

IL-32 up-regulation is associated with inflammatory cytokine production in allergic rhinitis

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Abstract

IL-32 is a described pro-inflammatory cytokine produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells. However, the specific mechanism of IL-32 on allergic rhinitis (AR) has not been elucidated. Here, we report a significant increase of IL-32 protein and mRNA in the nasal mucosa of AR patients. In addition, in nasal mucosa tissue from AR patients, the level of IL-32 production correlated with inflammation, IL-1 β , IL-18, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In an AR animal model, IL-32 significantly increased IgE and inflammatory cytokine levels. IL-32 expression was induced by recombinant human GM-CSF via activation of caspase-1 in eosinophils. In addition, depletion of IL-32 prevents the production of inflammatory cytokines in eosinophils. In conclusion, IL-32 is an important cytokine involved in the inflammation of AR. The regulation of IL-32 expression may form the basis of a new strategy for the treatment of AR.

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Introduction

Allergic rhinitis (AR) is a common manifestation of allergic diseases, affecting approximately 500 million people worldwide [1]. It is a major chronic inflammatory condition in the upper airways characterized by hypersensitivity, exudation, hypersecretion, inflammatory cell infiltration, and remodelling [2]. Patients with AR present an inflammatory IgE-mediated response characterized by a Th2 immunological pattern with mast cells and eosinophil activation and release of inflammatory mediators in response to exposure to allergens [3–5]. Eosinophils are innate effector cells that are important in immune responses against helminth parasitic infections and contribute to the pathology associated with allergic inflammatory conditions. Their recruitment to inflammatory sites occurs in response to chemotactic and activation signals, such as eotaxin and interleukin (IL)-5, and is a tightly controlled process [6]. Several cytokines are known to influence eosinophil function. In particular, granulocyte-macrophage colony-stimulating factor (GM-CSF) is a major survival and activating factor for hematopoietic cells that primes mature macrophages, eosinophils,

and neutrophils, and is known as a pleiotropic and pro-inflammatory cytokine [7].

IL-32 is a recently described cytokine produced by T lymphocytes, natural killer cells, epithelial cells, mast cells, keratinocytes, and blood monocytes [8–11], and was discovered in the synovial fluid of patients with rheumatoid arthritis [12]. The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons. IL-32 lacks sequence homology to presently known cytokine families. There are six isoforms (α , β , γ , δ , ϵ , and ξ) caused by alternative mRNA splicing, resulting in proteins with a molecular weight ranging from 14.9 to 26.7 kD [10,13]. Receptors of isoforms have yet to be identified, although proteinase 3 was recently identified as a specific IL-32 binding protein. All isoforms were biologically active but the recombinant IL-32 γ isoform showed the highest biological activity, producing inflammatory cytokines both *in vivo* and *in vitro* [14]. IL-32 was induced by *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacillus Calmette–Guérin as well as by either lipopolysaccharide (LPS) or mycobacteria [15]. Moreover, IL-32 production induced by *M. tuberculosis* is dependent on endogenous interferon- γ (IFN- γ) [15]. A recent study demonstrated that overexpression of the

inflammatory mediator cyclooxygenase 2 resulted in increased IL-32 levels [16]. IL-32 induces the production of TNF- α , IL-1 β , IL-6, and IL-8 by means of the activation of NF- κ B and p38 mitogen-activated protein kinase [8,9]. IL-32 also modulates the signals induced by specific Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD) ligands. IL-32 synergized with NOD1 and NOD2 ligands for the synthesis of IL-1 β and IL-6 via activation of caspase-1 [9].

Here, we demonstrate IL-32 expression in the nasal mucosa of AR patients. IL-32 significantly increases the production of inflammatory cytokines in AR animal models. This study identifies IL-32 for the first time as an important inflammatory cytokine involved in the inflammation of AR.

Materials and methods

Patient

Sixty five patients participated in this study (see Supporting information, Supplementary methods).

Cell culture

Human EoL-1 cells were a kind gift from Dr Bae H (Kyung Hee University). The cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; JRH Biosciences, Kansas, USA) at 37 °C under 5% CO₂ in air.

Assay of cytokine production

IgE, IL-1 β , IL-4, IL-6, IL-8, IL-18, TNF- α , VEGF, and GM-CSF production were measured according to the manufacturer's specifications (R&D Systems Inc, Minneapolis, MN, USA). IL-32 α and IL-32 α/β (BioLegend Inc, CA, USA) and IL-32 ($\alpha/\beta/\gamma/\delta$) (YbdY Biotech, Seoul, Korea) were analysed according to the manufacturers' specifications. Protein in tissue homogenates was determined using a bicinchoninic acid protein assay method (Sigma, MO, USA). The levels of cytokine were expressed as a ratio to the total protein in tissue homogenates.

RT-PCR

Total RNA was isolated from cells and nasal mucosa according to the manufacturer's specifications using the easy-BLUETM RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final eluates was determined by spectrophotometry. Total RNA (2.5 μ g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using a cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). We performed a PCR with the following primers: human (h) IL-32 α (5' CTG AAG GCC CGA ATG CAC CA 3'; 5' CCG TAG GAC TTG TCA CAA

AA 3'); hIL-32 β (5' CTG AAG GCC CGA ATG CAC CAG 3'; 5' GCA AAG GTG GTG TCA GTA TC 3'); hIL-32 γ (5' TGA CAT GAA GAA GCT GAA GGC 3'; 5' CAT GAC CTT GTC ACA AAA GCT C 3'); hIL-32 δ (5' TCT CTG ATG ACA TGA AGA AGC T 3'; 5' GCA AAG GTG GTG TCA GTA TC 3'), and hGAPDH (5' CAA AAG GGT CAT CAT CTC TG 3'; 5' CCT GCT TCA CCA CCT TCT TG 3'). To verify if equal amounts of RNA were used for the reverse transcription and PCR amplification from different experimental conditions, we also used GAPDH (5'CAA AAG GGT CAT CAT CTC TG 3'; 5'CCT GCT TCA CCA CCT TCT TG 3'). The annealing temperature was 60 °C for hIL-32 α , hIL-32 β , hIL-32 γ , hIL-32 δ , and hGAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Immunohistochemical analysis of the AR nasal mucosa tissue

Tissue samples were immediately fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with normal swine serum followed by 60 min of incubation with the IL-32 antibody (YbdY Biotech, Seoul, Korea) at a concentration of 3 μ g/ml. The secondary antibody, anti-goat IgG-horse radish peroxidase, was incubated for 30 min. Slides were developed with diaminobenzidine and stained with haematoxylin and eosin (H&E) for 30 s. Sections were coded and randomly analysed by two blinded observers (HJJ and HAO).

