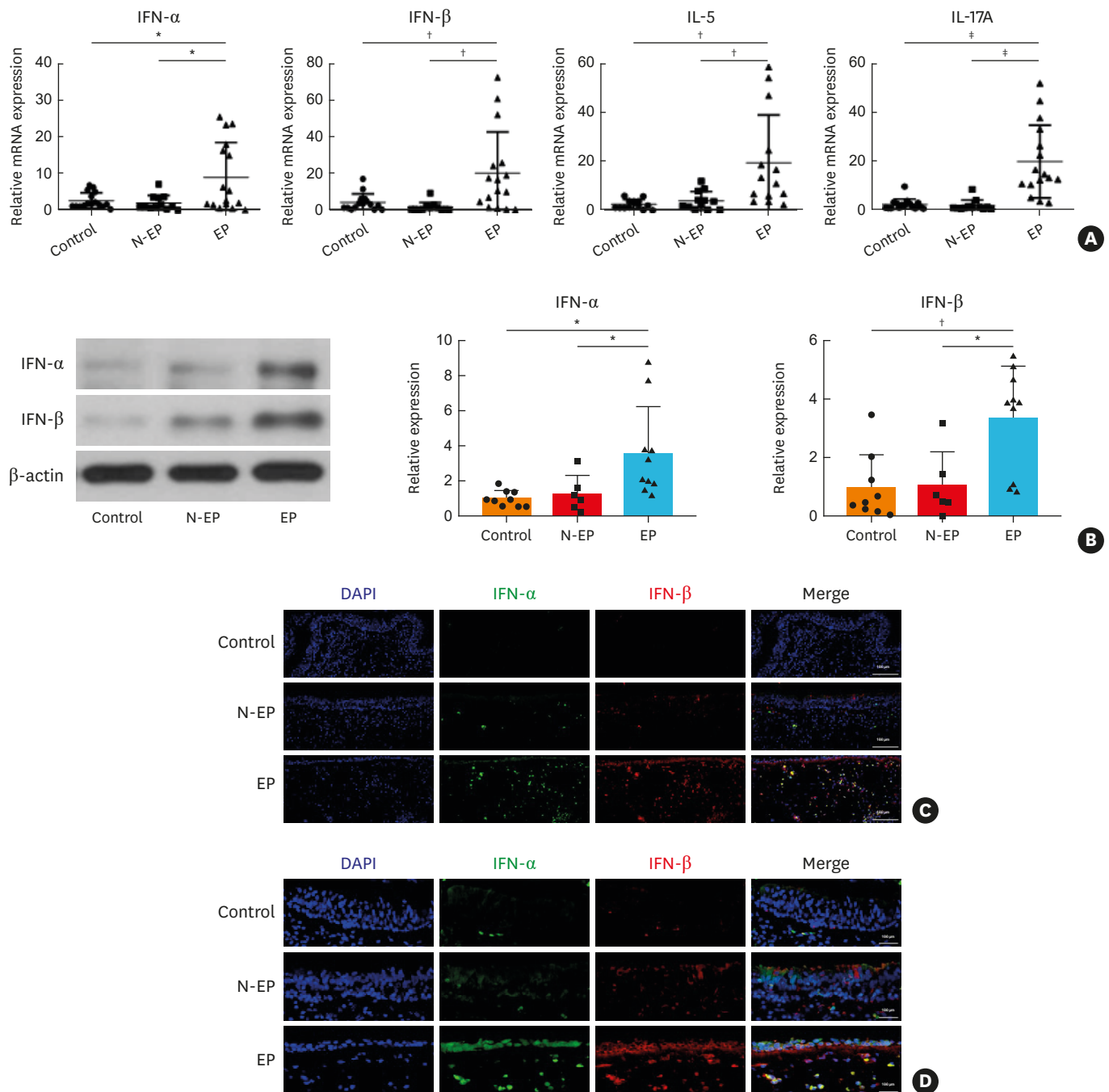


Fig. 1. Expression levels of type I IFNs are elevated in nasal polyps of patients with eosinophilic CRSwNP. Expression levels of type I IFNs were evaluated in nasal tissues from patients with CRS using qPCR, western blotting, and immunofluorescence. (A) The mRNA expression levels of IFN- α/β , IL-5, and IL-17A were significantly higher in EPs than in control unciniate tissues or N-EPs. (B) IFN- α and IFN- β protein levels were higher in the EP group than in the control or N-EP groups. (C, D) Representative immunofluorescence images showing that IFN- α - and IFN- β -positive cells were present in the epithelial and subepithelial layers of EPs (original magnification, $\times 200$ in C and $\times 400$ in D). IFN, interferon; CRSwNP, chronic rhinosinusitis with nasal polyposis; CRS, chronic rhinosinusitis; qPCR, quantitative polymerase chain reaction; IL, interleukin; N-EP, non-eosinophilic nasal polyp; EP, eosinophilic nasal polyp; DAPI, 4',6-diamidino-2-phenylindole. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

(Supplementary Fig. S1B). None of the surviving mice showed significant weight loss compared with their initial weights (day 0) (Supplementary Fig. S1C). Therefore, we used chloroquine at 50 mg/kg for further experiments.

Preparation of mouse tissue

Twenty-four hours after the final ovalbumin challenge, all mice were sacrificed. The heads of 15 mice from each group were resected *en bloc*, then used for protein and RNA extraction or paraffin embedding. To obtain nasal lavage fluid (NALF), the mouse trachea was exposed and partially cut under deep anesthesia. A 24G IV catheter (Becton-Dickinson, Sandy, UT, USA) was inserted into the posterior choana through the cutting end of the trachea. The nasal cavities were gently lavaged with 350 μ L of cold PBS twice, and the fluid from the nostrils was collected and centrifuged. Supernatants were stored at -80°C for further studies. For protein and RNA extraction ($n = 10$ mice per group), the nasal mucosa was removed meticulously using a small curette and a microforceps under a microscope. The heads of the remaining mice in each group were fixed in 4% paraformaldehyde for 2 days ($n = 5$ mice per group). After specimens had been fixed, they were washed in running tap water and then decalcified in 1 M ethylenediaminetetraacetic acid (pH 8.0) for 2 weeks at room temperature. The decalcified specimens were embedded in paraffin and sectioned coronally (4- μ m thickness), approximately 5 mm from the nasal vestibule, using a Leica microtome (Leica, Wetzlar, Germany).

Quantitative polymerase chain reaction (qPCR)

Total RNA from mouse tissues was isolated using the TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. For complementary DNA synthesis, 2 μ g of total RNA was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen), in accordance with the manufacturer's instructions. The mRNA expression was analyzed on a CFX Connect™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA) using the Power SYBR® Green PCR master mix (Life Technologies Ltd., Warrington, UK). The primers were purchased from GenoTech (Daejeon, Korea); sequences of the primers are listed in **Supplementary Table S2**.

