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Research Article

Ginsenoside F1 Attenuates Eosinophilic Inflammation in Chronic Rhinosinusitis by Promoting NK Cell Function

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ABSTRACT

Background: Ginsenosides have beneficial effects on several airway inflammatory disorders primarily through glucocorticosteroid-like anti-inflammatory activity. Among inflammatory cells, eosinophils play a major pathogenic role in conferring a risk of severe refractory diseases including chronic rhinosinusitis (CRS). However, the role of ginsenosides in reducing eosinophilic inflammation and CRS pathogenesis is unexplored.

Methods: We investigated the therapeutic efficacy and underlying mechanism of ginsenoside F1 (G-F1) in comparison with those of dexamethasone, a representative glucocorticosteroid, in a murine model of CRS. The effects of G-F1 or dexamethasone on sinonasal abnormalities and infiltration of eosinophils and mast cells were evaluated by histological analyses. The changes in inflammatory cytokine levels in sinonasal tissues, macrophages, and NK cells were assessed by qPCR, ELISA, and immunohistochemistry.

Results: We found that G-F1 significantly attenuated eosinophilic inflammation, mast cell infiltration, epithelial hyperplasia, and mucosal thickening in the sinonasal mucosa of CRS mice. Moreover, G-F1 reduced the expression of IL-4 and IL-13, as well as hematopoietic prostaglandin D synthase required for prostaglandin D_2 production. This therapeutic efficacy was associated with increased NK cell function, without suppression of macrophage inflammatory responses. In comparison, dexamethasone potently suppressed macrophage activation. NK cell depletion nullified the therapeutic effects of G-F1, but not dexamethasone, in CRS mice, supporting a causal link between G-F1 and NK cell activity.

Conclusion: Our results suggest that potentiating NK cell activity, for example with G-F1, is a promising strategy for resolving eosinophilic inflammation in CRS.

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1. Introduction

Chronic rhinosinusitis (CRS) is a persistent inflammatory disorder of the paranasal sinuses and upper airways with heterogeneous clinical symptoms (e.g., congestion, stuffiness, nasal discharge, facial pain, impairment or loss of smell, cough, and fatigue) [1–3]. Inflamed tissues from patients with CRS are characterized by a range of inflammatory mediators (e.g., prostaglandins and interleukins) and cell types (e.g., eosinophils, neutrophils, mast cells, and macrophages) [2,4,5]. Eosinophilic inflammation is one of the major pathologic hallmarks of CRS, most prominently CRS with nasal polyps, and is dominant in patients with refractory CRS [6,7]. Although the etiology of CRS is multifactorial and incompletely understood, there is increasing appreciation that specific deficiencies in the innate immune system are associated with initiation and persistence of inflammation in CRS [1,2,5].

Natural killer (NK) cells are cytotoxic innate lymphoid cells that use germline-encoded receptors to rapidly recognize and lyse target cells, such as cells that have undergone neoplastic transformation or viral infection [8,9]. Moreover, NK cells produce several chemokines and cytokines (e.g., IFN-γ) that recruit and activate various immune cells (e.g., T cells and macrophages) to produce coordinated immune responses to target cells. In addition, NK cells regulate the function of other immune and inflammatory

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cells, including eosinophils, either via direct cell–cell contact or cytokine production [10–13]. NK cells regulate eosinophilic inflammation by activating and causing apoptosis of eosinophils [12]. We previously reported that NK cells help to resolve eosinophilic inflammation in the sinuses and blood in an eosinophilic CRS (ECRS) mouse model [14]. NK cells also recognize and clear eosinophils in the airway of asthmatic mice [13]. Of note, NK cells from patients with CRS have impaired effector function, and the extent of such dysfunction correlates inversely with blood and sinonasal eosinophilia [14,15]. In this regard, strategies that restore or enhance NK cell function may hold promise for the treatment of CRS, particularly ECRS. However, such a strategy has not yet been addressed, and routine first-line treatment for ECRS is topical or oral glucocorticosteroids, which is often ineffective and associated with systemic side effects [16–19].

Ginsenosides are the core molecular components of ginseng, the root of *Panax ginseng* Meyer, and have various biomedical efficacies including anti-cancer, anti-fatigue, anti-aging, and immunomodulatory effects [20–24]. The difference in the number, position, and type of sugar moieties underlies the diverse pharmacological potential of ginsenosides [25–28]. Given their favorable safety profiles, ginsenosides are promising candidates for treating allergic and inflammatory disorders [29]; for example, ginsenosides Rh2 and Rg3 attenuate allergic airway inflammation by inhibiting NF-κB activation in a murine model of asthma and in human asthmatic airway epithelial tissues [30,31]. Moreover, ginsenosides Rd and Rg1 suppress ovalbumin-induced expression of Th2 cytokines (e.g., IL-4 and IL-13) and infiltration of eosinophils and mast cells in a murine model of allergic rhinitis [32,33]. Despite studies supporting the effectiveness of ginsenosides against allergic airway disorders, their therapeutic potential in CRS, especially ECRS, has not yet been investigated. Moreover, previous studies focused on the anti-inflammatory mechanisms of action of ginsenosides. Thus, the immune-stimulating efficacy of ginsenosides such as NK cell potentiation by G-F1 [22] in airway inflammatory disorders including CRS remains unclear. Given the causal relation between NK cell dysfunction and ECRS pathogenesis [14,15], we hypothesized that G-F1 may have therapeutic effects on the treatment of ECRS.

In this study, using a murine model of ECRS, we revealed that G-F1, a deglycosylated metabolite of G-Rg1, alleviates eosinophilic inflammation and disease pathogenesis by promoting NK cell function. This mechanism is distinct from the immunosuppressive action of glucocorticosteroids and suggests an alternative strategy for CRS treatment.

2. Materials and methods

2.1. Ginsenoside F1

Standard grade G-F1 was obtained commercially from Nanjing Zelang Medical Technology Co., Ltd. (Jiangsu, China) or was obtained by enzymatic conversion from G-Rg1 as previously described [34]. The purity of G-F1 used was > 98%.

2.2. Animals

BALB/c mice (6 to 7 weeks old) were obtained from Orient Bio Inc. (Sungnam, Korea). All mice were housed and fed under specific pathogen-free conditions. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Science.