Caspase-1 assay

Caspase-1 activity was measured according to the manufacturer's specifications using a caspase assay kit (R&D Systems). A recombinant caspase-1 enzyme was used as a positive control.

Western blot analysis

Protein was resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes. Blots were blocked for at least 2 h with 1 $\times$ PBS containing 0.05% Tween 20 containing 5% non-fat dry milk and then incubated with primary antibodies (anti-IL-32 α/γ antibody, 1 μ g/ml, YbdY Biotech; anti-IL-32 α/β antibody, 0.5 μ g/ml, BioLegend Inc; anti-caspase-1 antibody, 0.4 μ g/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, USA).

Transient transfection and luciferase assay

For the transfection, we seeded the EoL-1 cells in a 100 mm culture dish. We then used LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) to transiently transfect reporter gene constructs into EoL-1 cells.

After 48 h, transfected EoL-1 cells (5×10^5) were plated and stimulated with GM-CSF (10 ng/ml). Cells were harvested 24 or 48 h after stimulation and washed in cold PBS before lysis in 100 μ l of lysis buffer (Luciferase assay kit; Promega, Madison, WI, USA). After vortex mixing and centrifugation at $12\,000 \times g$ for 1 min at 4°C , the supernatant was stored at -70°C until used in the luciferase assay. We mixed 20 μ l of cell extract and 100 μ l of the luciferase assay reagent at room temperature. To measure the luciferase activity, we used a luminometer (1420 Luminescence Counter, Perkin Elmer, Waltham, MA, USA) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of *firefly* luciferase activity to *renilla* luciferase activity.

Ovalbumin-induced AR animal model

We maintained 6-week-old female BALB/c mice (Charles River Technology Gapyung-Gun, Korea) under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the Animal Care Committee of Kyung Hee University. We sensitized mice on days 1, 5, and 14 by intraperitoneal injection of 100 μ g of ovalbumin (OVA) emulsified in 20 mg of aluminum hydroxide (Sigma) and we challenged mice with 1.5 mg of OVA. Mice received 100 ng of human IL-32 before intranasal OVA challenge for 10 days. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA intranasal provocation at 7 days after challenge.

Statistical analysis

The clinical data were compared between groups by the Mann–Whitney *U*-test. The experiments reported are a summary of the data from at least three experiments and are presented as the mean $\pm$ SD. Statistical evaluation of the results was performed by ANOVA with the Tukey *post-hoc* test and independent *t*-test. All statistical analyses were performed using SPSS v12.00 statistical analysis software (SPSS Inc, Chicago, IL, USA). The results were considered significant at a value of $p < 0.05$.

Results

IL-32 is significantly expressed in the nasal mucosa tissue, adenoid tissue, and serum of the AR groups

Supplementary Table 1 (see Supporting information) shows the baseline characteristics for the normal and AR groups. We used nasal mucosa tissue, adenoid tissue, and serum for analysis of the IL-32 isoforms. IL-32 levels in all the tissues of the AR group were significantly higher than those in all the tissues of the

normal group (Figures 1A, 1C, and 1D; $p < 0.05$). IL-32 has four isoforms, α , β , γ , and δ . In addition, two more IL-32 isoforms, ϵ and ξ , were reported recently, but they were not abundantly expressed in other cell types except activated T cells [9,14]. We therefore investigated the protein and mRNA levels of the IL-32 isoforms in the nasal mucosa tissues using western blot and RT-PCR analysis. Expression of the IL-32 isoforms in protein and mRNA was increased in the AR group compared with the normal group (Figure 1B). IL-32 α and γ protein levels were significantly up-regulated in the nasal mucosa tissue of the AR group. Immunohistochemistry of nasal mucosa sections in the AR group revealed that IL-32 is highly expressed in the AR nasal mucosa biopsies (brown colour), whereas less IL-32 was observed in the normal tissue (Figure 1E, arrows). The most positive cells for IL-32 staining were seen in inflammatory cells such as eosinophils and mast cells (Figure 1E). Additionally, quantitative scores for IL-32 correlated significantly with those for inflammation on H&E staining (Table 1, $R = 0.543$, $p = 0.004$). Serum total IgE levels, peripheral blood eosinophil counts, and IL-1 β were significantly higher in the AR group than in the normal group (Figure 2, $p < 0.05$). The inflammatory mediator ECP was measured in the AR and normal groups to assess degranulation of eosinophils as a marker of mucosal inflammation. ECP levels were significantly increased in the AR group ($p = 0.014$). The AR grade also increased significantly in the AR group compared with the normal group.

IL-32 levels correlated with inflammatory cytokines in the nasal mucosa tissue of the AR group

IL-1 β , IL-18, and GM-CSF were measured in the nasal mucosa tissue of the AR and normal groups. Significantly increased IL-1 β and GM-CSF were found in the AR group compared with the normal group (Figure 3, $p < 0.05$), while no statistically significant difference was noted between the two groups with regard to IL-18 levels. The levels of IL-32 in the nasal mucosa tissues correlated significantly with the levels of IL-1 β , IL-18, and GM-CSF (Table 1, $R = 0.603$, $p = 0.002$ for IL-1 β ; $R = 0.559$, $p = 0.005$ for IL-18; $R = 0.795$, $p = 0.000$ for GM-CSF).

IL-32 increased the nasal IgE and inflammatory cytokine levels in AR animal models

We confirmed the regulatory effect of IL-32 in an AR animal model. Mice received 100 ng of human IL-32 before the intranasal OVA challenge. The number of nasal rubs after the OVA challenge in the control and IL-32 groups was significantly higher than that in the OVA-unsensitized mice (see Supporting information, Supplementary Figure 1). IgE and inflammatory cytokines (IL-1 β , IL-4, IL-6, IL-18, and TNF- α) were increased in the control and IL-32 groups compared with OVA-unsensitized mice (Figure 4). The IgE and inflammatory cytokine levels in the IL-32 group were