All PCR assays were performed in triplicate. For each sample, the differences in threshold cycles between the target gene and the glyceraldehyde 3-phosphate dehydrogenase reference gene (*i.e.*, $\Delta\text{Ct}_{\text{target gene}}, \Delta\text{Ct}_{\text{reference gene}}$) were determined; a calibrated delta Ct value ($\Delta\Delta\text{Ct}, \Delta\text{Ct}_{\text{target gene}} - \Delta\text{Ct}_{\text{reference gene}}$) was calculated. The relative quantitation values were then calculated using the following equation: relative quantitation = $2^{-\Delta\Delta\text{Ct}}$.

Western blotting analysis

Tissues were lysed in Pro-Prep solution (Intron, Seongnam, Korea). Total protein was measured using the Bradford protein assay (BioRad Laboratories). Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Burlington, MA, USA). Membranes were blocked with the Universal WB blocking buffer (JUBIOTECH, Deajeon, Korea) and incubated with the appropriate primary antibodies. Blots were then incubated with the appropriate peroxidase-conjugated secondary antibodies. The membranes were developed using a chemiluminescent reagent (enhanced chemiluminescent; GE Healthcare, Chicago, IL, USA) and subsequently exposed to X-ray film to visualize the signal. The following primary antibodies were used in western blotting analysis: IFN- α (F-7; Santa Cruz, Dallas, TX, USA), IFN- β (ab85803; Abcam, Cambridge, MA, USA), BAFF (ab16081; Abcam), p65 (F-6; Santa Cruz), and anti- β actin (sc-47778; Santa Cruz).

Immunofluorescence staining

Paraffin-embedded tissues were deparaffinized and dehydrated using a graded ethanol series. Antigen retrieval was performed by boiling the slides in 10 mM citrate buffer (pH

6.0) for 4 minutes. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 10 minutes, followed by washing in PBS. Sections were incubated in streptavidin-biotin blocking solution for 1 hour (Vector Laboratories, Burlingame, CA, USA). Sections were stained with the anti-IFN- α monoclonal antibody (E-7; Santa Cruz), anti-IFN- β monoclonal antibody (ab85803; Abcam) and anti-TLR9 monoclonal antibody (ab37154; Abcam). Alexa 488-conjugated anti-mouse IgG and Alexa 594-conjugated anti-rabbit IgG (Invitrogen) were then applied as secondary antibodies. 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) was used at a concentration of 300 nM for nuclear counterstaining. The resulting stained specimens were mounted in VectaShield Antifade Mounting Medium (CH-1000; Vector Laboratories). The slides were then observed under a fluorescence microscope (Leica).

Hematoxylin and eosin staining

Prior to hematoxylin and eosin staining, all sections were deparaffinized with xylene and then rehydrated in a graded ethanol/distilled water series. The slides were scanned using the Panoramic MIDI digital slide scanner (Panoramic software 2.0; 3DHISTECH Ltd., Budapest, Hungary). Five coronal sections that were similar to the sinus cavity were chosen for evaluation to minimize processing errors and confirm the presence of mucosal lesions. Ten areas of nasal mucosal sections in the maxillary sinus or lateral wall of the nasal cavity were chosen randomly for the evaluation under high-power fields ($\times 400$) and measured by 2 examiners who were blinded regarding the groups to which the animals had been assigned. Polypoid lesions were defined as distinct mucosal elevations with inflammatory cell infiltration around the microcavities. The thickness of edematous mucosa was measured as the distance between the apices of epithelial cells and the upper border of the subepithelial glands zone. For the assessment of mucosal thickness, at least 3 measurements at random points were made in appropriate areas of each high-power field; the mean values from 4 different high-power fields were recorded for comparison. Thickness was measured using the CaseViewer program (v: 1.15.3; 3DHISTECH Ltd.).

Immunohistochemistry

The paraffin-embedded tissues were cleansed of paraffin and rehydrated using graded alcohols. Endogenous peroxidase activity was blocked with 3% H₂O₂ at 10 minutes and was washed using PBS. Antigen retrieval was performed by boiling the slides in 10 mM citrate buffer (pH 6.0) for 5 minutes. Anti-high mobility group box 1 (HMGB1) (ab79823; Abcam) was used as the primary antibody and incubated at 4°C overnight. Negative controls were prepared using PBS in place of the primary antibodies. After that, the slides were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours. Sections were developed with 3, 3'-diaminobenzidine solution (DAKO, Brüsseler, Germany) at room temperature for 1 minute. The slides were finally observed under an optical microscope (Olympus, Tokyo, Japan) at a magnification of $\times 400$.

Enzyme-linked immunosorbent assay

NALF was collected from the mice and used for analysis of IFN- α (42120-1; PBL Assay Science, Piscataway, NJ, USA), IFN- β (DY42400; R&D Systems, Minneapolis, MN, USA), and BAFF (DY2106; R&D Systems) and Anti-dsDNA ELISA (ORG604; Orgentec Diagnostika GmbH, Mainz, Germany) by enzyme-linked immunosorbent assay, in accordance with the manufacturer's instructions. Optical absorbance values at 450 nm were determined using a microplate reader. The lower detection limits of the enzyme-linked immunosorbent assay kits used were 12.5 IU/mL, 15.6 pg/mL, and 62.5 pg/mL for IgG autoantibodies to IFN- α , IFN- β , and BAFF, respectively.

Statistical analysis

All data were analyzed using GraphPad Prism, version 8.00 (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as the mean \pm standard error of the mean. Differences with $P < 0.05$ were considered statistically significant; the degrees of significance were presented as follows: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

RESULTS

Expression of type I IFNs is elevated in EPs

We previously demonstrated that type I IFNs were up-regulated in polyp tissues from patients with CRSwNP. In the present study, we evaluated the expression levels of type I IFNs, IL-5, and IL-17A in patients with each of the CRS endotypes by using qPCR, western blotting, and immunofluorescence. The mRNA expression levels of both IFN- α and IFN- β were significantly higher in the EP group than in the control (uncinate tissue) or N-EP groups (**Fig. 1A**; $P < 0.05$ for IFN- α and $P < 0.01$ for IFN- β). The expression levels of IL-5 and IL-17A were also significantly up-regulated in the EP group compared with the control or N-EP groups (**Fig. 1A**; $P < 0.005$ for IL-5 and IL-17A). IFN- α and IFN- β protein levels were higher in the EP group than in the control ($P < 0.05$ for IFN- α and $P < 0.01$ for IFN- β) or N-EP groups ($P < 0.05$ for all) (**Fig. 1B** and **Supplementary Fig. S2**). Immunofluorescence staining revealed that cells that expressed both IFN- α and IFN- β were present in the subepithelial area of NPs in the EP group (**Fig. 1C and D**).

