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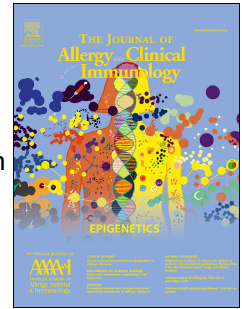
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Interleukin-32 γ suppressed atopic dermatitis through inhibition of miR-205 expression via inactivation of nuclear factor-kappa B

Yong Sun Lee, MSc^{1*}, Sang-Bae Han, PhD^{1*}, Hyeon Joo Ham, BSc¹, Ju Ho Park, MSc¹, Jong Sung Lee, BSc¹, Dae Yeon Hwang, PhD², Young Suk Jung, PhD³, Do Young Yoon, PhD^{4,#} and Jin Tae Hong, PhD^{1,#}

¹College of Pharmacy and Medical Research Center, Chungbuk National University, Osongsaengmyeong 1-ro 194-21, Osong-eup, Heungduk-gu, Cheongju, Chungbuk, 28160, Republic of Korea

²Department of Biomaterial Science, College of Natural Resources and Life Science/Life and Industry Convergence Research Institute, Pusan National University, Miryang 50463, Republic of Korea

³College of Pharmacy, Pusan National University, Busandaehag-ro, Geumjeong-gu, Busan 46241, Republic of Korea

⁴Department of Bioscience and Biotechnology, Konkuk University, Gwangjin-gu, Seoul 05029, Republic of Korea

* The authors are equally contributed.

#Correspondence: Dr. Jin Tae Hong (jinthong@chungbuk.ac.kr), College of Pharmacy and Medical Research Center, Chungbuk National University, Osongsaengmyeong 1-ro, Osong-eup, Heungdeok-gu, Cheongju, Chungbuk, 28160, Korea, Tel: +82-42-261-2813, Fax: +82-42-268-2732; Dr. Do Young Yoon (ydy4218@konkuk.ac.kr), Department of Bioscience and Biotechnology, Konkuk University, Gwangjin-gu, Seoul 05029, Republic of Korea

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ABSTRACT

Background: Interleukin 32 (IL-32) is a novel cytokine involved in many inflammatory diseases. However, the role of IL-32 γ , an isotype of IL-32, in atopic dermatitis has not been reported.

Objective: We investigated the effects of IL-32 γ on the atopic dermatitis development and its action mechanisms.

Methods: We used phthalic anhydride (PA) and MC903-induced atopic dermatitis model using wild type and IL-32 γ transgenic mice. We conducted the therapy experiments using recombinant IL-32 γ protein in reconstructed human skin model and PA-induced model. We conducted the receiver operating characteristic analysis of IL-32 γ with new atopic dermatitis biomarkers, IL-31 and IL-33, in serum from atopic dermatitis patients.

Results: Dermatitis severity and epidermal thickness were significantly reduced in PA and MC903-induced IL-32 γ transgenic mice compared to wild type mice. The concentration of AD-related cytokines was reduced in PA and MC903-induced IL-32 γ transgenic mice compared to wild type mice. Subsequent analysis showed that IL-32 γ inhibits miR-205 expression in PA and MC903-induced skin tissues and TNF- α /IFN- γ -treated HaCaT cells. IL-32 γ reduced NF- κ B activity in skin tissues from PA and MC903-induced mice and TNF- α /IFN- γ -treated HaCaT cells. NF- κ B inhibitor treatment with IL-32 γ expression further suppressed expression of inflammatory mediators as well as miR-205 in TNF- α /IFN- γ -treated HaCaT cells. Furthermore, recombinant IL-32 γ protein alleviated atopic dermatitis-like inflammation in *in vivo* and reconstructed human skin model. Spearman's correlation analysis showed that serum levels of IL-32 γ and miR-205 were significantly concordant in patients with atopic dermatitis.

Conclusion: Our results indicate that IL-32 γ reduces atopic dermatitis through the inhibition of miR-205 expression via inactivation of NF- κ B.

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90 CAPSULE SUMMARY

91 Our results demonstrate that IL-32 γ can suppress the development of atopic
92 dermatitis by prevention of pro-inflammatory cytokine release through inhibition of
93 NF- κ B-mediated miR-205 expression. IL-32 γ could be useful for treatment of
94 atopic dermatitis.

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96 CLINICAL IMPLICATIONS

97 This study provides the new insight of IL-32 γ as a potential therapeutic molecule for
98 treatment of atopic dermatitis.