2.3. Murine CRS model

ECRS was induced by intranasal challenge with a mixture of 0.7 U protease from *Aspergillus oryzae* (Sigma-Aldrich, St. Louis, MO) and 75 μg ovalbumin (Sigma-Aldrich), diluted in sterile Dulbecco's phosphate-buffered saline (DPBS) to a total volume of 20 μL three times a week for 5 weeks [14,35]. Control mice were intranasally challenged with DPBS. To assess the therapeutic effect of G-F1, mice were administered with an intraperitoneal injection of 50 mg/kg G-F1 or an intranasal injection of 3.5 mg/kg G-F1 three times a week during CRS modeling. Dexamethasone (Sigma-Aldrich) was used as a positive control at a dosage of 2 mg/kg intranasally as described in previous studies using murine model of ECRS and asthma [36,37]. After CRS development and treatment, nasal tissue sections were trimmed from euthanized mice and placed in 4% formaldehyde solution (Thermo Fisher Scientific, Waltham, MA).

2.4. NK cell depletion

For depletion of NK cells, mice were intraperitonially injected with 10 μL rabbit anti-asialo ganglio-N-tetraosylceramide (anti-ASGM1; Cedarlane Laboratories Ltd., Burlington, Canada) while control mice were injected with rabbit serum (Sigma-Aldrich). Mice were injected with anti-ASGM1 or rabbit serum 1 day before an intranasal challenge with protease and ovalbumin, and injected once every 5 days until euthanasia. To evaluate NK cell depletion, splenocytes were isolated from each group of mice; stained with anti-CD3ε-PerCP (145-2C11; BD Biosciences, Franklin Lakes, NJ) and anti-NKp46-PE (29A1.4; BD Biosciences); and analyzed by flow cytometry.

2.5. Histological analysis

After fixation, the paraffin blocks were cut into 3 μm serial sections and stained with hematoxylin and eosin (H&E) by a pathologist in the Department of Pathology, Asan Medical Center in a blinded manner for the group assignments. Epithelial hyperplasia was scored as none (0), minimal (1), mild (2), moderate (3), or severe (4). Minimal was defined as barely detectable, mild as slightly detectable, moderate as easily detectable, and severe as very evident as previously described [38]. Maximal mucosal thickness in nasal tissue was determined at the transition zone of the olfactory and respiratory epithelia by using an image analysis system (CellSens Standard 1.7; Olympus, Tokyo, Japan). For eosinophil detection, slides were deparaffinized, rehydrated, and stained with Sirius Direct Red 80 (Sigma-Aldrich). Infiltration of eosinophils into the lamina propria was measured by counting the number of cells per high-powered field. To detect mast cells, slides of sinonasal tissue from CRS mice were deparaffinized, rehydrated, and stained with acidic toluidine blue (Sigma-Aldrich). The density of the positive area was analyzed with ImageJ software (NIH, Bethesda, MD) in a total of six randomly chosen fields.

2.6. Immunohistochemistry

To detect levels of hematopoietic prostaglandin D synthase (H-PGDS) in the sinonasal tissues of ECRS mice, slides were first incubated with peroxidase blocking solution (Bloxall blocking solution; Vector Labs, Burlingame, CA), boiled in a microwave oven in citrate buffer (pH 6, Sigma-Aldrich) diluted in distilled water for 3 min for epitope retrieval, and then washed in PBS. Thereafter, slides were incubated with a blocking solution containing 1% goat serum and 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature, stained with Sirius Red for identifying eosinophils, and then incubated with a primary antibody against H-PGDS

(1:100; Cayman Chemicals, Ann Arbor, MI) for 1 h. Next slides were stained with Vectastain ABC kits (peroxidase, rabbit IgG; Vector Labs, Burlingame, CA) according to the manufacturer's instructions. The diaminobenzidine signal was observed using a diaminobenzidine substrate kit (Roche Diagnostics, Basel, Switzerland) and analyzed using ImageJ software. After immunohistochemical staining, slides were assessed by light microscopy (LEICA, Wetzlar, Germany) to detect histologic abnormalities and inflammation in CRS.

2.7. Immunofluorescence

To detect production of IL-1β by macrophages in the sinonasal tissues of ECRS mice, slides were first subjected to peroxidase blocking and epitope retrieval before incubation with primary antibodies against IL-1β (1:100, 3A6; Cell Signaling Technology, Beverly, MA) and CD68 (1:100, FA-11; Bio-Rad, Hercules, CA) for 1 h, followed by Alexa Fluor 488-conjugated goat anti-mouse F(ab′)2 (1:250; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 647-conjugated goat anti-rabbit F(ab′)2 (1:250; Jackson ImmunoResearch) for 30 min in PBS containing 1% BSA and 1% goat serum. To detect IFN-γ expression by NK cells, the processed slides were incubated with primary antibodies against IFN-γ (1:100, XMG1.2; Biolegend, San Diego, CA) and ASGM1 (1:200; Cedarlane Labs, Ontario, Canada) for 1 h, followed by Alexa Fluor 647-conjugated goat anti-rat F(ab′)2 (1:250; Jackson ImmunoResearch) and Alexa Fluor 488-conjugated goat anti-rabbit F(ab′)2 (1:250; Jackson ImmunoResearch) for 30 min in PBS containing 1% BSA and 1% goat serum. All incubations were conducted under coverslips at room temperature, followed by three washes with PBS. Coverslips were mounted with ProLong Gold anti-fade reagent (Molecular Probes, Eugene, OR). The fluorescence signal of stained slides was imaged with an LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) in a total of 6 randomly chosen field images (420 × 420 μm). Based on analysis of pixel fluorescence intensities, which ranged from 0 to 255, the positive signal was distinguished from background by empirically counting only those pixels above a threshold value, thus maximizing the inclusion of only those pixels with specific antigen staining. The fluorescent area was calculated as the percentage of total pixels with a fluorescent intensity value greater than the background threshold.