Chloroquine attenuates mucosal inflammation and polyp formation in the CRS mouse model

We previously showed that the type I IFN-mediated TLR9 signaling pathway is involved in the pathogenesis of CRSwNP.¹⁴ To confirm this in our mouse model, we evaluated the effects of the TLR9 signaling inhibitor chloroquine on polyp formation and mucosal inflammation. Previous reports indicated that this mouse model exhibits elevated eosinophilic infiltration and up-regulation of Th2 cytokines in the sinonasal mucosa, representative of ECRS.¹⁹

The polyp score was significantly higher in the NP group than in the control ($P < 0.005$), NP + Chlq ($P < 0.05$), or NP + Dexam groups ($P < 0.05$) (**Fig. 2A and B**). The epithelial thickness of nasal mucosa was also significantly greater in the NP group than in the control, NP + Chlq, or NP + Dexam groups (**Fig. 2A and C**, $P < 0.005$ for all). The mucosal thickness was also significantly greater in the NP group than in the control, NP + Chlq, or NP + Dexam groups (**Fig. 2A and D**, $P < 0.005$ for all). The number of eosinophils in nasal mucosa was also significantly higher in the NP group than in the control, NP + Chlq, or NP + Dexam groups (**Fig. 2E and F**; $P < 0.001$ vs. control, $P < 0.005$ vs. NP + Chlq or NP + Dexam groups). These results suggested that treatment with chloroquine suppressed NP formation and mucosal inflammation; moreover, the therapeutic effects of chloroquine were similar to those of dexamethasone.

Chloroquine suppresses the induction of type I IFNs and BAFF in the CRS mouse model

The mRNA expression levels of type I IFNs and BAFF were significantly up-regulated in the NP group compared with the control ($P < 0.005$ for IFN- α , and $P < 0.001$ for IFN- β and BAFF; **Fig. 3A**). The NP + Chlq or NP + Dexam groups significantly suppressed the expression levels of type I IFNs and BAFF ($P < 0.005$ for all; **Fig. 3A**). The protein expression levels of type I IFNs and BAFF in the sinonasal mucosa of mice were consistent with the mRNA expression results

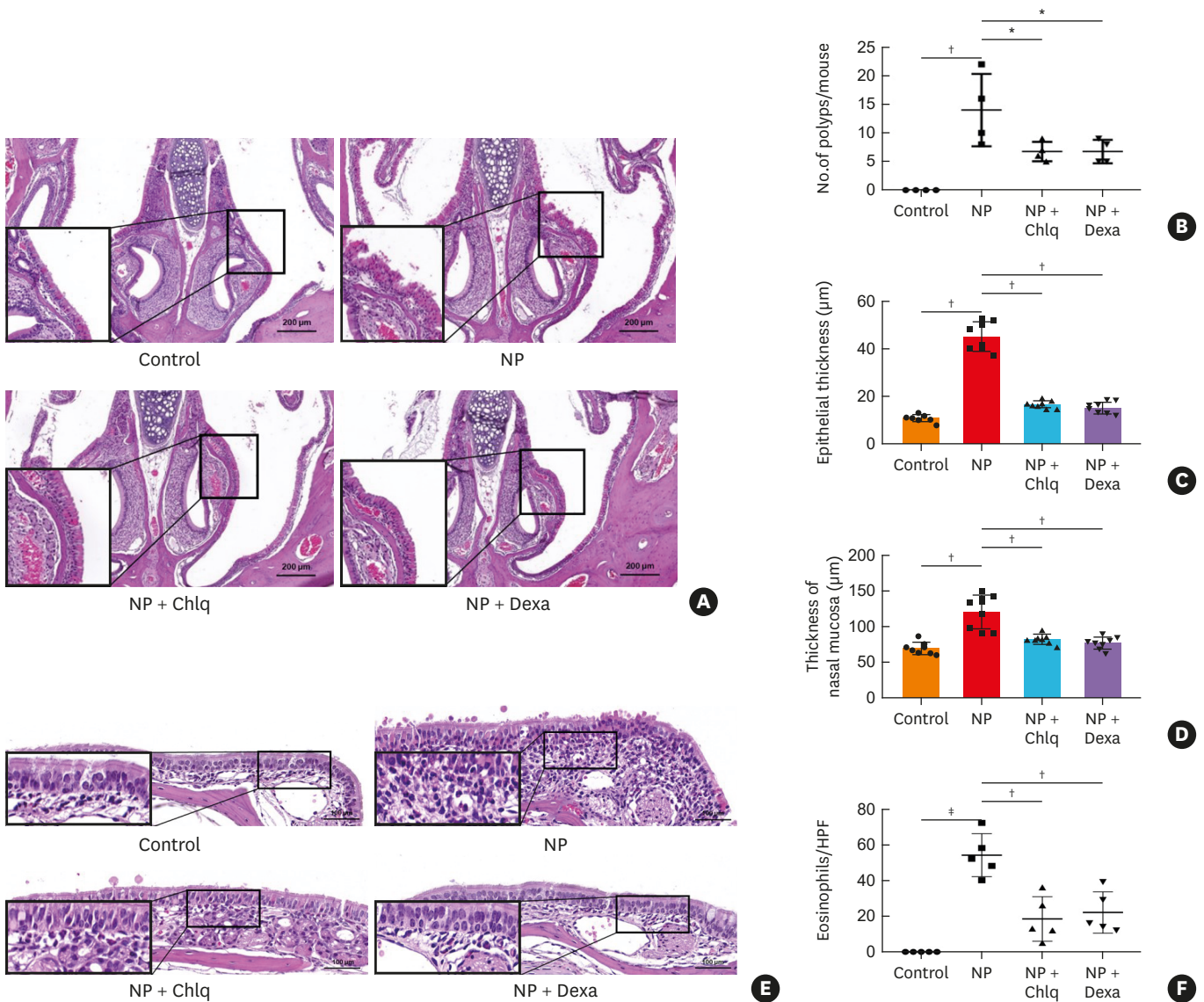


Fig. 2. Chloroquine exhibits an anti-polyp effect in the mouse model of nasal polyps. (A, B) The polyp score was significantly higher in the NP group than in the control, NP + Chlq, and NP + Dexa groups. (A, C) The thickness of nasal mucosa epithelium was significantly greater in the NP group than in the control, NP + Chlq, and NP + Dexa groups. (A, D) Mucosal thickness was significantly greater in the NP group than in the control, NP + Chlq, and NP + Dexa groups (original magnification, ×40). (E, F) The number of eosinophils in nasal mucosa was also significantly higher in the NP group than in the control, NP + Chlq, or NP + Dexa groups (original magnification, ×400). NP, nasal polyp; NP + Chlq, nasal polyp treated with chloroquine; NP + Dexa, nasal polyp treated with dexamethasone; N-EP, non-eosinophilic nasal polyp; EP, eosinophilic nasal polyp; HPF, high-power field. * $P < 0.05$; † $P < 0.005$; ‡ $P < 0.001$.