99

100 Key words

101 Atopic dermatitis; IL-32 γ ; miR-205; NF- κ B

102

103 Abbreviations used

104 **AD:** atopic dermatitis

105 **AUC:** area under the curve

106 **CCL:** chemokine (C-C motif) ligand

107 **IFN:** interferon

108 **IHC:** immunohistochemistry

109 **IL:** interleukin

110 **IgE:** immunoglobulin E

111 **I κ B α :** nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,
112 alpha

113 **miRNA:** microRNA

114 **NF- κ B:** nuclear factor-kappa B

115 **PA:** phthalic anhydride

116 **qPCR:** quantitative real time polymerase chain reaction

117 **RHS:** reconstructed human skin
118 **ROC:** receiver operating characteristic
119 **Tg:** transgenic
120 **TNF:** tumor necrosis factor
121 **TSLP:** thymic stromal lymphopoietin
122 **WT:** wild type

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142 **INTRODUCTION**

143 Atopic dermatitis (AD) is a chronic inflammatory skin disease, which is
144 characterized by complex pathophysiology accompanied by an itchy and scaly rash ¹.
145 AD is induced by an impairment of the skin-barrier function, dysfunctional immune
146 system, genetic, and environmental risk factors ². AD pathogenesis consists of acute
147 and chronic phases ³. Acute phase of AD is mediated by T helper type 2 (Th2) cells,
148 which produce various cytokines such as IL-4, IL-5, and IL-13 in lesioned skin of AD
149 ⁴. These cytokines play a critical role in the promotion of IgE synthesis to initiate
150 immunoglobulin E (IgE)-mediated allergic inflammation by activating mast cells and
151 eosinophils ^{4, 5}. In addition, Th2 cytokines directly impact epidermal keratinocytes,
152 which produce pro-inflammatory cytokines that induce an inflammatory response
153 through infiltration of immune cells into the skin lesions. Keratinocytes in the skin are
154 the crucial providers or initiators of AD ⁶. Activated keratinocytes express various
155 pro-inflammatory cytokines and chemokines such as thymic stromal lymphopoietin
156 (TSLP), tumor necrosis factor (TNF)- α , IL-1, IL-6, IL-25, IL-33, CCL17, and CCL22,
157 which contribute in infiltration of inflammatory cells and allergic inflammation in AD
158 pathogenesis^{3, 7, 8}. After acute phase, chronic phase of AD is mediated by Th1 cells.
159 Infiltrated and accumulated eosinophils and macrophages actively secrete IL-12,
160 which stimulates the accumulation and development of IFN- γ expressing Th1 cells in
161 the lesion ⁴. The increased level of IFN- γ in the lesion exacerbates AD by stimulating
162 the homing of immune cells and inducing spongiosis of the skin epidermis.

163 IL-32 γ was cloned as a gene induced by IL-18 and was formerly known as natural
164 killer (NK) cell transcript 4 ⁹. IL-32 has six splice variants, IL-32 α , IL-32 β , IL-32 γ , IL-
165 32 δ , IL-32 ϵ and IL-32 ζ , but the functional differences between these isoforms remain
166 unknown ^{10, 11}. IL-32 modulates the generation of TNF- α , IL-1 β , IL-6 and IL-10 in
167 inflammatory diseases ¹². It also has been implicated in many inflammatory disorders,
168 and its expression was correlated with disease severity in patients with Crohn's
169 disease, rheumatoid arthritis, colitis, inflammatory bowel disease and chronic
170 obstructive pulmonary disease ¹³⁻¹⁵. It is reported that IL-32 contributes to AD
171 pathogenesis by inducing keratinocyte apoptosis ¹⁶. In addition, serum levels of IL-32
172 were increased in patients with AD compared to controls ¹⁶. Our previous study has

shown that IL-32 γ inhibits the expression of TNF- α and IL-1 β via inactivation of NF- κ B signaling in colon cancer¹⁷. In addition, IL-32 β reduces expression of TNF- α , IL-1 β and IL-6 in tumor and spleen tissues of mouse xenograft model¹⁸. It is also reported that IL-32 γ acts as an anti-inflammatory cytokine that inhibits TPA-induced skin inflammation¹⁹. However, the direct roles of IL-32 γ in AD pathogenesis have not been studied.