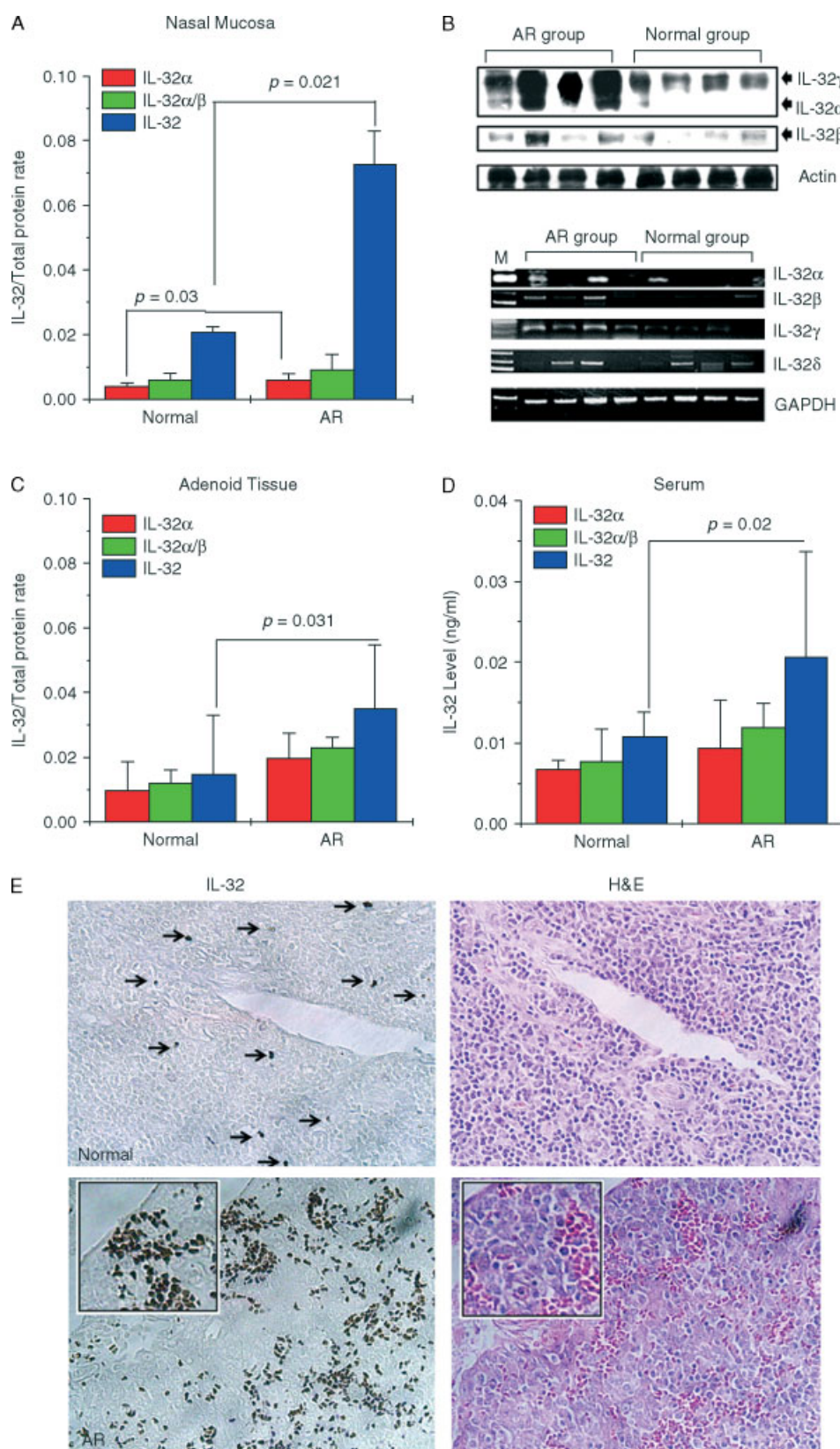


Figure 1. IL-32 expression is up-regulated in the AR group. Isoforms of IL-32 protein and mRNA were analysed by ELISA (A), western blot analysis (B, upper), and RT-PCR (B, lower) in the nasal mucosa tissues. (C, D) IL-32 protein levels in the adenoid tissue and serum from the AR and normal groups. (E) IL-32 expression in the nasal mucosa tissue. IL-32 is indicated by arrows. The IL-32 level measured in the tissue homogenate is presented as a ratio to the total protein level in the tissue.

higher than those in the control group. IgE, IL-4, IL-6, IL-18, and TNF- α increased significantly in the IL-32 group compared with the control group ($p < 0.05$). IL-32 and OVA synergistically increased the production of IgE and inflammatory cytokines in the OVA-sensitized mice. The respective numbers of inflammatory cells in the nasal mucosa in the control and IL-32 groups were significantly higher than those in OVA-unsensitized mice, but there was no difference between the IL-32 group and the control group in terms of the number of inflammatory cells (Figure 4).

IL-32 was expressed by GM-CSF in the human eosinophilic leukaemia cell line EoL-1

Eosinophils play a major role in the inflammatory reaction of AR [3]. GM-CSF is known to be an activator of eosinophils [7]. The GM-CSF level also increased significantly in the AR group (Figure 3). To assess the regulatory effect of GM-CSF on IL-32 expression in the *in vitro* model, we performed ELISA, luciferase

Table 1. IL-32 correlation with inflammatory cytokines in the nasal mucosa tissue

Parameter	R value	p value
Inflammation	0.543	0.004
IL-1 β	0.603	0.002
IL-18	0.559	0.005
GM-CSF	0.795	0.000

Expression of IL-32 was correlated with microscopic inflammation of the biopsies. Correlations are expressed by using Spearman's rank correlation coefficient. $p < 0.005$.

assay, western blotting, and RT-PCR. IL-8 production was assayed to test the activity of GM-CSF. In this study, we observed the kinetics of IL-32 production after stimulation with GM-CSF (10 ng/ml). The production of IL-32 was increased up to 24 h after GM-CSF treatment and declined thereafter from 48 to 90 h (Figure 5A). IL-32 production was significantly increased by 10 ng/ml GM-CSF (Figure 5B, $p = 0.032$). GM-CSF and IL-32 γ also significantly increased IL-8 production (Figure 5C, $p < 0.05$). We