(**Fig. 3B**). The protein expression levels of type I IFNs and BAFF were significantly higher in the NP group than in the control group ($P < 0.05$ for IFN- β , $P < 0.01$ for IFN- α and BAFF); the induction of these proteins was significantly suppressed in both the NP + Chlq group ($P < 0.01$ for IFN- α , $P < 0.05$ for IFN- β and BAFF) and the NP + Dexa group ($P < 0.005$ for IFN- α , and $P < 0.01$ for IFN- β and BAFF; **Fig. 3B**).

The levels of type I IFNs and BAFF secreted in NALF were consistent with the findings regarding mRNA and protein expression levels. The levels of type I IFNs and BAFF secreted were significantly higher in the NP group than in the control group ($P < 0.001$ for IFN- α and

IFN- β ; $P < 0.005$ for BAFF); the levels of secretion elevated were significantly suppressed in both the NP + Chlq group ($P < 0.001$ for IFN- α and IFN- β ; $P < 0.005$ for BAFF) and the NP + Dexa group ($P < 0.01$ for IFN- β , $P < 0.005$ for BAFF, and $P < 0.001$ for IFN- α ; **Fig. 3C**). The levels of anti-dsDNA IgG secreted were significantly higher in the NP group than in the control group ($P < 0.01$), the NP + Chlq group ($P < 0.05$), and the NP + Dexa group ($P < 0.05$) (**Fig. 3D**).

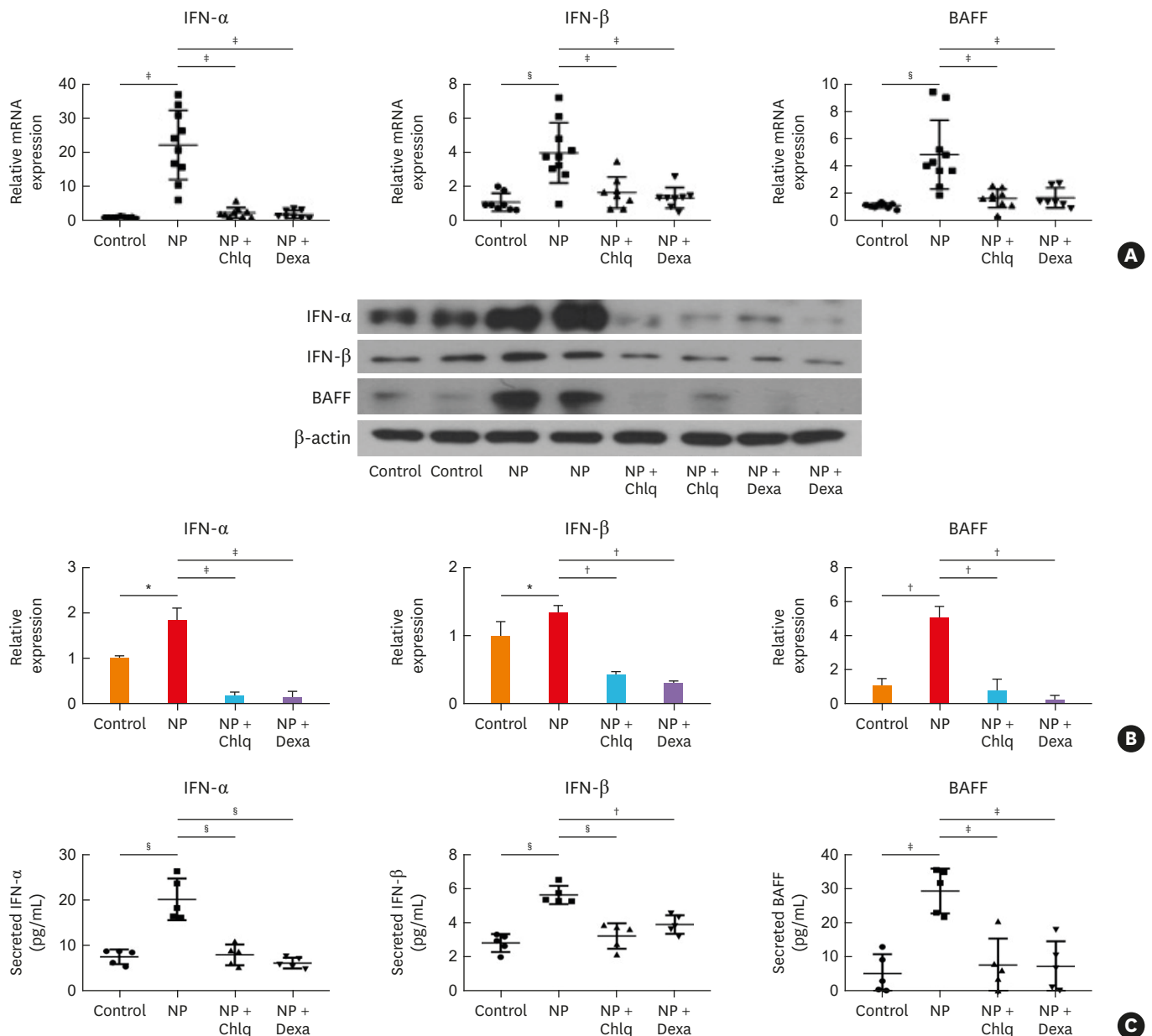


Fig. 3. Chloroquine treatment suppresses the production of type I IFNs and BAFF in the murine model of nasal polyps.

(A) Treatment with chloroquine (NP + Chlq group) or dexamethasone (NP + Dexa group) significantly suppressed the induction of type I IFNs and BAFF in the NP group. (B) Elevated type I IFN and BAFF protein levels in the NP group were significantly suppressed in the NP + Chlq and NP + Dexa groups. (C) Secretion of type I IFNs and BAFF in nasal lavage fluid was significantly suppressed in the NP + Chlq and NP + Dexa groups. (D) The secreted levels of anti-dsDNA IgG were significantly higher in the NP group than in the control, NP + Chlq, and the NP + Dexa groups. In addition, there were significant positive correlations between the protein levels of anti-dsDNA IgG and BAFF. (E) Significant positive correlations were also observed between mRNA levels of IFN- α/β and the BAFF. IFN, interferon; BAFF, B-cell activating factor; NP, nasal polyp; NP + Chlq, nasal polyp treated with chloroquine; NP + Dexa, nasal polyp treated with dexamethasone; IgG, immunoglobulin G.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