It is known that miRNAs are involved in the pathogenesis of many diseases including atopic dermatitis²⁰⁻²². miR-155 may contribute in development of atopic dermatitis through upregulation of T cell activation²³. It is also reported that miR-29b promoted keratinocyte apoptosis via suppression of anti-apoptotic protein BCL2L2 in AD²⁴. Recent studies revealed that miR-302e attenuated release of inflammatory cytokines such as IL-1 β , IL-6, TNF- α and TSLP, in HMC-1 human mast cell line²⁵. Previous studies demonstrated that NF- κ B plays a critical role in development of atopic dermatitis²⁶. NF- κ B inhibitors adequately inhibit allergic inflammation including inflammatory cell infiltration and AD-related cytokine release in NC/Nga mice AD model^{27, 28}. In addition, miR-146a inhibits NF- κ B signaling to prevent inflammatory responses in keratinocytes and atopic dermatitis²⁹. Yang *et al* reported that miR-124 suppresses chronic skin inflammation in AD via inhibition of NF- κ B signaling in keratinocytes³⁰. Further, miR-205 is upregulated in serum and urine of AD patients compared to healthy controls and IL-32 α suppresses miR-205 biogenesis to inhibit endothelial cell inflammation^{21, 31}. miR-205 is increased due to activation of NF- κ B signaling in lung cancer³², and it is regulated by NF- κ B signaling in breast cancer³³.

In this study, to investigate whether IL-32 γ inhibits AD pathogenesis via suppression of miR-205 through inactivation of NF- κ B. We compared AD development in WT and IL-32 Tg mice by two type of chemicals induced mode, and analysis the relationship between IL-32 γ level and atopic dermatitis patients. Our data provide the significant suppressive effect of IL-32 γ in the development of AD.

METHODS

Cell culture and transfection

HaCaT cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) in humidified atmosphere of 5% CO₂. HaCaT cells were transfected with pcDNA3.1(+)-6xMyc-IL-32γ vector or control vector using the Lipofectamine[®] 3000 transfection reagent in OPTI-MEM, according to the manufacturer's specifications (Invitrogen, Waltham, MA, USA). For miR-205 transfection, HaCaT cells were transfected with miR-205 mimic, miR-205 inhibitor or its controls (Qiagen GmbH, Hilden, Germany) using Lipofectamine[®] RNAiMAX reagent in OPTI-MEM, according to the manufacturer's specifications (Invitrogen, Waltham, MA, USA). Transfected cells were stimulated with TNF-α/IFN-γ (20 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA) for indicated time points.

Animals

The experimental protocols were carried out according to the guidelines for animal experiments of the Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Research Center at Chungbuk National University, Korea (Ethic approval No. CBNUA-1146-18-01). All efforts were made to minimize animal suffering, and to reduce the number of animals used. All mice were housed in four mice cage with automatic temperature control (21-25 °C), relative humidity (45-65%), and 12 h light-dark cycle. Food and water were provided *ad libitum*. Wild type (WT, C57BL/6) mice were purchased from DBL (Eumsung, Korea). Generation of hIL-32γ transgenic (Tg) mice has been described in the previous study¹⁷. In brief, a 705-base pair fragment of the hIL-32γ gene was sub-cloned into the EcoRI sites of the pCAGGS expression vector. IL-32γ insertion was confirmed by amplification of genomic DNA isolated from the tails of transgenic mice using WizPure[™] Taq DNA polymerase kit (Wizbiosolutions, Seongnam, Korea) and the following specific primer set: sense, 5'-GAAGGTCCTCTCTGATGACA-3' and antisense, 5'-GAAGAGGGACAGCTATGACTG-3'. Genomic DNA samples were extracted from

tails of transgenic mice and PCR analysis was performed for IL-32 γ gene expression. WT mice do not express IL-32 γ . IL-32 γ Tg mice have no overt phenotype compared with WT mice. IL-32 γ Tg mice were viable, fertile, and had no tissue or organ abnormalities.

PA- or MC903-induced AD model

Age-matched male mice (8-weeks old) were used in all of the experiments. To induce AD skin, PA or MC903 (Vitamin D3 analog) were used. PA-induced AD model were developed as previously described³⁴. A 5% PA solution or AOO (vehicle, acetone olive=4:1) was applied to the dorsal skin 3 times a week for 4 weeks. MC903-induced AD model was developed as previously described³⁵. MC903 solution (45 μ M) or ethanol was applied on dorsal skin for 17 days (5 days treatment, 2 days interruption, 5 days treatment, 2 days interruption, 2 days treatment). Clinical score was evaluated as none (0), mild (1), moderate (2) or severe (3) according to the AD symptoms. At the end of the study period, blood specimens were collected.