2.8. Quantitative real-time PCR (qRT-PCR)

Turbinated mucosal tissues were harvested and placed in RNAlater solution (Invitrogen, Waltham, MA), and then RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized from 1μg total RNA using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Real-time PCR analysis were performed using a SYBR Green real-time PCR master mix (Toyobo) and LightCycler 480 real-time PCR system (Roche Diagnostics). GAPDH mRNA was used as a normalization control. The relative mRNA levels of IL-4, IL-5, IL-13, IL-1β, IL-17A, and IFN-γ were assessed using qRT-PCR. To analyze the effect of G-F1 or dexamethasone on inflammatory cytokine production by macrophages, primary peritoneal macrophages were treated with LPS (10 ng/mL; InvivoGen, San Diego, CA) for 4 h in the absence or presence of G-F1 or dexamethasone. The relative mRNA levels of IL-1β, TNF-α, IL-6, and IL-12p40 were assessed using qRT-PCR. The PCR primers used were detailed in .Supplementary Table 1

2.9. ELISA

Primary macrophages were obtained from the intraperitoneal cavity of mice after intraperitoneal injection of 3.85% thioglycollate medium. After 3 days, the peritoneal cavity was washed using Dulbecco's modified Eagle's medium (Gibco, Waltham, MA) with 10 % fetal bovine serum (Gibco), and cells recovered from the peritoneal cavity were collected. After washing, cells were seeded in a culture plate and incubated for 3 h. Macrophages were pretreated with G-F1 or dexamethasone 15 min before stimulation with LPS either for 8 h to release TNF-α, IL-6, and IL-12p40, or for 4 h followed by addition of 5 mM ATP to release IL-1β. The amount of IL-1β, TNF-α, IL-6, and IL-12p40 released into the supernatants of cultured macrophages was measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's recommendation (R&D Systems, Minneapolis, MN).

2.10. Statistical analysis

All data were analyzed with GraphPad Prism software (version 5.00; GraphPad, San Diego, CA). Treatment groups were analyzed using the two-tailed Mann–Whitney U-test or student's t-test. P-values < 0.05 were regarded as statistically significant.

3. Results

3.1. Ginsenoside F1 alleviates eosinophilic inflammation in a murine model of CRS

We previously identified that G-F1 is the most potent of 15 ginsenosides in enhancing NK cell activity, and that it improves NK cell-mediated cancer surveillance in two distinct mouse tumor models [22]. Given the causal relation between NK cell dysfunction and ECRS pathogenesis [14,15], we tested if G-F1 could have therapeutic benefits in ECRS. To address this question, we used a previously established ECRS mouse model [14] to assess the effect of G-F1 on ECRS development. Ginsenosides often undergo metabolic transformation in the intestinal tract, such as stepwise deglycosylation of sugar moieties, which results in different and adverse pharmacological potentials [26,28,39]. Thus, G-F1 was administered to mice through local intranasal or systemic intraperitoneal injection. Treatment with G-F1 significantly and effectively alleviated eosinophilic inflammation in the sinonasal mucosa of ECRS mice following either intranasal injection of 3.5 mg/kg or intraperitoneal injection of 50 mg/kg (Fig. 1A and B). G-F1 treatment also reduced epithelial hyperplasia and maximal mucosal thickness in ECRS mice, particularly when intranasally injected (Fig. 1A and B). The infiltration of mast cells, which produce various inflammatory mediators including PGD_2 that drive eosinophilic inflammation [40,41], was reduced by G-F1 treatment (Fig. 1A and B). Collectively, these results indicate that G-F1 has a therapeutic effect on eosinophilic inflammation and disease progression in a murine model of ECRS. Because local intranasal injection (3.5 mg/kg) was more efficacious than intraperitoneal injection (50 mg/kg), we chose intranasal injection for subsequent experiments in consideration of effective dose, safety, and systemic disposition and metabolic transformation.

3.2. Ginsenoside F1 alleviates eosinophilic inflammation related to the PGD_2 pathway

Next, we compared the therapeutic efficacy of G-F1 and dexamethasone, the most widely used drug for the treatment of allergic inflammatory disorders including ECRS. Mice were intranasally administered G-F1 or dexamethasone during the development of