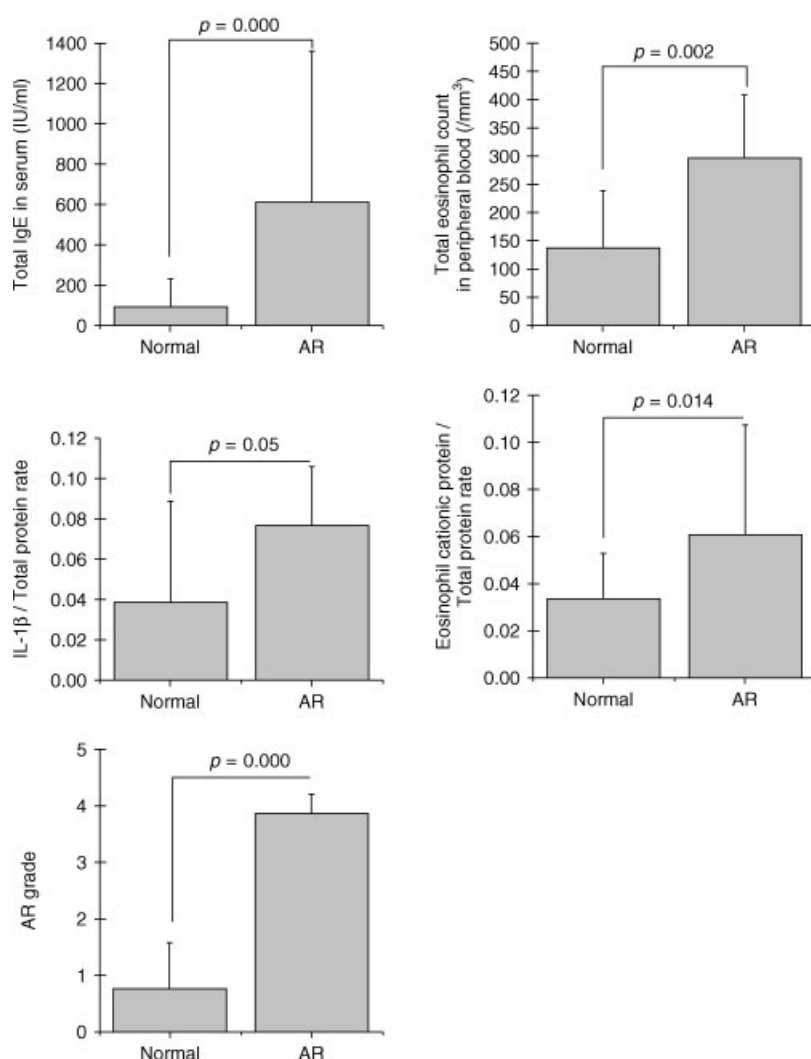


Figure 2. Serum, blood, and adenoid levels of total IgE, total eosinophil number, ECP, and IL-1 β . Total IgE was measured in serum. Total eosinophil numbers were measured in peripheral blood. ECP and IL-1 β were measured in the adenoid tissue. AR grade was also scored for the AR and normal groups. IL-1 β and ECP measured in the tissue homogenate are presented as a ratio to the total protein level in the tissue.

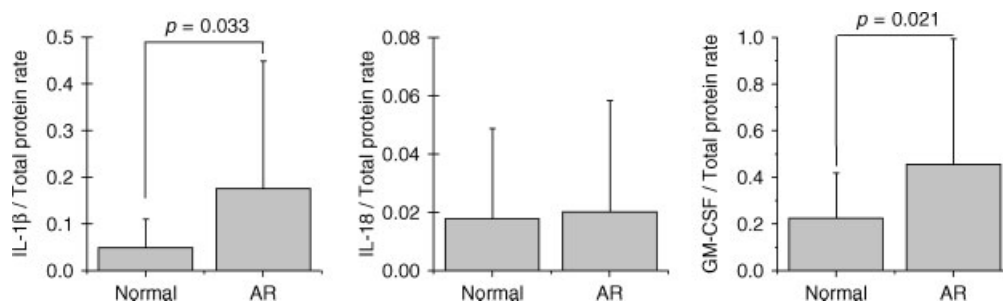


Figure 3. Inflammatory cytokine levels were increased in the AR group. The nasal mucosa levels of IL-1 β , IL-18, and GM-CSF were measured in 25 AR patients and 25 normal people. All parameters measured in the tissue homogenate are presented as a ratio to the total protein level in the tissue.