AD-like reconstructed human skin model

A reconstructed human skin model (RHS model) was purchased from TEGO Science Inc. (Neoderm®-ED, Seoul, Korea). RHS model contains epidermis and dermis. The RHS model generated AD-like inflammation using an inflammatory cocktail as previously described method with some modifications³⁶⁻³⁸. In brief, an inflammatory cocktail (AD cocktail; IL-4: 30 ng/mL; IL-13: 30 ng/mL; TNF- α : 3.5 ng/mL; PeproTech Inc.) was supplemented with or without recombinant human IL-32 γ (100 ng/mL; YbdY Biotech, Seoul, Korea) to the culture medium for 6 days. Culture medium was changed every 48 h.

Immunohistochemistry (IHC)

Skin tissues and RHS tissues were removed, fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then sectioned into 5 μ m thick slices.

Hematoxylin and eosin (H&E) staining and immunohistochemistry were performed as described previously³⁹. The slides were incubated with the following specific primary antibodies: F4/80 (Santa Cruz Biotechnology, CA, USA); Arginase-1 (Cell Signaling, Beverly, MA, USA); CD86 (GeneTex, Taiwan, R.O.C); Ly6G (Invitrogen, Waltham, MA, USA); p-p65 and FOXP3 (Abcam, Cambridge, MA, USA). Toluidine blue was used for mast cell staining. Detail information was described in Table E1.

Enzyme-linked immunosorbent assay (ELISA) assay

Cytokines and IgE levels were measured by ELISA kits provided by KOMA Biotech (Seoul, Korea), R&D systems (Minneapolis, MN, USA) and Abcam (Cambridge, MA, USA) following the manufacturer's protocol. Human serum samples were analyzed using IL-31, IL-32 β , IL-32 γ and IL-33 ELISA kits purchased from myBioSource (IL-32 β and IL-32 γ ; San Diego, CA, USA) and KOMA Biotech (IL-31 and IL-33; Seoul, Korea) following the manufacturer's instructions. Detail information was described in Table E1.

Western blot analysis

Western blot analysis was performed as described previously¹⁹. The membranes were incubated with the following specific primary antibodies: IL-32 (BioLegend, San Diego, CA, USA); p50, Histone H1 and β -actin (Santa Cruz Biotechnology, CA, USA), iNOS, COX-2 and p65 (Abcam, Cambridge, MA, USA), p-I κ B α and I κ B α (Cell Signaling, Beverly, MA, USA). Detail information was described in Table E1.

Quantitative real-time PCR (qPCR)

Total RNA from skin tissues, RHS tissues and cells was extracted by Ribo^{EX} RNA Extraction Kit (GeneAll Biotechnology, Seoul, Korea) and cDNA was synthesized using High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using specific primers in a StepOnePlusTM PCR System (Applied Biosystems, Foster City, CA, USA) (see Table

E2). The values obtained for the target gene expression were normalized to 18S and quantified relative to the expression in control samples. For miRNA experiment, miRNA of skin tissues and cells was extracted by miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) and miRNA of serum was extracted by miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany). cDNA was generated with the miScript II RT Kit (Qiagen GmbH, Hilden, Germany). Pre-mature and mature miR-205 primers were purchased from Qiagen. Rnu6B was used as an internal control for skin tissues and cells. miR-39 (*C. elegans*) was used as an internal control for serum miRNA analysis.

Luciferase assay

HaCaT cells were transiently co-transfected with IL-32 γ plasmid vector and luciferase-expressing NF- κ B plasmid vector (Promega; Madison, WI, USA) for 24 h. The transfected cells were treated with TNF- α /IFN- γ (20 ng/mL) for indicated time points. Luciferase activity was measured using a luciferase assay kit (Promega; Madison, WI, USA) following the manufacturer's instructions.

Human samples

Human serum samples from AD patients and normal controls (20 samples, respectively) were obtained from Chungbuk National University Hospital and Korea Institute of Radiological & Medical Sciences (KIRAMS) Radiation Biobank. All studies using human samples were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of Chungbuk National University Medical Center (IRB No. CBNU-201902-BR-786-01).

Gene-disease/miRNA-gene network analyses

The gene-disease network of IL-32 was analyzed using Disease-Connect (www.disease-connect.org)⁴⁰. Web server analyzed gene-disease network based on various sources, genome-wide association studies (GWAS), online mendelian inheritance in man (OMIM), GeneRIF and GeneWays. The strength of the

connections between an input gene and diseases is quantified as the p-value of a hypergeometric enrichment test. The miRNA-gene network of miR-205 and RELA (p65) were analyzed using ONCO.IO (<https://onco.io>). ONCO.IO database tool is based on the manually curated annotation of experimentally verified molecular interactions described in the literatures.