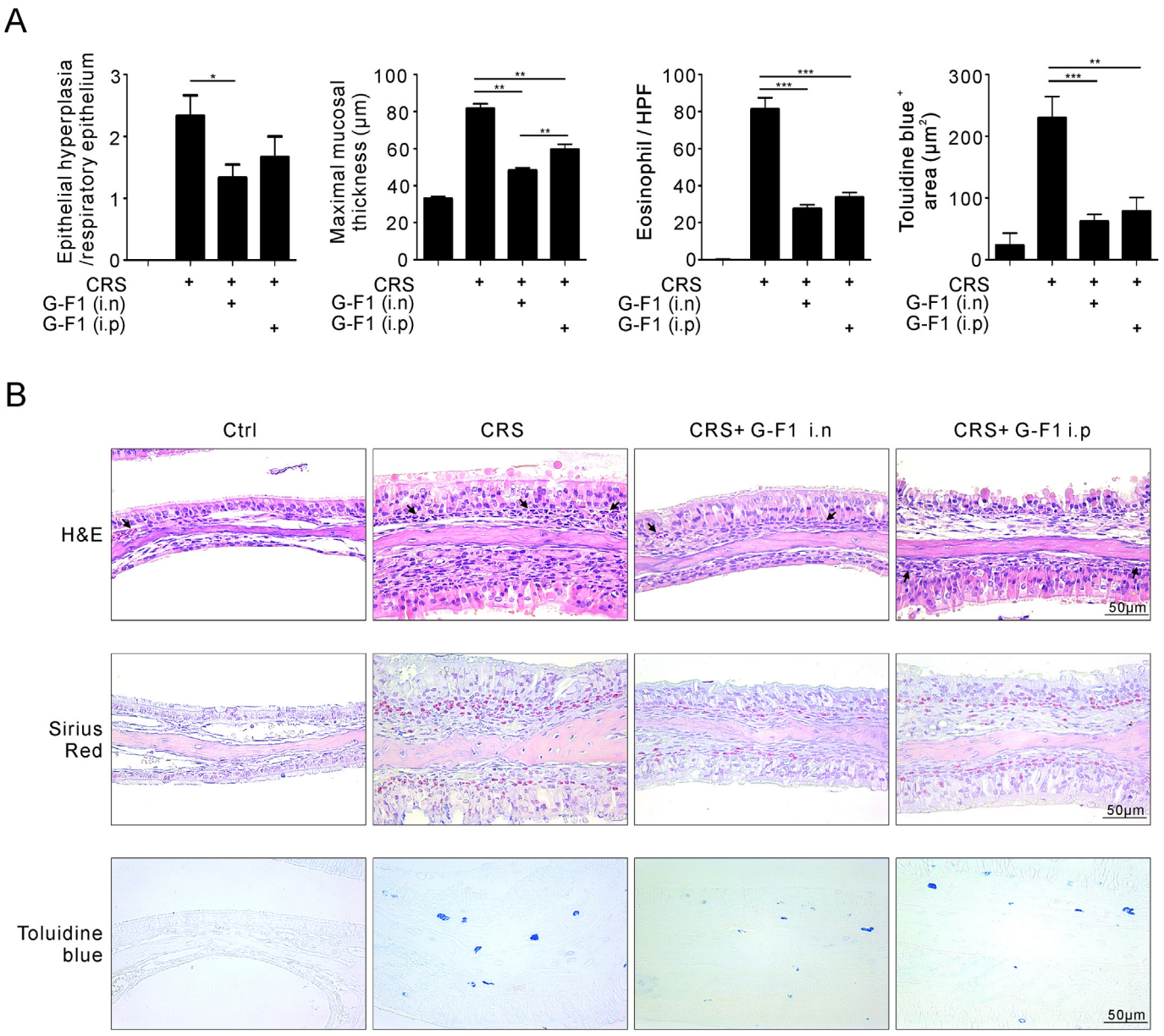

Fig. 1. Ginsenoside F1 (G-F1) has therapeutic potential in a murine model of eosinophilic chronic rhinosinusitis (ECRS). (**A**) Effect of G-F1 administered through intranasal (i.n.) or intraperitoneal (i.p.) injection on scores of epithelial hyperplasia, maximal mucosal thickness in hematoxylin and eosin (H&E)-stained tissue sections, eosinophil counts of the lamina propria per high-powered field (HPF) in Sirius Red-stained tissue sections, and the area of toluidine blue-positive infiltrated mast cells. (**B**) Representative photographs of H&E (top)-, Sirius Red (middle)-, and acidic toluidine blue (bottom)-stained sections. The arrows in H&E stained sections indicate the area of leukocyte infiltration along with the changes in epithelial hyperplasia and mucosal thickness. Scale bars = 50 μm. Data are expressed as means ± SEMs (n = 6 per group). *P < .05, **P < .01, and ***P < .001 by Mann–Whitney U-test.

ECRS. The therapeutic efficacy of G-F1 at a dose of 3.5 mg/kg was comparable to that of dexamethasone at a dose of 2 mg/kg in terms of reducing epithelial hyperplasia, maximal mucosal thickness, and the numbers of eosinophils and mast cells infiltrating the sinonasal mucosa of ECRS mice (Fig. 2A and B). Based on this finding, we next investigated whether G-F1 or dexamethasone modulated levels of H-PGDS, an enzyme that is required for PGD_2 production and is implicated in ECRS pathophysiology [42]. Immunohistochemical staining demonstrated that G-F1 significantly decreased H-PGDS protein levels, which correlated with reduced tissue eosinophilia (Fig. 2C). As expected, dexamethasone similarly reduced H-PGDS levels. These results suggest that G-F1 reduces eosinophilic inflammation by modulating the PGD_2 pathway.

3.3. Ginsenoside F1 promotes NK cell function in sinonasal tissue of ECRS mice

To understand the mechanism of action of G-F1, we assessed the expression of selected genes related to allergic inflammation in the sinonasal tissue of ECRS mice. Using qRT-PCR, we observed that both G-F1 and dexamethasone caused a significant reduction in the mRNA expression of Th2-related IL-4 and IL-13 but not of IL-5 (Fig. 3A); this effect likely accounts for the common therapeutic efficacy of G-F1 and dexamethasone. Moreover, mRNA expression of CCL11 (eotaxin-1), but not CCL5 (RANTES), linked to eosinophil recruitment was markedly upregulated in ECRS mice, which was significantly reduced by both G-F1 and dexamethasone treatment,