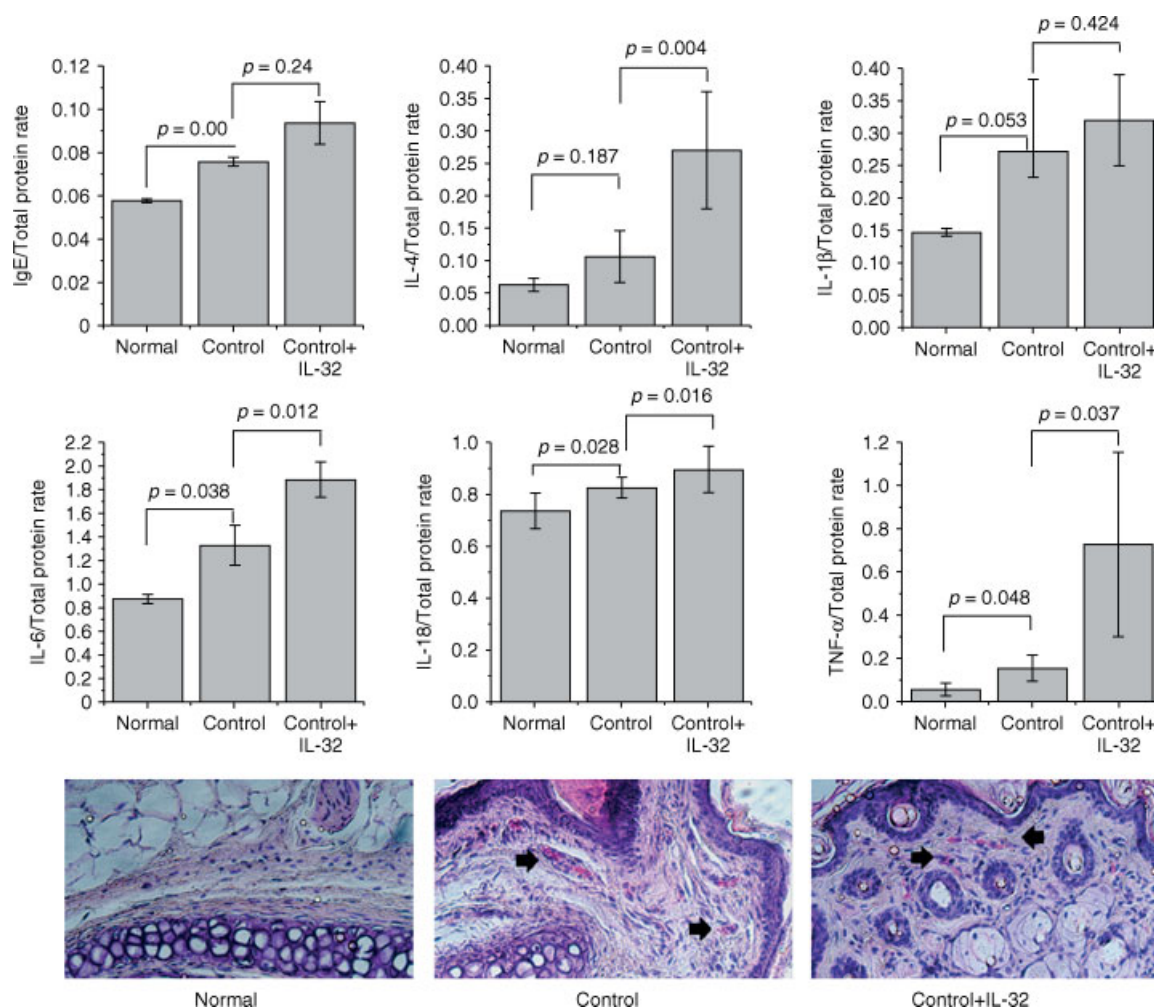


Figure 4. IgE and inflammatory cytokine levels in the nasal mucosa tissue from AR mice. We sensitized mice on days 1, 5, and 14 by intraperitoneal injection of 100 μ g ovalbumin (OVA) emulsified in 20 mg of aluminum hydroxide (Sigma) and we challenged mice with 1.5 mg of OVA. Mice received 100 ng of human IL-32 before intranasal OVA challenge for 10 days. IgE, IL-1 β , IL-4, IL-6, IL-18, and TNF- α were measured by the ELISA method. All parameters measured in the tissue homogenate are presented as a ratio to the total protein level in the tissue. Nasal mucosa stained with H&E. Arrows indicate inflammatory cells. Normal, OVA-unsensitized mice ($n = 10$); control, OVA-sensitized mice ($n = 10$); control + IL-32, OVA sensitization + 100 ng IL-32-treated mice ($n = 10$). Original magnification $\times 400$.

next examined whether GM-CSF can modulate IL-32 expression. Reporter plasmid pIL-32-Luc (containing an IL-32 promoter region) was generously provided by Ying Zhu (Wuhan University). As shown in Figure 5D, GM-CSF (10 ng/ml) significantly increased reporter gene activity ($p = 0.01$). GM-CSF also induced IL-32 (α , β , γ , and δ) mRNA and protein expression (Figures 5E and 5F).

GM-CSF increased IL-32 production through activation of caspase-1

Caspase-1 activates the inactive precursors of the IL-1 β , IL-18, and IL-32 cytokines involved in inflammation. IL-32 is known to be an activator of caspase-1. To evaluate whether GM-CSF increased caspase-1 activity, we performed a caspase-1 assay and western

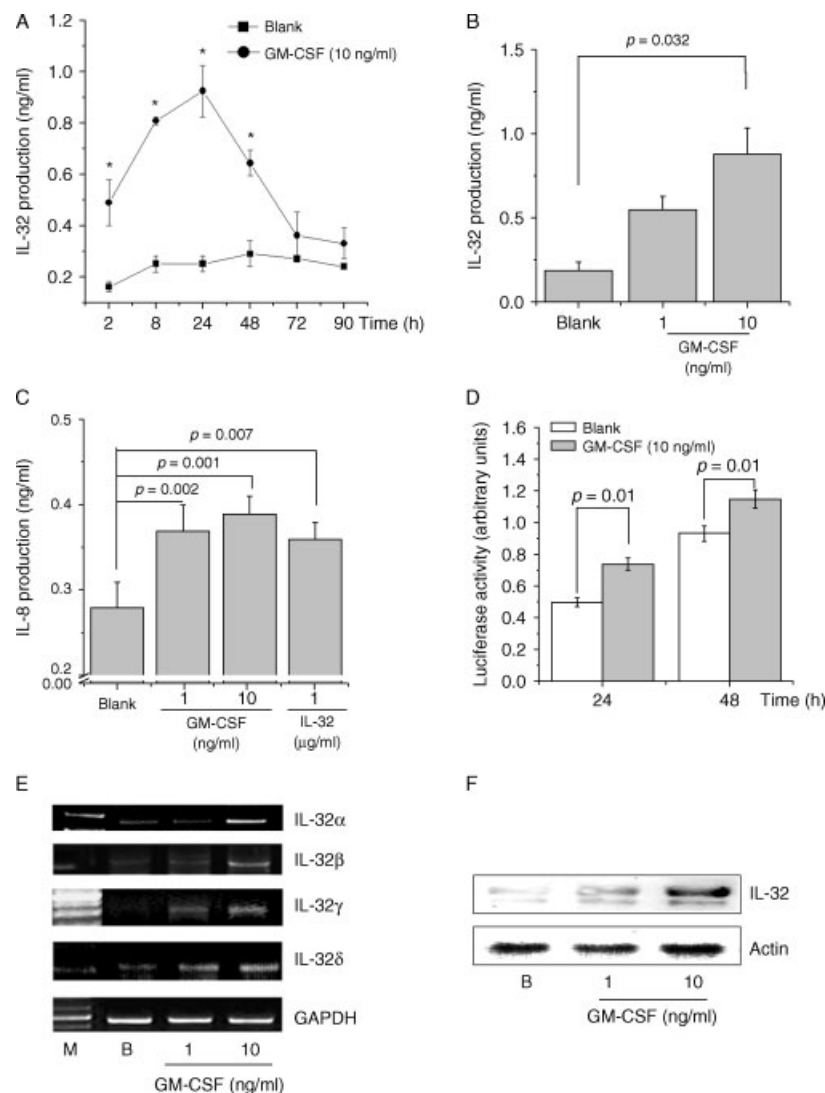


Figure 5. IL-32 was expressed by GM-CSF in EoL-1 cells. (A) Cells (5×10^5) were treated with GM-CSF (10 ng/ml) for various times. (B, C) Cells (5×10^5) were treated with GM-CSF (1 and 10 ng/ml) for 24 h. IL-32 and IL-8 production was determined by the ELISA method. (D) pIL-32-Luc activity was assayed by luciferase assay. (E) Cells (5×10^6) were treated with GM-CSF (1 and 10 ng/ml) for 4 h. IL-32 isoforms were analysed by RT-PCR. (F) Cells (5×10^6) were treated with GM-CSF (1 and 10 ng/ml) for 24 h. IL-32 was analysed by western blot analysis. Results are representative of three independent experiments. * $p < 0.01$, different from the unstimulated cells. M = marker; B = blank. Blank: unstimulated cells.

blot analysis. As shown in Figures 6A and 6B, GM-CSF increased caspase-1 activity. Increased caspase-1 activity by GM-CSF or IL-32 was inhibited by caspase-1 inhibitor (Figure 6C). Increased IL-32 production by GM-CSF was decreased by caspase-1 inhibitor treatment (Figure 6D).

IL-32 is required for the production of inflammatory cytokines on EoL-1 cells

In order to confirm whether up-regulation of inflammatory cytokines was mediated by IL-32, we depleted IL-32 in EoL-1 cells using siRNA (IL-32 pool; iNtRON Biotech, Korea). EoL-1 cells were transfected with IL-32 siRNA and stimulated with GM-CSF (10 ng/ml) for 24 h. Messenger RNA and protein analysis by RT-PCR and western blotting revealed that IL-32 was depleted (Figures 7A and 7B). The protein levels of IL-1 β , IL-6, IL-8, TNF- α , and VEGF in

the culture medium were measured using the ELISA method. GM-CSF significantly increased the production of inflammatory cytokines IL-6, IL-8, TNF- α , and VEGF but not IL-1 β (Figure 7C, $p < 0.05$). However, the production of inflammatory cytokines was significantly decreased by IL-32 siRNA (Figure 7C, $p < 0.05$).