Statistical analysis

All experiments were conducted in triplicates, and were repeated at least three times with similar results. Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Group differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Spearman's test was used to analyze the correlation between IL-31, IL-32 γ , IL-33 or miR-205 in patient samples. All values were presented as mean + S.D. Significance was set at $p < 0.05$ for all tests.

RESULTS

IL-32 γ suppresses experimentally induced AD pathogenesis

IL-32 has been known to have a pro-inflammatory or an anti-inflammatory role in various diseases¹³. Using gene-disease relation analysis, we found that IL-32 is closely associated with many skin diseases including atopic dermatitis (Fig 1, A). To explore the relationship between IL-32 γ and atopic dermatitis, we evaluated the role of IL-32 γ on experimentally induced AD pathogenesis by treatment with PA. PA-treated WT mice showed symptoms indicative of erythema, edema and erosion in dorsal skin. These changes were impeded in PA-induced IL-32 γ Tg mice (Fig 1, B). To investigate the suppressive effect of IL-32 γ on AD, histological analysis of the dorsal skin was performed (Fig 1, B). Clinical score and epidermal thickness were also significantly reduced in the PA-induced IL-32 γ Tg mice compared to PA-induced WT mice (Fig 1, C). We determined mRNA expression and protein level of IL-32 γ in PA-induced skin tissues. The results showed that mRNA expression and protein level of IL-32 γ were increased in PA-induced skin tissues from IL-32 γ Tg mice (Fig 1, D). To confirm the role of IL-32 γ , we further developed the vitamin D3 analog MC903-induced experimental AD model. AD symptoms were reduced in MC903-induced IL-32 γ Tg mice compared to MC903-induced WT mice (see Fig E1, A). Morphological and histological analyses of AD lesions from skin showed reduction of hyperkeratosis, parakeratosis, and acanthosis, along with the reduction of the infiltration of dermal inflammatory cells in MC903-induced IL-32 γ Tg mice compared to MC903-induced WT mice (see Fig E1, A). In addition, clinical score, epidermal thickness and the number of mast cells (see Fig E1, B) as well as serum IgE level (see Fig E1, D) were significantly reduced in MC903-induced IL-32 γ Tg mice compared to MC903-induced WT mice. These results thus showed that IL-32 γ could suppress experimental AD pathogenesis.

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377 **IL-32 γ reduces inflammatory responses**

378 To determine if IL-32 γ could reduce allergic inflammation in PA-induced AD skin
379 tissues, we primarily evaluated the changes of auricular lymph node weight. Lymph
380 node weight of PA-induced IL-32 γ Tg mice was significantly lower than PA-induced
381 WT mice (see Fig E2, A). In addition, expression levels of inflammation markers,
382 COX-2 and iNOS, were significantly reduced in skin tissues of PA-induced IL-32 γ Tg
383 mice compared to PA-induced WT mice (see Fig E2, B). Previous studies indicated
384 that inflammatory cells mediate atopic dermatitis. We thus investigated the infiltration
385 of inflammatory cells in AD skin tissues. We found a decreased infiltration of F4/80+
386 macrophages and Arginase-1+ macrophages in PA-induced IL-32 γ Tg mice
387 compared with PA-induced WT mice (see Fig E3, A and B). But, CD86+
388 macrophages were not changed in both unstimulated and PA-induced skin tissues
389 (see Fig E3, A and B). Furthermore, Ly6G+ neutrophils and mast cells were also less
390 infiltration in the skin tissues of PA-induced IL-32 γ Tg mice compared to PA-induced
391 WT mice (see Fig E3, A and B). Previous research indicated that FOXP3+ regulatory
392 T (Treg) cells control atopic dermatitis by suppression of inflammatory cell
393 recruitment in OVA-induced atopic dermatitis mouse model ⁴¹. We thus analyzed
394 whether IL-32 γ controls skin inflammation by FOXP3+ Treg cells. Increased number
395 of FOXP3+ cells were accumulated in the skin tissues of PA-induced IL-32 γ Tg mice
396 compared to PA-induced WT mice (see Fig E3, A and B). In addition, we further
397 analyzed the mRNA levels of eosinophil and type 2 innate lymphoid cell (ILC2)
398 markers. The results showed that the expression of eosinophil (Siglec-F, Epx and
399 Prg2) and ILC2 (Sca-1, CD25 and KLRG1) markers were decreased in PA-induced
400 IL-32 γ Tg mice compared with PA-induced WT mice (see Fig E4, A and B). Th2-
401 related cytokines are critical factors for AD pathogenesis ⁷. We analyzed Th2-related
402 cytokines in PA-induced skin tissues. ELISA analyses showed that the levels of Th2-
403 related cytokines, including IL-4, IL-5, IL-13, IL-31, IL-33, TSLP, were reduced in skin
404 tissues from PA-induced IL-32 γ Tg mice compared to PA-induced WT mice (Fig 2, A).
405 qPCR analyses also showed that mRNA levels of Th2-related cytokines were
406 significantly reduced in skin tissues from PA-induced IL-32 γ Tg mice compared to
407 PA-induced WT mice (see Fig E5, A). The protein levels and mRNA expression of