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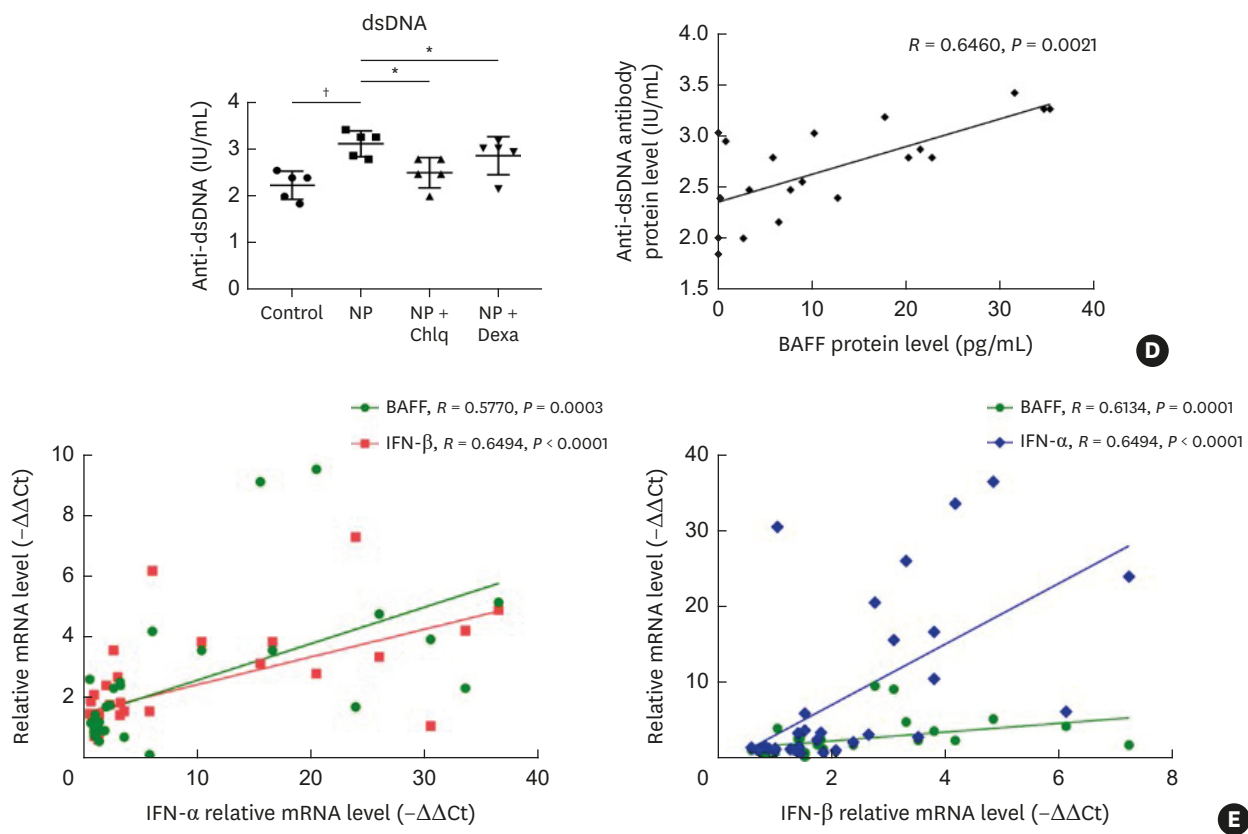


Fig. 3. (Continued) Chloroquine treatment suppresses the production of type I IFNs and BAFF in the murine model of nasal polyps.

(A) Treatment with chloroquine (NP + Chlq group) or dexamethasone (NP + Dexa group) significantly suppressed the induction of type I IFNs and BAFF in the NP group. (B) Elevated type I IFN and BAFF protein levels in the NP group were significantly suppressed in the NP + Chlq and NP + Dexa groups. (C) Secretion of type I IFNs and BAFF in nasal lavage fluid was significantly suppressed in the NP + Chlq and NP + Dexa groups. (D) The secreted levels of anti-dsDNA IgG were significantly higher in the NP group than in the control, NP + Chlq, and the NP + Dexa groups. In addition, there were significant positive correlations between the protein levels of anti-dsDNA IgG and BAFF. (E) Significant positive correlations were also observed between mRNA levels of IFN- α/β and the BAFF. IFN, interferon; BAFF, B-cell activating factor; NP, nasal polyp; NP + Chlq, nasal polyp treated with chloroquine; NP + Dexa, nasal polyp treated with dexamethasone; IgG, immunoglobulin G.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

Associations were evaluated between the anti-dsDNA IgG and BAFF protein levels. There were significant positive correlations between the protein levels of anti-dsDNA IgG and BAFF (Fig. 3D). Significant positive correlations were also observed between mRNA levels of IFN- α/β and the BAFF (Fig. 3E). These results indicated that autoantibodies could be involved in type I IFN-related BAFF up-regulation in a mouse CRS model.

The distribution of mucosal IFN- α/β expression was examined using immunofluorescence imaging; IFN- α and IFN- β were predominantly expressed in the epithelial and subepithelial layers of NP mice (Supplementary Fig. S3). Their expression levels were reduced in the NP + Chlq and NP + Dexa groups.

Chloroquine suppresses the expression of TLR9 signaling-induced molecules and cytokines

The expression levels of TLR9 and HMGB1 were reportedly elevated in NPs of patients with CRSwNP.¹⁴ Therefore, we investigated the expression levels of these molecules in our mouse model using qPCR; TLR9 and HMGB1 mRNA levels were significantly greater in the NP group than in the control group ($P < 0.005$ for TLR9 and $P < 0.01$ for HMGB1). The induction

of these molecules was significantly suppressed in both the NP + Chlq group ($P < 0.005$ for TLR9 and HMGB1) and the NP + Dexa group ($P < 0.001$ for TLR9; $P < 0.005$ for HMGB1; Fig. 4A). Expressions of TLR9 and HMGB1 were examined using immunofluorescence and