Discussion

In this study, we have shown that the patients with AR differ significantly from normal healthy control in having increased expression of IL-32 in the adenoid tissue, nasal mucosa tissue, and serum. IL-32 correlates with the production of inflammatory cytokines in the nasal mucosa tissue. Furthermore, we found that GM-CSF strongly induced IL-32 expression via activation of caspase-1 in EoL-1 cells.

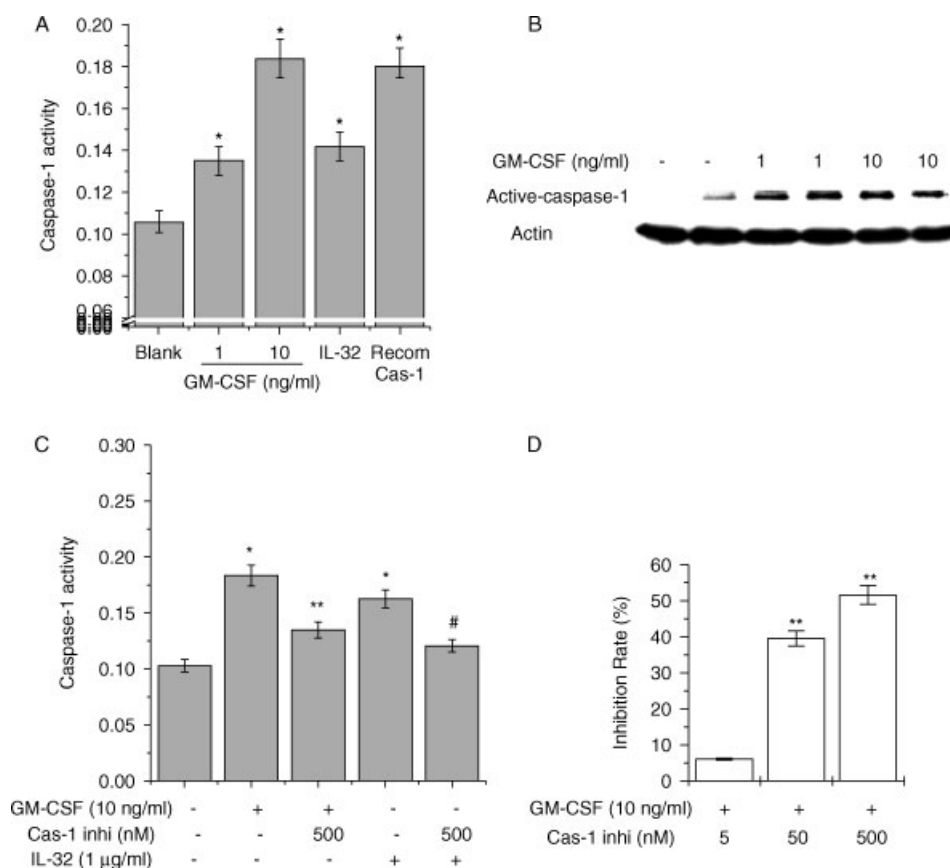


Figure 6. GM-CSF increased IL-32 expression via activation of caspase-1. (A, C) Cells (5×10^6) were treated with caspase-1 inhibitor (500 nM) for 1 h and then stimulated with GM-CSF or IL-32 for 2 h. Caspase-1 activity was determined by a colorimetric kit. (B) Caspase-1 was determined by western blot analysis. (D) Cells (5×10^5) were treated with caspase-1 inhibitor (5, 50, and 500 nM) for 1 h and then stimulated with GM-CSF for 24 h. IL-32 production was determined by ELISA. Recomb Cas-1 = recombinant caspase-1; Cas-1 inhi = caspase-1 inhibitor. * $p < 0.01$, different from the unstimulated cells. ** $p < 0.01$, different from GM-CSF. # $p < 0.01$, different from IL-32.

IL-32 plays a role in many inflammatory disorders, and its expression has been related to disease severity in patients with Crohn's disease, rheumatoid arthritis, chronic obstructive pulmonary diseases, and atopic dermatitis [10,12,17,18]. IL-32 stimulates monocytes to produce pro-inflammatory cytokines such as IL-6, TNF- α , VEGF, and chemokines [8,19], and induces the differentiation of monocytes into macrophage-like cells [20]. IL-32 induced the release of IL-4 and IFN- γ , two known inhibitors of osteoclast formation and activation [21]. In this study, we found that IL-32 isoforms, α , β , γ , and δ , are expressed in the nasal mucosa tissue. We also showed that IL-32 α and IL-32 γ in the protein increased significantly in the nasal mucosa of the AR group. However, the levels of α and α/β isoforms were not significantly different from those in the adenoid tissue and serum (Figure 1). IL-32 also correlated with IL-1 β , IL-18, and GM-CSF in nasal mucosa tissue (Figure 3 and Table 1). In addition, IL-32 increased the IgE and IL-4 levels in AR animal models (Figure 4). From this, we suggest that IL-32 plays an important role in the inflammation of AR.

IL-32 was initially detected in T cells and NK cells activated by IL-2 or IFN- γ [8,22] and induced in peripheral blood mononuclear cells and monocytes

by *Mycobacterium tuberculosis* [15]. IL-32 has six isoforms as a result of alternative splicing [8,10,13].

Alsaleh *et al* reported that IL-32 mRNA transcription was induced by IFN- γ , TNF- α , LPS, BLP (TLR2 ligand), and poly I:C [23]. However, all the isoforms are not secreted; this depends on the cell types. Intracellular accumulation of IL-32 α protein was detected in human pancreatic periacinar myofibroblasts but was not detected in culture supernatants [24]. IL-32 β was also produced by T cells but was not secreted into the culture medium [13]. IL-32 was expressed at high levels in human epidermal keratinocytes after stimulation with IFN- γ and TNF- α but was not secreted by keratinocytes [10]. In this study, we demonstrated that GM-CSF induced IL-32 mRNA transcription (α , β , γ , and δ) and protein secretion on EoL-1 cells (Figure 5). Although it was recently suggested that IL-32 may play a role as a cytoplasmic protein [13,24], our results are consistent with several other reports which indicate that IL-32 is secreted from primary cells as well as several cell lines [8,17,25]. Therefore, IL-32 is increased in physiological fluids under certain disease conditions, suggesting that IL-32 can also be extracellularly secreted under certain physiological conditions. Overall, our report suggests that IL-32 can exert differential roles in cytosol and extracellular environments. We did