Th1 and Th17 cytokines (TNF- α , IL-6, IL-1 β and IL-17) were also markedly reduced in skin tissues from PA-induced IL-32 γ Tg mice compared to PA-induced WT mice (Fig 2, B and see Fig E5, B). Furthermore, serum levels of IL-4, IL-13, TNF- α , and IgE were also reduced in PA-induced IL-32 γ Tg mice compared to PA-induced WT mice (Fig 2, C and D). Like the PA-induced model, the protein levels of TNF- α , IL-4, IL-13, IL-31, IL-33 and TSLP in skin tissues were also significantly reduced from MC903-induced IL-32 γ Tg mice compared to MC903-induced WT mice (see Fig E1, C). The mRNA levels of TNF- α , IL-4, IL-13, IL-31, IL-33 and TSLP in skin tissues were also decreased from MC903-induced IL-32 γ Tg mice compared to MC903-induced WT mice (see Fig E5, C). Previous studies indicated that IL-32 γ contributed the IL-10 production^{17, 19}. Thus, we analyzed the IL-10 levels in PA-induced skin tissues. The concentration of IL-10 at basal level were higher in skin tissues from IL-32 γ Tg mice compared to WT mice. In AD condition, the concentration of IL-10 was decreased in both WT and IL-32 γ Tg mice compared to unstimulated condition (Fig 2, E).

IL-32 γ inhibits miR-205 expression

Our previous study has revealed that IL-32 α suppressed endothelial cell inflammation by inhibition of miR-205 expression³¹. Serum and urine levels of miR-205 are significantly upregulated in children with AD compared with the healthy controls⁴². We therefore analyzed whether IL-32 γ could suppress atopic dermatitis through regulation of miR-205 expression. miR-205 expression was upregulated in PA and MC903-induced skin tissues from WT mice compared to non-treated WT mice, and significantly decreased in PA and MC903-induced skin tissues from IL-32 γ Tg mice (Fig 3, A and see Fig E1, E). We further identified that IL-32 γ suppressed TNF- α /IFN- γ -induced miR-205 expression in HaCaT cells (Fig 3, B). miRNA processing is regulated by Drosha, DGCR8 (Pasha) and Dicer1, which are miRNA biogenesis enzymes. We confirmed that IL-32 γ inhibited miR-205 expression by suppression of DGCR8 and Dicer1 in *in vivo* (Fig 3, C). To further investigate the role of miR-205 in inflammation, we inhibited miR-205 in TNF- α /IFN- γ -stimulated keratinocytes and analyzed the expression of pro-inflammatory mediators. The results showed that inhibition of miR-205 significantly reduced the mRNA expression

of TNF- α , IL-1 β , IL-6 and TSLP in TNF- α /IFN- γ -induced HaCaT cells (Fig 3, D). Moreover, miR-205 mimic treatment synergistically induced these pro-inflammatory cytokines in TNF- α /IFN- γ -induced HaCaT cells (Fig 3, D). However, TNF- α /IFN- γ -induced IL-32 γ expression was not altered by miR-205 mimic or inhibitor themselves. These results suggested that IL-32 γ inhibited AD-like skin inflammation via suppression of miR-205 expression.

IL-32 γ suppresses activation of NF- κ B in PA-induced skin tissues and keratinocytes