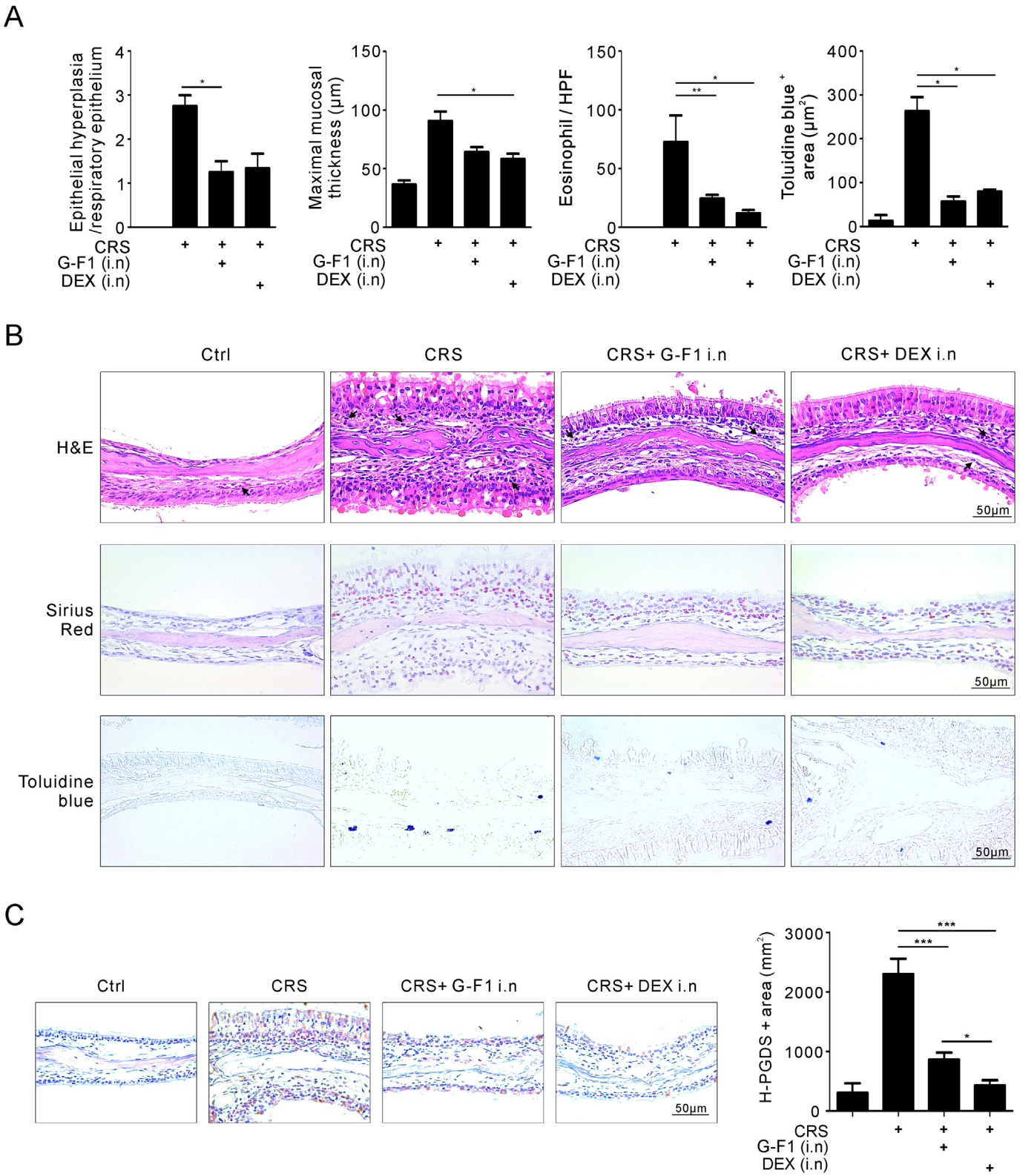

Fig. 2. Comparison of the therapeutic efficacy of ginsenoside F1 (G-F1) and dexamethasone (DEX) against eosinophilic chronic rhinosinusitis (ECRS). (A) Effect of G-F1 or dexamethasone administered via intranasal (i.n.) injection on scores of epithelial hyperplasia, maximal mucosal thickness in H&E-stained tissue sections, eosinophil counts of the lamina propria in Sirius Red-stained tissue sections, and the area of toluidine blue-positive infiltrated mast cells. (**B**) Representative photographs of H&E (top)-, Sirius Red (middle)-, and acidic toluidine blue (bottom)-stained sections. The arrows in H&E stained sections indicate the area of leukocyte infiltration along with the changes in epithelial hyperplasia and mucosal thickness. (**C**) Representative immunostaining for hematopoietic prostaglandin D synthase (H-PGDS) and Sirius Red counterstaining in the sinonasal tissue from each group of mice (left). The bar chart (right) shows the H-PGDS-positive area. Scale bars = 50 μm. Data are expressed as means ± SEMs (n = 4–5 per group). *$P < .05$, **$P < .01$, and ***$P < .001$ by Mann–Whitney U-test.

correlating with the decrease in eosinophilic inflammation (Supplementary Fig. 1). In addition, we found a significant increase in the mRNA levels of CCL5 (RANTES) upon treatment with G-F1 but not dexamethasone. CCL5 is constitutively expressed in NK cells and is highly upregulated upon stimulation [43,44], compatible with our observation of NK cell potentiation by G-F1. Importantly, G-F1 and dexamethasone differentially regulated the mRNA expression of IL-1β and IFN-γ; dexamethasone (but not G-F1) reduced IL-1β mRNA, whereas G-F1 (but not dexamethasone) upregulated IFN-γ mRNA (Fig. 3A). G-F1 treatment also increased IL-17A mRNA levels. IL-1β is a pro-inflammatory cytokine preferentially produced by macrophages and monocytes that potentiates the production of PGD_2 and Th2 cytokines by mast cells [45,46]. IFN-γ is secreted predominantly by activated lymphocytes including NK and T cells, and orchestrates Th1-mediated immune responses [47]. Immunohistochemical analysis revealed a marked increase in IL-1β staining, which was associated with the presence of $CD68^+$ macrophages in the sinonasal mucosa of ECRS mice; dexamethasone but not G-F1 reduced the level of IL-1β staining (Fig. 3B). By contrast, G-F1 (but not dexamethasone) treatment significantly increased the level of IFN-γ staining colocalized with $ASGM1^+$ cells, including NK cells, in the sinonasal tissue of ECRS mice (Fig. 3C). Collectively, these results suggest that the mechanism of therapeutic efficacy of G-F1 against ECRS is distinct from that of dexamethasone and likely involves promotion of NK cell function.

3.4. Ginsenoside F1 does not have anti-inflammatory activity

To further understand the mechanism of action of G-F1 under inflammatory conditions, we assessed the effect of G-F1 on the production of inflammatory cytokines by activated macrophages. In CRS, autophagic dysregulation of macrophages can promote and perpetuate eosinophilic inflammation [35]. Stimulation of peritoneal macrophages caused a marked increase in IL-1β, TNF-α, IL-6, and IL-12 production, which was reduced by dexamethasone but not G-F1 (at concentrations of up to 40 μM; Fig. 4A). This result was confirmed at the level of mRNA expression using qPCR (Fig. 4B). Thus, these results suggest that, unlike dexamethasone, the therapeutic efficacy of G-F1 against ECRS is not mediated by anti-inflammatory activity.

3.5. G-F1-mediated alleviation of ECRS is NK cell-dependent

Finally, we explored the contribution of NK cells to the therapeutic efficacy of G-F1 against ECRS by depleting NK cells with anti-ASGM1 treatment. As expected, the population of CD3ε-NKp46+ NK cells in the spleen was significantly decreased in mice intraperitoneally injected with anti-ASGM1 (Supplementary Fig. 2). Consistent with our previous report showing an inhibitory effect of NK cells on eosinophilia in CRS [14], epithelial hyperplasia and eosinophilic inflammation in the sinonasal mucosa were exacerbated in ECRS mice treated with anti-ASGM1 (Fig. 5A and B). Although not significant, the maximal mucosal thickness and infiltration of mast cells were also increased by NK cell depletion in ECRS mice. Of note, G-F1 treatment did not improve the changes in aggravated maximal mucosal thickness and the number of eosinophils in the sinonasal mucosa caused by NK cell depletion (Fig. 5A and B), suggesting no or little therapeutic effect of G-F1 in the absence of NK cells. By comparison, dexamethasone significantly decreased tissue abnormalities and infiltration of eosinophils and mast cells in the sinonasal mucosa of CRS mice independently of anti-ASGM1 injection. Thus, these results indicate that the therapeutic efficacy of G-F1 against ECRS is primarily mediated by NK cells.

4. Discussion

Ginsenosides, principal active components of ginseng, have attracted considerable attention as promising therapeutic agents for the treatment of inflammatory disorders due to their immunomodulatory effects and favorable safety profiles [48,49]. Although more than 100 types of ginsenosides with differential composition of sugar moieties have been identified [50], the immuno-pharmacological effects of each ginsenoside are less clear. Numerous studies have revealed that ginsenosides exhibit either immune-suppressive or immune-stimulatory properties. This complication remains a major challenge for therapeutic development of ginsenosides. In this study, we present evidence for the first time that G-F1, a deglycosylated metabolite of G-Rg1, alleviates eosinophilic inflammation and disease pathogenesis by potentiating NK cell function in a murine model of ECRS. Moreover, the therapeutic mechanism of G-F1 is probably distinct from other anti-inflammatory ginsenosides (e.g., Rh2, Rg3, Rd), which are effective in allergic airway disorders (i.e., allergic rhinitis and asthma). This notion is supported by the comparison of G-F1 and dexamethasone, which has established immunosuppressive effects, in this study.