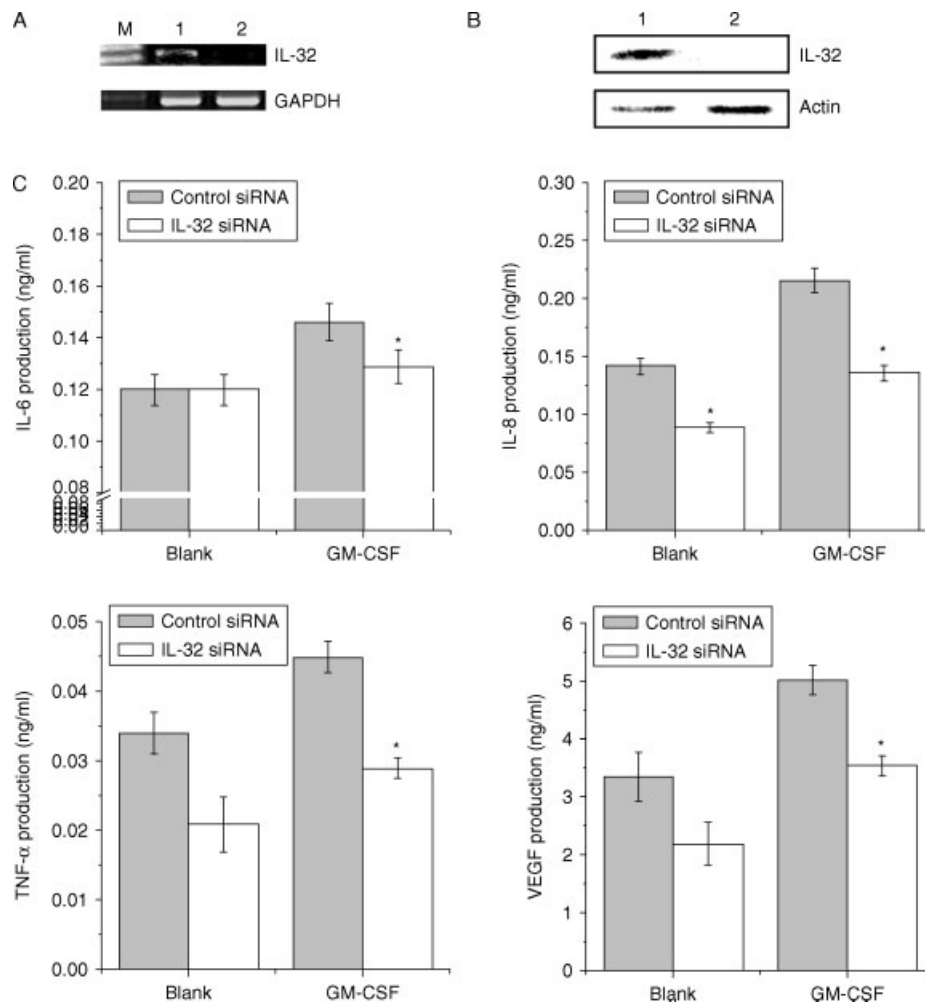


Figure 7. Essential role of IL-32 for inflammatory cytokine expression of EoL-1 cells. EoL-1 cells were transfected with IL-32 siRNA. Total RNA and protein were assayed by RT-PCR (A) and western blot analysis (B) for IL-32. (C) Cells (5×10^5) were treated with GM-CSF (10 ng/ml) for 24 h. IL-6, IL-8, TNF- α and VEGF production were assayed by using ELISA. M = marker; 1 = control siRNA; 2 = IL-32 siRNA. * $p < 0.01$, significantly different from the control siRNA transfected cells.

not measure respectively IL-32 α , β , γ , and δ production. Further investigation is necessary to investigate secreted isoforms from EoL-1 cells.

Nasal biopsies of patients with active AR show accumulations of mast cells, eosinophils, and basophils in the epithelium and an accumulation of eosinophils in the deeper lamina propria [26]. Recruitment of inflammatory cells, including eosinophils, basophils, and T cells, results in the further release of histamine and leukotrienes, as well as other compounds including pro-inflammatory cytokines and chemokines, sustaining the allergic response and promoting the late phase response [26,27]. In this study, IL-32 did not significantly affect the number of inflammatory cells in the AR animal model. From this, we suggest that IL-32 had no effect on the migration of eosinophils. Eosinophils, prominent leukocytes in AR, are characterized by an abundance of intracellular granules that contain preformed proteins including distinct cationic proteins, such as ECP, and a wide range of preformed cytokines, chemokines, and growth factors [28,29]. GM-CSF is a major survival and activating factor for eosinophils and is known as a pleiotropic and pro-inflammatory cytokine [7].

The respiratory epithelium expresses GM-CSF, and infiltrating leukocytes can be induced to synthesize GM-CSF as an autocrine growth factor by inflammatory and chemotactic stimuli. GM-CSF also greatly enhances leukocyte oxidative burst activity and mediator release and serves as a major leukocyte survival factor in inflammatory lesions [30,31]. GM-CSF and IL-3 activated eosinophils, transforming the storage form of ECP into the secreted form [7]. In this study, GM-CSF induced IL-32 protein and mRNA expression (Figure 5). GM-CSF also stimulated IL-6, IL-8, TNF- α , and VEGF production (Figure 7). In addition, IL-32 increased IL-8 production. However, when the depletion of IL-32 by IL-32 siRNA was achieved in EoL-1 cells, the production of inflammatory cytokines induced by GM-CSF partially decreased (Figure 7). From this, we can presuppose that IL-32 produced by GM-CSF partially plays a role in the expression of inflammatory cytokines in eosinophils. These properties suggest that IL-32 might play an important role in the amplification of inflammatory reactions.

Inflammasomes are multi-protein cytoplasmic complexes that mediate the activation of inflammatory

caspase-1 [32]. Caspase-1 cleaves pro-IL-1 β to IL-1 β and also activates IL-18 and IL-33. In this manner, caspase-1 controls the maturation of some of the pro-inflammatory cytokines. In a very elegant study, Netea *et al* reported that IL-32 is a cell-associated pro-inflammatory cytokine which is specifically stimulated by mycobacteria through caspase-1- and IL-18-dependent production of IFN- γ [15]. IL-32 also synergizes with NOD 1 and NOD2 ligands for IL-1 β and IL-6 production through a caspase-1-dependent mechanism [9]. IL-32 potentiates one or more of the intracellular pathways induced by NOD ligands. Synthetic muramyl dipeptide engagement of NOD2 results in the activation of two signalling pathways. First, NOD2 activates the serine/threonine kinase receptor-interacting protein 2/RICK/CARDIAK, and this leads to NF- κ B activation and transcription of pro-inflammatory cytokine genes such as those encoding TNF- α , IL-1 β , and IL-6 [33]. Second, the CARD domain of NOD-like receptor proteins interacts with caspase-1 and -5, and this interaction results in the processing of cytokine precursors such as pro-IL-1 β and pro-IL-18 [34,35]. We showed that GM-CSF increased IL-32 production and mRNA expression. From this, we found that GM-CSF increased the inflammatory reaction through an intracellular pathway such as IL-32. In this study, GM-CSF and IL-32 also increased caspase-1 activation and IL-8 production in EoL-1 cells. Caspase-1 inhibitor reduced GM-CSF- or IL-32-induced caspase-1 activation. Taken together, these results imply that GM-CSF is an important mediator of the inflammation of AR. However, further study is necessary to clarify the reaction mechanism between IL-32 and caspase-1 in GM-CSF-stimulated eosinophils.