NF- κ B signaling has been known to play a central role in inflammation and inhibition of NF- κ B activity suppresses AD pathogenesis²⁶⁻²⁸. Previous studies indicated that miR-205 was regulated by NF- κ B signaling in lung and breast cancers^{32, 33}. Also, web-based miRNA-gene interaction network analysis showed that miR-205 is a downstream target of RELA (p65) (see Fig E6, A and B). To determine whether IL-32 γ inhibits AD pathogenesis via suppression of NF- κ B dependent miR-205 expression, we analyzed the NF- κ B signaling by Western blot and IHC analysis in PA-induced skin tissues. IL-32 γ inhibited nuclear translocation of p50 and p65 and activation of I κ B α in PA-induced skin tissues (Fig 4, A and B). MC903-induced NF- κ B activation was also suppressed in IL-32 γ Tg mice (see Fig E7). We found that phosphorylation of I κ B α and nuclear translocation of p50 and p65 were inhibited in TNF- α /IFN- γ -induced IL-32 γ overexpressed HaCaT cells compared with TNF- α /IFN- γ -induced HaCaT cells (Fig 4, C). We examined the effect of IL-32 γ on TNF- α /IFN- γ -induced NF- κ B transcription activity. IL-32 γ expression resulted in the inhibition of NF- κ B luciferase activity induced by TNF- α /IFN- γ treatment (Fig 4, D). NF- κ B inhibitor Bay 11-7082 (BAY) treatment alleviated the expression of inflammatory factors and miR-205 in TNF- α /IFN- γ -induced HaCaT cells (Fig 4, E and F). In addition, IL-32 γ showed a synergistic effect with BAY to inhibit inflammation and miR-205 expression (Fig 4, E and F). These data indicated that inhibition of NF- κ B is implicated in IL-32 γ -mediated inhibition of AD pathogenesis by suppression of miR-205 expression.

Knockdown of IL-32 increases skin inflammation in keratinocytes

We showed that IL-32 γ has anti-inflammatory effect on AD-like skin inflammation in AD mouse model and keratinocytes. We tested whether knockdown of IL-32 enhances skin inflammation in keratinocytes. The results showed that inhibition of IL-32 by siRNA increased activation of NF- κ B in TNF- α /IFN- γ -induced HaCaT cells (see Fig E8, A). In addition, mRNA expression of inflammatory cytokines, TNF- α , IL-1 β , IL-6 and TSLP, was much higher in TNF- α /IFN- γ -induced IL-32 knockdown HaCaT cells compared to control cells (see Fig E8, B). Furthermore, miR-205 expression was significantly increased in TNF- α /IFN- γ -induced IL-32 knockdown HaCaT cells compared to control cells (see Fig E8, C). Further analysis indicated that miR-205 mimic transfection in TNF- α /IFN- γ -treated IL-32 γ overexpressed HaCaT cells showed decreased miR-205 expression (see Fig E8, D).

IL-32 γ attenuates the inflammation in AD-like reconstructed human skin model

To confirm the anti-inflammatory effect of IL-32 γ on AD development, we used AD-like reconstructed human skin model (AD-RHS model). We found that rhIL-32 γ treatment significantly inhibited epidermal thickness in AD-RHS model (Fig 5, A). AD cocktail-induced NF- κ B activation was also reduced in rhIL-32 γ -treated RHS tissues (Fig 5, B). Increased expression of miR-205 and AD-related cytokines, TNF- α , IL-1 β , IL-6, TSLP and IL-32 γ , were suppressed by rhIL-32 γ treatment (Fig 5, C and D).

Recombinant IL-32 γ protein treatment alleviates AD pathogenesis in PA-induced experimental AD model

We examined the therapeutic effect of IL-32 γ in PA-induced experimental AD model. The administration of recombinant human IL-32 γ protein (rhIL-32 γ) reduced PA-mediated AD development (Fig 6, A). Histological analysis showed that rhIL-32 γ treatment was reduced dermal infiltration of mast cells and epidermal thickness (Fig 6, A). The protein levels of AD-related cytokines including TNF- α , TSLP, IL-4, IL-13, IL-31 and IL-33 in skin tissues and serum IgE levels were significantly reduced by rhIL-32 γ treatment (Fig 6, B and C). Moreover, the treatment of rhIL-32 γ reduced the mRNA levels of AD-related cytokines were also decreased from PA-induced AD skin tissues (see Fig E5, D). We also found that administration of rhIL-32 γ inhibited miR-

205 expression and NF- κ B activity in PA-induced mice (Fig 6, D and E).