Eosinophilic inflammation in CRS is frequently associated with severe refractory disease and is thus a major therapeutic target [51,52]. In this respect, strategies that promote the resolution of eosinophilic inflammation by reducing the survival and migration of eosinophils have been actively pursued [53,54]. Using patient samples and a murine model, we previously demonstrated that there is a causal link between eosinophilic inflammation in CRS and impaired function of NK cells, which normally have an intrinsic capacity to induce eosinophil apoptosis [12,14]. In support of this finding, a recent study revealed that the function of NK cells from patients with severe refractory asthma is significantly impaired, and there is a concomitant increase in the number of target leukocytes including eosinophils [11]. In addition, NK cell function is further disabled by *ex vivo* exposure to dexamethasone, which likely accounts for the resistance to glucocorticosteroid therapy and the persistent inflammation seen in severe asthma [11]. Hence, selective targeting and activation of NK cells may provide an alternative therapeutic modality to treat eosinophilic inflammation in airway inflammatory disorders, particularly those resistant or refractory to glucocorticosteroid therapy. In this regard, our results showing that G-F1-mediated resolution of eosinophilia in CRS occurs via potentiation of NK cell function suggest G-F1 as an eligible and alternative candidate for ECRS treatment. We previously demonstrated that NK cell depletion after CRS development leads to persistent eosinophilic inflammation [14], an observation compatible with an important role of NK cells in the timely resolution of allergic airway inflammation [55]. Thus, we speculate that G-F1-mediated potentiation of NK cells has a therapeutic effect on ECRS even after CRS induction, which merits further investigation in various therapeutic settings. Moreover, it will be interesting to investigate whether G-F1 can alleviate other eosinophilic inflammatory disorders such as hypereosinophilic syndromes, which are associated with significant morbidity and mortality [54]. In terms of safety, doses of G-F1 up to 50 mg/kg were not associated with any systemic toxicity in ECRS mice, consistent with our previous study using a murine tumor model [22]. Moreover, owing to easy access to sinus mucosa, it was possible to administer G-F1 via the intranasal route, where it was therapeutically effective at a much lower dose (3.5 mg/kg) than via intraperitoneal delivery (50 mg/kg), further ensuring favorable efficacy at a safe dose. This dose was also comparable to the dose effective in patients with allergic rhinitis (3 mg/kg/d; the adult dose recommended by the Korea Ginseng Corporation) [56]. However, a systematic dose range-finding study

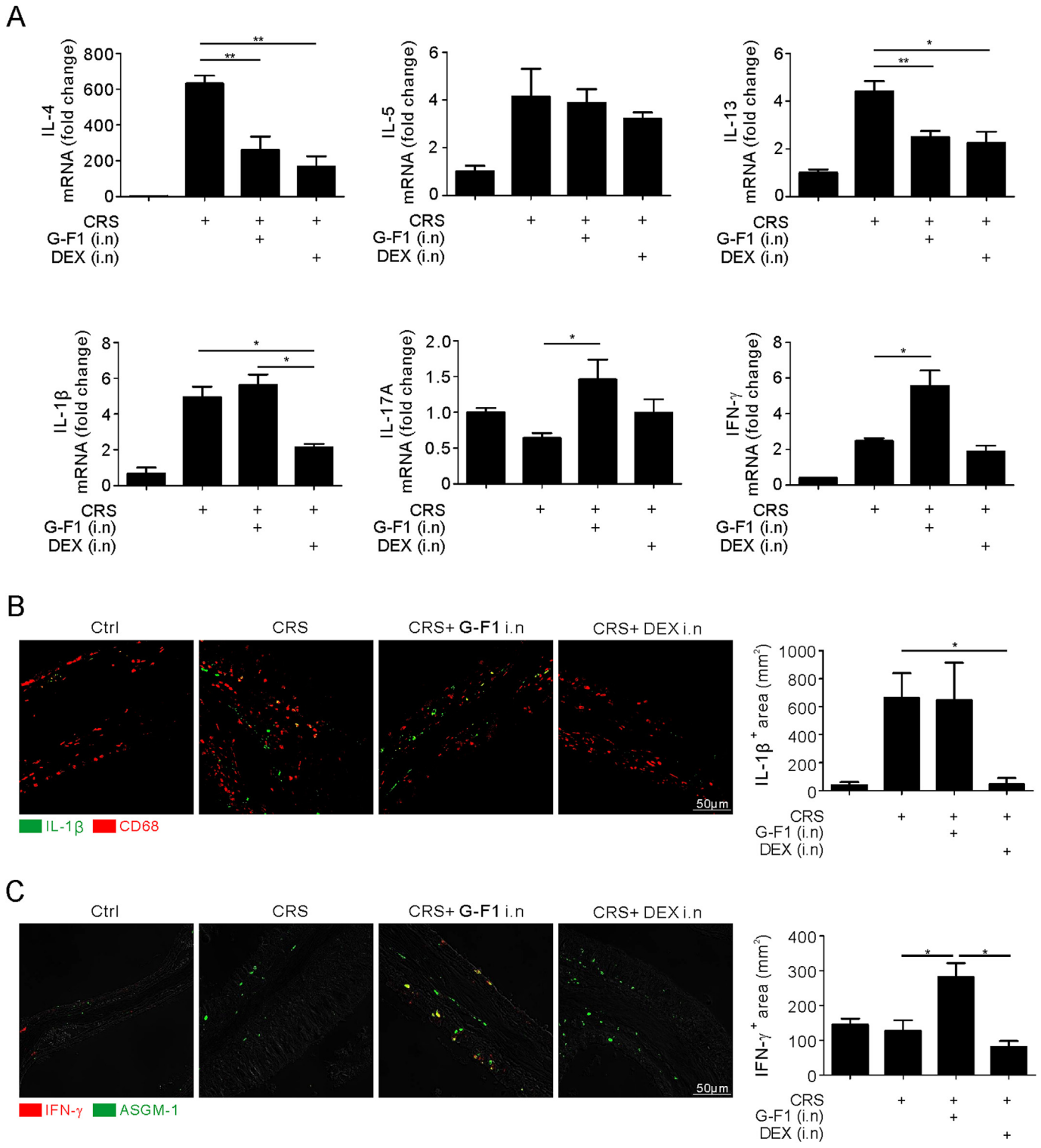

Fig. 3. Ginsenoside F1 (G-F1) and dexamethasone (DEX) have distinct mechanisms of action. (**A**) Effect of G-F1 or dexamethasone on relative mRNA levels of IL-4, IL-5, IL-13, IL-1β, IL-17A, and IFN-γ, as determined using qRT-PCR and normalized to GAPDH mRNA. (**B, C**) Representative dual-immunofluorescence staining for IL-1β and CD68 (B) and for IFN-γ and ASGM1 (C) in the sinonasal tissue from each group of mice (left). Yellow signals indicate colocalization of two marker proteins. The bar charts show the IL-1β-positive (B) and IFN-γ-positive (C) area (right). Scale bars = 50 μm. Data are expressed as means ± SEMs. *$P < .05$ and **$P < .01$ by two-tailed student's *t*-test.

would be required to establish the therapeutic effect of G-F1 on ECRS in association with NK cells depending upon the route of administration.