In conclusion, our results imply that IL-32 increases significantly in AR and that IL-32 expression is induced via the activation of caspase-1 by GM-CSF in eosinophils. The involvement of IL-32 in the pathogenic mechanisms causing human AR remains to be demonstrated. However, given that the level of IL-32 expression correlated with clinical and histological markers of inflammation, as well as the presence of cytokines known to be important in the pathogenesis of AR, IL-32 plays an important role in the amplification of inflammatory reactions. It is therefore tempting to speculate that inhibitors of IL-32 could be useful for attenuating inflammation-related clinical symptoms. However, in designing such clinical interventions for blocking IL-32, one should take into consideration not only the advantageous effects, but also the risk of potential deleterious consequences for the host. Therefore, further investigation is necessary to determine other possible mechanisms of IL-32 expression and activation prior to clinical use in AR patients.

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Abbreviations

AR	allergic rhinitis
ECP	eosinophil cationic protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL	interleukin
MAST-CLA	multiple allergosorbent chemiluminescence assay
NOD	nucleotide oligomerization domain
TLR	Toll-like receptor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

Author contribution statement

HJJ performed the majority of the experiments and wrote the manuscript. SYS performed sample analysis. HAO performed immunohistochemistry. MHK and JSC provided comments on the manuscript. HMK supervised the research and co-wrote the manuscript.

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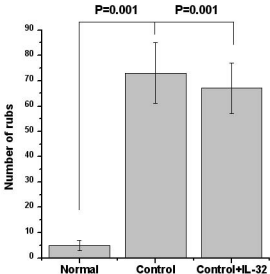
SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary methods. Patients.

Table S1. Baseline characteristics for the normal and AR groups.

Figure S1. Clinical symptoms in the AR animal models.



Supplementary Figure 1

Supplementary Table 1. Baseline characteristics for the normal and AR groups

Sample	Group	Sex profile	Age (y), Mean±SD
Adenoid (n=80)	Normal (n = 40)	26 <i>M</i> , 14 <i>F</i>	6.3±2.5
	AR (n = 40)	31 <i>M</i> , 9 <i>F</i>	7.4±3.1
Nasal mucosa (n=50)	Normal (n = 25)	13 <i>M</i> , 12 <i>F</i>	38.8±16.8
	AR (n = 25)	19 <i>M</i> , 6 <i>F</i>	34.2±15.0

F, Female; *M*, Male

Sensitivity to allergens was assessed by detection of serum specific IgE.

Supporting information

Supplementary methods

Patients

Sixty five patients participated in this study (Supplementary Table 1). All subjects were examined at the Ears, Nose, and Throat (ENT) Department of the Kyunghee University Hospital in Seoul, Korea between of June 2007 and of July 2007. The local ethics committee approved the study, and informed consent was obtained. Baseline characteristics for normal and the AR group are shown in Supplementary Table 1. All patients were referred to the ENT department out of their need for an operation. Before surgery, all patients underwent a routine ENT examination and a skin prick test (SPT), which included eight common inhalant allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, cat fur, dog fur, tree mixture, grass mixture, mugwort, and ragweed; Allergopharma, Hamburg, Germany). The result of each test was reported as the A/H ratio (the mean weal diameter for the allergen compared with that for histamine). An A/H ratio >2 to the common inhalant allergens on the SPT was regarded as a positive response. Blood samples obtained from patients during the operation were used for a multiple allergosorbent chemiluminescence assay (MAST-CLA) (Hitachi Chemical Diagnostics Inc., CA, USA), to determine the serum total IgE concentration, and to count eosinophils. For the MAST-CLA, antigens attached to threads were sequentially incubated with patient serum, enzyme-absorbed anti-IgE, and a photoresponse reagent. The chemical fluorescence generated by each was developed on Polaroid film and scored according to a five-point system, from 0 to 4. All reactions of grade 2 or higher were scored as positive. All patients with allergic rhinitis had typical symptoms of perennial nasal allergy, nasal eosinophilia, and a positive skin test or positive MAST-CLA result to house dust mites. Patients with allergy and exacerbation of upper respiratory

infection during the 6 weeks preceding the study were not included in the study. Other exclusion criteria included current smoking; a medical history of recurrent infections of the respiratory tract; and use of topical or oral corticosteroids, leukotriene modifiers, or antihistamines during the 8 weeks that preceded the study. The adenoid tissues were obtained from children patients admitted for an adenotonsillectomy due to recurrent infections, such as pharyngotonsillitis, rhinosinusitis, adenitis, and otitis media or problems owing to adenotonsillar hypertrophy, such as upper airway obstruction, sleep disorder, abnormal dentofacial growth, and dysphagia. The normal nasal mucosa was obtained from the inferior turbinates of healthy control subjects who had normal nasal mucosa and who were admitted for augmentation rhinoplasty. Allergic nasal mucosa was obtained from patients with severe persistent allergic rhinitis during septoturbinate surgery; their turbinate mucosa showed an edematous and congested appearance. These patients had been previously treated with medical management (antihistamine and intranasal steroid spray) without satisfactory improvement for at least 3 months. They had 1 or more of the following symptoms: sleep disturbance; impairment of daily activities, leisure and/or sports; and impairment of school or work. All tissue samples were divided into 2 portions. One portion was frozen in liquid nitrogen and stored at -70°C for protein isolation. For immunohistochemistry, another sample was also frozen in liquid nitrogen and stored at -70°C for a subsequent procedure.

Supporting information

Supplementary figure legend

Supplementary Figure 1. Clinical symptoms in the AR animal models. The number of the nasal rubs that occurred in the 10 min after the OVA intranasal provocation. Normal, OVA-unsensitized mice ($n = 10$); Control, OVA-sensitized mice ($n = 10$); Control + IL-32, OVA-sensitization + 100 ng IL-32-treated mice ($n = 10$).