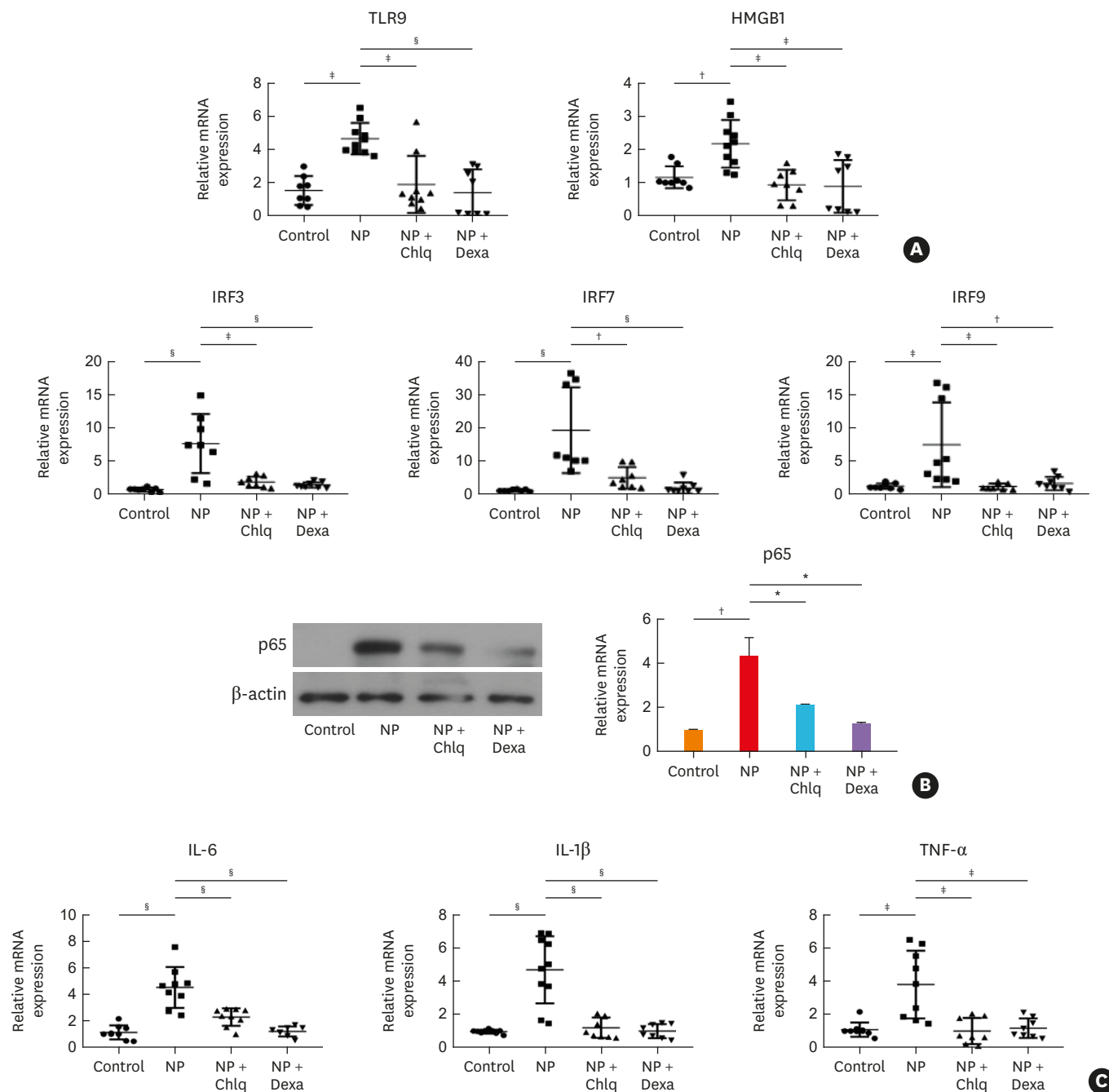


Fig. 4. Chloroquine treatment suppresses the expression of TLR9 signaling-related molecules and cytokines.

(A) The expression levels of TLR9 and HMGB1 were significantly up-regulated in the NP group compared with the control group. This up-regulation was significantly suppressed in the NP + Chlq and NP + Dexa groups. (B) The expression levels of IRFs and p65 were significantly up-regulated in the NP group, compared with the control group; this up-regulation was significantly suppressed in the NP + Chlq and NP + Dexa groups.

(C) Proinflammatory cytokines were significantly up-regulated in the NP group compared with the control group. The expression of these proinflammatory cytokines was significantly suppressed in the NP + Chlq and NP + Dexa groups.

TLR, Toll-like receptor; HMGB1, high mobility group box 1; NP, nasal polyp; NP + Chlq, nasal polyp treated with chloroquine; NP + Dexa, nasal polyp treated with dexamethasone; IRF, interferon regulatory factor.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

immunohistochemical stain, respectively. Mucosal expressions of TLR9 were higher in the NP group than the control, NP + Chlq, or NP + Dexa groups (**Supplementary Fig. S4**). The cytosolic or extracellular expressions of HMGB1 were increased in the NP groups, and these were suppressed by NP + Chlq or NP + Dexa (**Supplementary Fig. S5**).

Expression of IRFs is induced by the TLR9 signaling pathway; IRF genes were significantly up-regulated in the NP group compared with the control group ($P < 0.001$ for IRF3 and IRF5; $P < 0.005$ for IRF9). The induction of these genes was significantly suppressed in both the NP + Chlq group ($P < 0.005$ for IRF3 and IRF9; $P < 0.01$ for IRF5) and the NP + Dexa group ($P < 0.001$ for IRF3 and IRF5 and $P < 0.01$ for IRF9; **Fig. 4B**). The expression levels of p65, a component of NF- κ B, were also evaluated by western blotting. The protein levels of p65 were higher in the NP group than in the control ($P < 0.01$); protein expression levels of p65 were significantly suppressed in the NP + Chlq ($P < 0.05$) and NP + Dexa groups ($P < 0.05$; **Fig. 4B**).

Proinflammatory cytokines induced by the TLR9 signaling pathway were also significantly up-regulated in the NP group compared with the control group ($P < 0.001$ for IL-6 and IL-1 β ; $P < 0.005$ for TNF- α ; **Fig. 4C**). The induction of proinflammatory cytokines was significantly suppressed in both the NP + Chlq group ($P < 0.005$ for TNF- α and $P < 0.001$ for IL-6 and IL-1 β) and the NP + Dexa group ($P < 0.005$ for TNF- α , and $P < 0.001$ for IL-6 and IL-1 β ; **Fig. 4C**).

Chloroquine treatment suppresses the expression of T-helper cytokines.

The mRNA expression levels of C-C motif chemokine 11 (CCL11) and T-helper cytokines (IL-5, IL-13, IFN- γ , and IL-17A) were significantly higher in the NP group than in the control group ($P < 0.001$ for CCL11, IL-5, IL-13, and IFN- γ ; $P < 0.005$ for IL-17A; **Fig. 5A**). The induction of these cytokines was significantly suppressed in both the NP + Chlq group ($P < 0.005$ for CCL11; $P < 0.001$ for IL5, IL-13, and IFN- γ ; $P < 0.01$ for IL-17A) and the NP + Dexa group ($P < 0.001$ for CCL11, IL5, and IL-13; $P < 0.005$ for IFN- γ ; $P < 0.01$ for IL-17A; **Fig. 5A**). The expression of the neutrophil marker Ly6G did not significantly differ between the groups.

Associations were evaluated between the mRNA expression levels of IFN- α/β and other cytokines; significant positive correlations were observed between IFN- α/β levels and the levels of each of the following: CCL11, IL-5, IL-13, IFN- γ , and IL-17A; however, there was no correlation with Ly6G (**Fig. 5B**). These results suggested that TLR9 induced the up-regulation of type I IFNs, resulting in elevated Th-mediated mucosal inflammation, which is often observed in EPs; thus, mucosal inflammation and polypogenesis could be suppressed by TLR9 signaling inhibition.