A mechanistic study indicated that G-F1-mediated therapeutic effects are different from the typical glucocorticosteroids-like anti-inflammatory activity of other ginsenosides including G-Rg1 [57], a glycosylated precursor of G-F1, and were attributed to stimulation

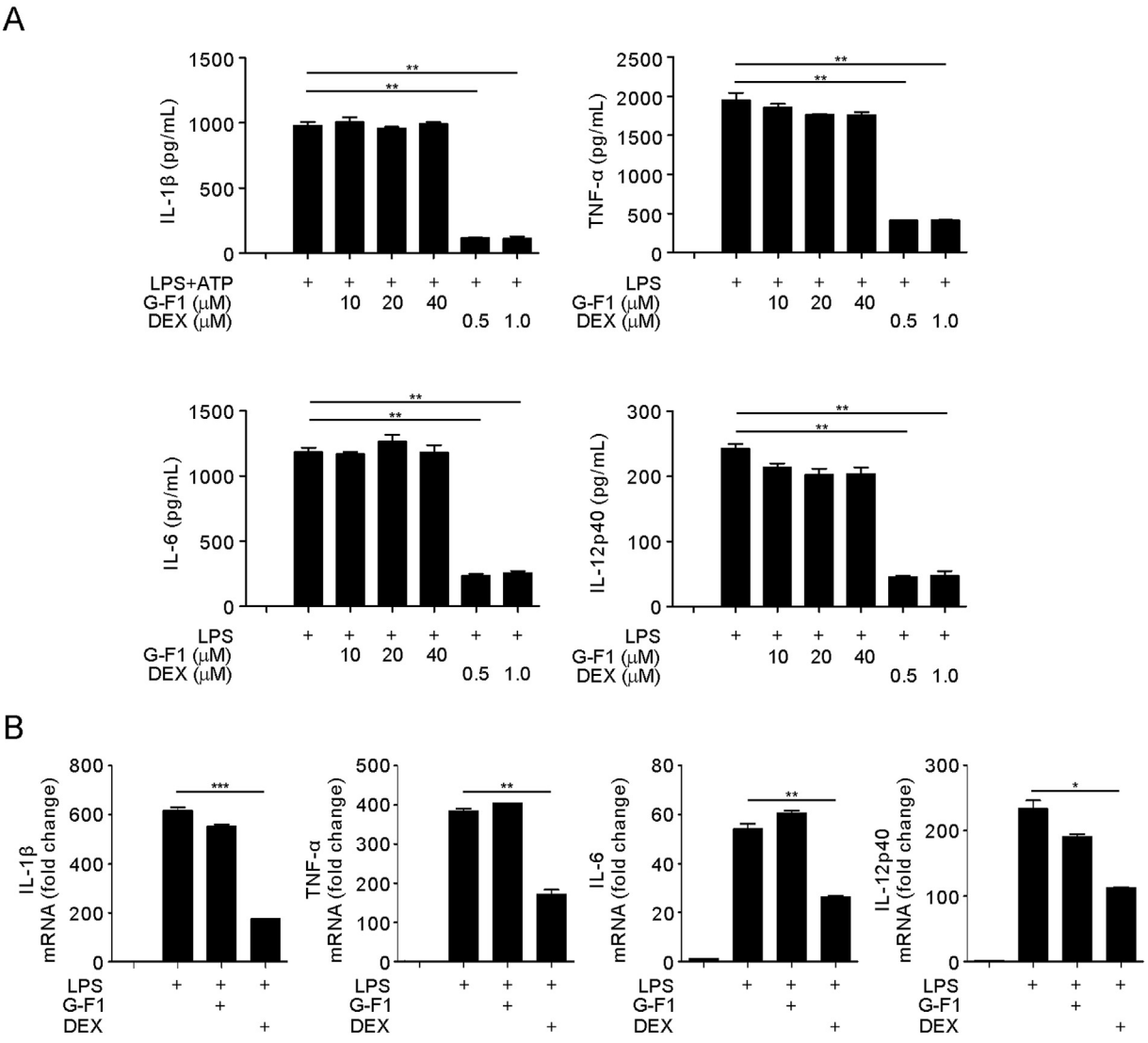

Fig. 4. Dexamethasone (DEX) but not ginsenoside F1 (G-F1) suppresses inflammatory cytokine production from LPS-stimulated macrophages. (**A**) Effect of G-F1 or dexamethasone at the indicated doses on the level of IL-1β, TNF-α, IL-6, and IL-12p40 in the culture supernatants of LPS-activated macrophages, as determined by ELISA. (**B**) Effect of G-F1 (40 μM) or dexamethasone (1 μM) on relative mRNA levels of IL-1β, TNF-α, IL-6, and IL-12p40, as determined by qRT-PCR and normalized to GAPDH mRNA. Data are expressed as means ± SDs. *$P < .05$, **$P < .01$, and ***$P < .001$ by two-tailed student's *t*-test.

of NK cell function. In support of a structure-dependent effects of ginsenosides on NK cell function, we previously demonstrated that G-F1 but not G-Rh1 and protopanaxatriol among the G-Rg1 metabolites significantly enhance NK cell function [22]. This substantial difference in the underlying mechanism of G-F1 was supported in this study by our comparison of the effects of G-F1 and dexamethasone. In support, the mRNA expression and protein levels of IL-1β and IFN-γ was regulated oppositely by the treatment with G-F1 and dexamethasone despite their common therapeutic effects converged at the levels of Th2 cytokines (i.e., IL-4 and IL-13) and eosinophilic inflammation. As expected, dexamethasone suppressed macrophage production of IL-1β, whereas G-F1 (but not dexamethasone) upregulated NK cell production of IFN-γ in sinonasal mucosa of ECRS mice. This differential regulation was further confirmed by assessing the effect of G-F1 or DEX on the production of various inflammatory cytokines from isolated macrophages and the disease severity of ECRS after NK cell depletion. Both IL-1β and IFN-γ contribute to the regulation of type 2 cytokine production and eosinophilic inflammation, and are implicated in CRS pathophysiology [15,35,46,47,58]. Thus, our results suggest that G-F1 and dexamethasone may target different cells or steps of the pathogenic cascade during ECRS development, eventually leading to common therapeutic outcomes. However, the involvement of other cytokines, mediators, and cells including mast cells affected by G-F1 or dexamethasone cannot be excluded, which deserves further investigation.