DISCUSSION

A previous study showed that only the expression of IFN- β was significantly elevated in tissues from patients with CRSwNP compared with those from the healthy individuals or patients with CRS who did not have NP; moreover, IFN- α expression did not significantly differ among the patient groups.¹⁴ A recent study also showed that IFN- β responses were elevated in patients with ECRS and that IFN- β levels were positively correlated with IL-5, IL-13, and CCL11 levels, as well as with the number of eosinophils in NP tissue; those findings suggested that type I IFNs may contribute to the development and pathogenesis of ECRS.²⁰ Herein, we investigated the expression levels of type I IFNs according to CRS endotypes: EP and N-EP. Our results indicated that both IFN- α and IFN- β were expressed at significantly higher levels

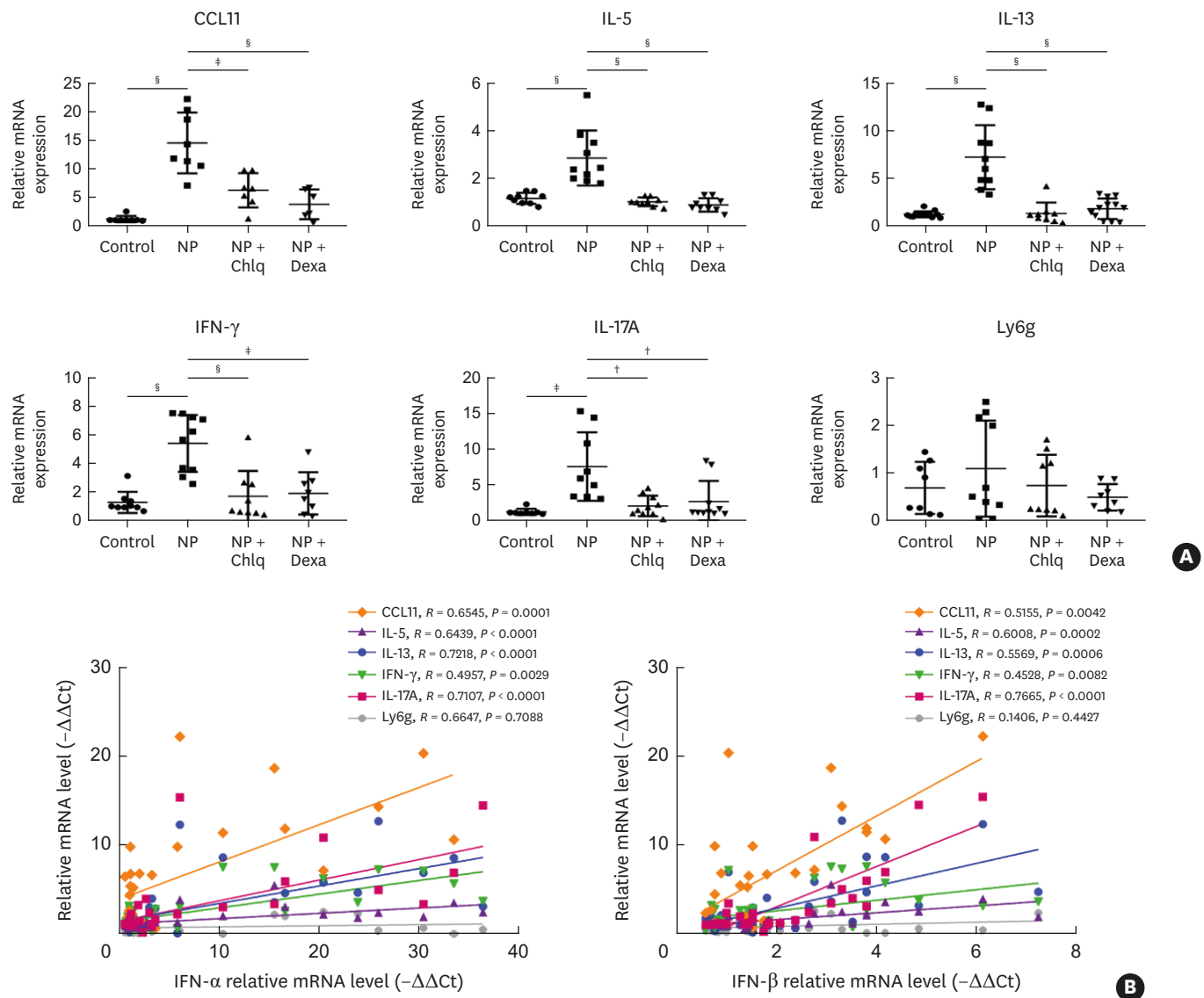


Fig. 5. Chloroquine treatment suppresses the expression of T-helper cell cytokines. (A) The mRNA expression levels of CCL11 and the T-helper cytokines IL-5, IL-13, IFN-γ, and IL-17A were significantly higher in the NP group than in the control group. The induction of these molecules in the NP group was significantly suppressed in the NP + Chlq and NP + Dexa groups. (B) The expression levels of the neutrophil marker Ly6G did not significantly differ among the groups. The expression levels of chemokines and cytokines, except for Ly6G, were significantly positively correlated with IFN-α/β expression levels. CCL11, C-C motif chemokine 11; IL, interleukin; IFN, interferon; NP, nasal polyp; NP + Chlq, nasal polyp treated with chloroquine; NP + Dexa, nasal polyp treated with dexamethasone.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.005$; § $P < 0.001$.

in the EP group than in the N-EP group, and that both IFN-α and IFN-β may contribute to the pathogenesis of EP rather than N-EP. On the basis of the results, we conducted animal study using the well-established mouse model of ECRS.^{17,18} Our results demonstrated that the increased polyp score and mucosal inflammation in ECRS mice were significantly reduced after treatment with chloroquine; the therapeutic effects of chloroquine were comparable to those of dexamethasone.