In sum, this study demonstrates that G-F1 has a previously unrecognized potential to promote the resolution of eosinophilic inflammation in CRS by promoting NK cell effector function. Moreover, the finding that G-F1 modulates the function of NK cells but not macrophages, and a comparison of these effects with dexamethasone, provides new insight into potential therapeutic mechanisms that could underlie ECRS treatment. Given the appreciation that G-F1 is pharmacologically active in the human systemic circulation [59], our findings may provide a rationale for developing G-F1 as a candidate for the treatment of refractory inflammatory disorders driven by eosinophils.

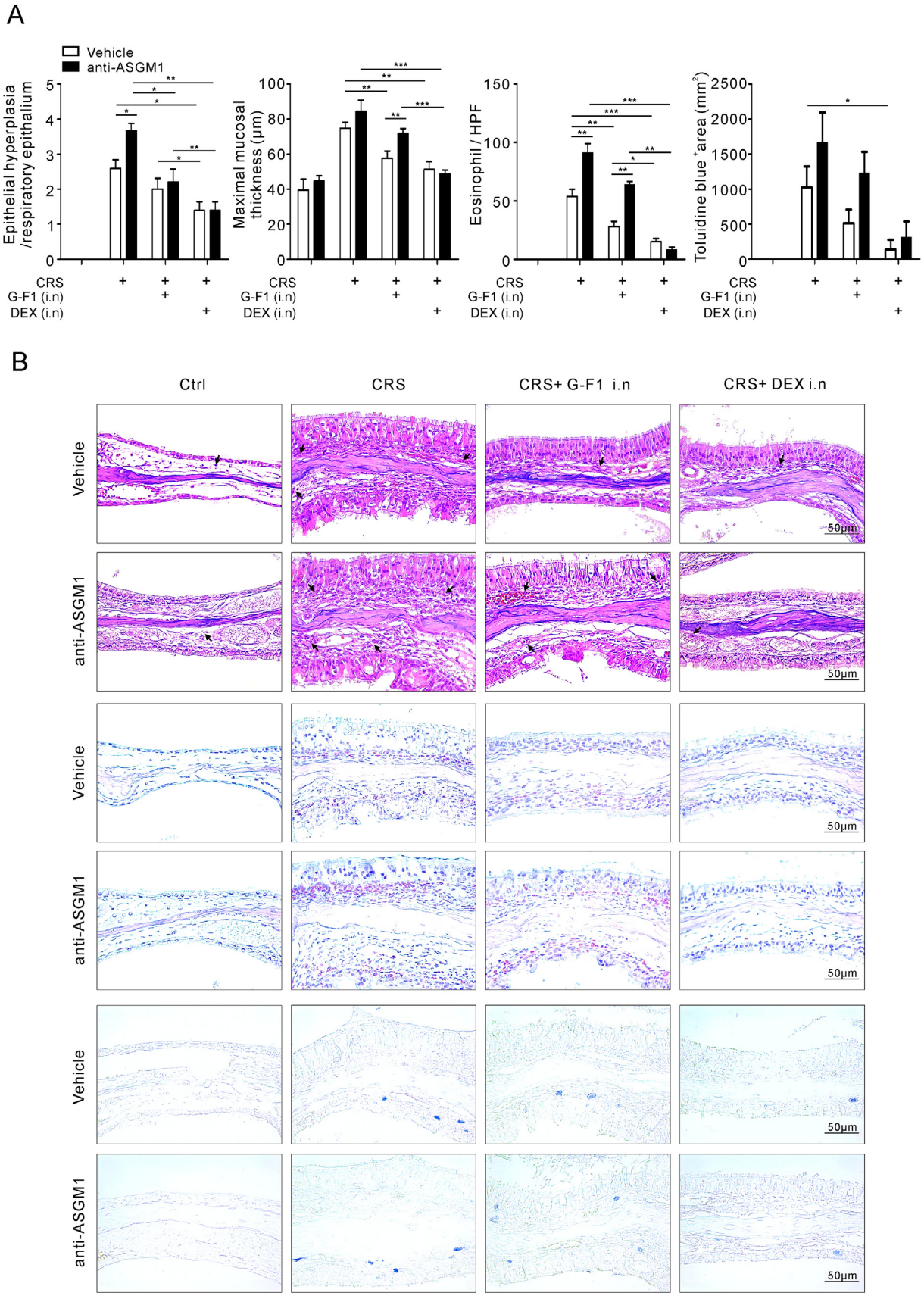

Fig. 5. NK cell depletion nullified the therapeutic efficacy of ginsenoside F1 (G-F1) but not dexamethasone (DEX). (**A**) Effect of G-F1 or dexamethasone after NK cell depletion with anti-ASGM1 on scores of epithelial hyperplasia, maximal mucosal thickness in H&E-stained tissue sections, eosinophil counts of the lamina propria in Sirius Red-stained tissue sections, and the area of toluidine blue-positive infiltrated mast cells. (**B**) Representative photographs of H&E (top)-, Sirius Red (middle)-, and acidic toluidine blue (bottom)-stained sections from chronic rhinosinusitis mice treated with vehicle (upper) or anti-ASGM1 (lower). The arrows in H&E stained sections indicate the area of leukocyte infiltration along with the changes in epithelial hyperplasia and mucosal thickness. Scale bars = 50 μm. Data are expressed as means ± SEMs (n = 5–6 per group). **P* < .05, ***P* < .01, and ****P* < .001 by Mann–Whitney *U*-test.

Author contributions

S.J.K. and J.L. designed and carried out most of the experiments and analyzed data. M.-Y.K. and W.S.C. performed *in vivo* experiments and analyzed data. M.-Y.K. and H.K. performed *in vitro* experiments and analyzed data. S.C.K. designed the experiment and analyzed data. H.S.K. conceived of the study, designed experiments, analyzed the data, and wrote the manuscript, with input from all coauthors.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.03.007.

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