The administration of CpG DNA, which is a ligand for TLR9, has been shown to induce high levels of IFN-α in mice, resulting in IFN-α-dependent activation of T cells, B cells, and natural killer cells.¹² The production of other inflammatory cytokines, such as IL-6, IFN-γ, IL-12,

and TNF- α , was also enhanced.^{12,21} The identification of plasmacytoid dendritic cells as the primary source of IFN- α , and the expression of TLR7 and TLR9 in these cells, suggested that the ligands of these receptors constitute the main inducers of IFN- α production *in vivo*.²² In the present study, we did not investigate cells that produce type I IFNs; however, our previous study of human samples revealed that the main source of IFN- β in NPs was plasmacytoid dendritic cells.¹⁴

Circulating DNA- and RNA-containing immune complexes can activate plasmacytoid dendritic cells through TLR7 and TLR9, thereby resulting in proinflammatory cytokine production and disease development.²³ Interestingly, similar results were found in the present study. The expression levels of TLR9, HMGB1, type I IFNs, IL-6, IL-1 β , and TNF- α were also increased in the ECRS mice (NP group) compared with the control group. The pathogenesis of CRSwNP was presumed to be similar to that of systemic lupus erythematosus; TLR9 agonists have been shown to increase the expression levels of type I IFNs and BAFF in dispersed NP cells. Up-regulation of these genes was inhibited by chloroquine *in vitro*.¹⁴

In the previous study, we demonstrated that BAFF could be regulated by type I IFN, and the strong positive correlation between the expression of BAFF and dsDNA associated molecule implies that BAFF might be involved in the TLR9 signaling pathway.¹⁴ In the present study, we also verified that there were significant positive correlations between BAFF and the protein levels of anti-dsDNA IgG/mRNA levels of IFN- α/β . These results indicated that autoantibodies could be involved in type I IFN-related BAFF up-regulation in a mouse CRS model.

Our results demonstrated that the expression levels of proinflammatory cytokines and type I IFNs were elevated in NP mice; moreover, chloroquine treatment suppressed the induction of these cytokines. These results suggested that chloroquine inhibits nasal polygenesis by suppressing the TLR9 signaling pathway. Interestingly, cytokines and chemokines involved in eosinophil attraction and survival were also suppressed by chloroquine treatment; their expression levels were associated with the expression levels of type I IFNs. It is reasonable that chloroquine suppressed eosinophilic inflammation in the nasal and paranasal sinus mucosa of ECRS mice as several studies have demonstrated relationships between type I IFNs and eosinophilic inflammation.^{20,24,25} In addition, IFN- γ and IL-17A, important cytokines in Th1 and Th17 responses, respectively, were elevated in the ECRS mice; these responses were also suppressed by chloroquine treatment.

CRS is characterized by heterogeneous and complex pathogenesis, and various T-helper responses have been associated with the pathogenesis of CRS, especially recalcitrant CRS.^{1,26} We found that Th responses were increased in ECRS mice and that chloroquine suppressed these responses. A previous study reported that chloroquine inhibited activation of all T-helper cell subsets, which was mediated by biological actions of JNK/AP-1 signaling in T-cells.²⁷ In this study, chloroquine directly suppressed proliferation, metabolic activity, and cytokine secretion of T-cells following anti-CD3/anti-CD28 activation.²⁷ We did not investigate whether chloroquine inhibits activation of T-helper cell subsets, but inhibition of the JNK/AP-1 signaling in T-cells may be involved in therapeutic effects of chloroquine in our murine polyp model.

Drug repositioning entails the redevelopment of a compound for use in a different disease; this approach is viable because approved drugs and abandoned compounds have been tested in humans, providing detailed information regarding pharmacology, formulation, dosage,

and potential toxicity.^{28,29} Chloroquine has been used for many years to treat patients with autoimmune diseases; it has also been widely administered as a prophylactic treatment for malaria.³⁰ From this point of view, chloroquine could also potentially be used as a therapeutic approach for patients with recalcitrant ECRS or as an alternative to systemic corticosteroid administration.

Chloroquine blocks TLR7 and TLR9 signaling through direct binding to nucleic acids and subsequent inhibition of endosomal acidification, which is necessary for the activation of endosomal TLRs.^{11,31} Additionally, chloroquine and hydroxychloroquine have been shown to exert anticancer effects and to boost the activities of conventional antineoplastic regimens by inhibiting the fusion of autophagosomes and lysosomes.^{32,33} Chloroquine has also been shown to target cancer stem cells by inhibiting Janus kinase 2 signaling.³⁴ Therefore, the effects of chloroquine reported in the present study are presumably mediated (at least in part) by the inhibition of Janus kinase 2 or the fusion of phagosomes and lysosomes; they are not simply mediated by inhibition of the TLR9 signaling pathway. Further studies are needed to elucidate the multidimensional mechanisms of action of chloroquine in the treatment of ECRS.

Despite its efficacy in TLR9-driven inflammatory diseases, chloroquine has a narrow therapeutic index. Prolonged, high-dose exposure to chloroquine can result in reversible cardiac muscle damage and irreversible retinopathy.^{7,9} Therefore, additional research is needed to identify alternative compounds that exhibit similar therapeutic effects with lower toxicity.

In conclusion, chloroquine treatment suppressed TLR9 signaling-related molecules and cytokines as well as controlling NP formation and mucosal inflammation in ECRS mice. Therefore, chloroquine may potentially be used as a therapeutic approach for patients with ECRS and future studies are needed to verify its exact action mechanisms on ECRS.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1

Participant characteristics

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Supplementary Table S2

Sequences of primers used for qPCR

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Supplementary Fig. S1

Experimental protocol for development of a murine model of NPs. (A) Seventy-two female BALB/c mice (age, 4 weeks; weight, 20–25 g) were divided into 4 groups: the control (PBS,

n = 18), NP (n = 20), NP + Chlq (n = 18), and NP + Dexa (n = 16) groups. (B) To verify the anti-inflammatory effects of chloroquine in the murine NP model, 3 doses (10, 50, 100 mg/kg) of chloroquine were used for the assessment of toxicity; based on these results, 50 mg/kg of chloroquine was selected. To determine the treatment dose of chloroquine, mice (7 weeks old, 18–20 g) were divided into 4 groups (3 mice per group); these mice received 5 doses of IP injection with PBS (control) or 3 different doses of chloroquine (10, 50, and 100 mg/kg) between days 0 and 4. One day after the first injection, 2 mice died in the high-dose (100 mg/kg) group. (C) One mouse in the high-dose group survived as did all mice in the other groups. None of the surviving mice showed significant weight loss.

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Supplementary Fig. S2

Protein expressions of IFN- α and IFN- β were evaluated using western blot assay from all samples of each group.

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Supplementary Fig. S3

Distribution of mucosal IFN- α/β expression was examined using immunofluorescence. IFN- α and IFN- β were mainly expressed in epithelial and subepithelial layers in mice in the NP group. Their expression levels were lower in the NP + Chlq and NP + Dexa groups (original magnification, $\times 400$).

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Supplementary Fig. S4

Expressions of TLR9 were examined using immunofluorescence stain. Mucosal expressions of TLR9 were higher in the NP group than the control, the NP + Chlq or the NP + Dexa group (original magnification, $\times 400$).

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Supplementary Fig. S5

Expressions of HMGB1 were examined using immunohistochemical stain. Cytosolic or extracellular expressions of HMGB1 were increased in the NP groups, and these were suppressed by NP + Chlq or NP + Dexa (original magnification, $\times 400$).

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