Clinically correlation of IL-32 γ and miR-205 in human atopic dermatitis

In this study, the expression of IL-32 γ resulted in inhibition of AD pathogenesis by suppression of miR-205 expression through the regulation of NF- κ B signaling. We analyzed the levels of IL-32 γ , miR-205 as well as recent discovered AD biomarkers, IL-31 and IL-33, in serum of AD patients using ELISA analysis. Serum levels of IL-32 γ , IL-33 and miR-205 were significantly increased in AD patients compared to those in healthy controls (Fig 7, A). However, serum IL-31 level was not different between AD patients and healthy controls (Fig 7, A). The serum level of IL-32 γ was 85.07 $\pm$ 21.14 pg/mL in healthy controls and 116.14 $\pm$ 32.62 pg/mL in AD patients, respectively. The serum level of IL-33 was 1056.856 $\pm$ 1244.015 pg/mL in healthy controls and 2080.446 $\pm$ 1485.41 pg/mL in AD patients, respectively. The expression level of miR-205 was 0.00029 $\pm$ 0.000133 in healthy controls and 0.000457 $\pm$ 0.000265 in AD patients, respectively. We further analyzed receiver operating characteristic (ROC) analysis for the diagnostic reference. The AD diagnosis cut-off value, sensitivity, specificity and area under the curve (AUC) of IL-31 were 1761, 60%, 60% and 0.5975, respectively (Fig 7, A). The AD diagnosis cut-off value, sensitivity, specificity and AUC of IL-32 γ were 88.45, 85%, 85% and 0.8850, respectively (Fig 7, A). The AD diagnosis cut-off value, sensitivity, specificity and AUC of IL-33 were 1524, 65%, 65% and 0.7275, respectively (Fig 7, A). The AD diagnosis cut-off value, sensitivity, specificity and AUC of miR-205 were 0.000344, 60%, 60% and 0.72, respectively (Fig 7, A). Thus, ROC analysis indicated that IL-32 γ is most significant and a good diagnostic reference for AD. The Spearman's correlation test was used to evaluate the correlation between and IL-31, IL-32 γ and IL-33 for miR-205 in AD patients. We found that serum miR-205 correlated with serum IL-32 γ ($r=0.5383$; $p=0.0143$; Fig 7, B). However, serum IL-31 and IL-33 were not correlated with serum miR-205 ($r=0.1579$; $p=0.5061$ for IL-31-miR-205; $r=0.2586$; $p=0.2709$ for IL-33-miR-205; Fig 7, B). Previous study indicated that IL-32 β is predominant isoform under inflammatory conditions⁴³. Thus, we further analyzed the IL-32 β level in AD patients. The results showed that serum IL-32 β level was not different between AD patients and healthy controls (521.26 $\pm$ 182.75 pg/mL in healthy controls and 446.04 $\pm$ 213.94

pg/mL in AD patients; see Fig E9). Moreover, ROC analysis indicated that the AD diagnosis cut-off value, sensitivity, specificity and AUC of IL-32 β were 430, 65%, 65% and 0.6725, respectively. These values are much less than those of IL-32 β (see Fig E9).

DISCUSSION

AD is characterized by inflammation and numerous inflammatory cell infiltration, mainly comprising of Th2 cells, macrophages, neutrophils, mast cells, and eosinophil infiltrates in AD skin lesions, and an increase of epidermal and dermal thickness⁴⁴. In the present report, IL-32 γ mitigated histological changes, such as severe ear thickness, epidermal hyperplasia and infiltration of inflammatory cells. Immunohistochemistry analysis showed accumulation of various inflammatory cells in PA-induced skin tissues, but IL-32 γ overexpression repressed inflammatory cell infiltration in skin tissues. Antigen specific-IgE contributes to the perpetuation of allergic inflammation, potentially through the promotion of IL-4, IL-5 and IL-13 secreting activated Th2 cells. IL-32 γ overexpression also reduced expression of AD-related cytokines, including IL-4, IL-5, IL-13, IL-25, IL-31, IL-33 and TSLP, and serum IgE levels in the skin lesions in AD. Thus, IL-32 γ has inhibitory effect on the development of AD by regulation of cytokine release and inflammatory cell infiltration.

IL-32, originally named NK cell transcript 4 (NK4), is a recently evaluated inflammatory cytokine⁴⁵. Its transcriptions are highly expressed in immune tissues⁴⁶. IL-32 was reported to induce diverse pro-inflammatory cytokines such as TNF- α , IL-1 α , IL-1 β and IL-6 especially in the early phase of the inflammatory process⁴⁷. However, a previous study has demonstrated that IL-32 isoforms have an anti-inflammatory role in various inflammatory diseases¹³. Recent studies revealed that IL-32 γ overexpression inhibited the production of inflammatory cytokines in skin cancer, colon cancer, and liver injury^{17, 19, 48}. In addition, IL-32 γ overexpression increased IL-10 production in normal or inflammation conditions^{14, 19, 49}. IL-10 has an immunosuppressive function to allergic inflammation that regulated Th2-type cytokine production^{50, 51}. Thus, IL-32 γ could in part suppress AD-like inflammation

through an increase of IL-10 expression. On the other hands, previous studies revealed that IL-32 contributed to the pathogenesis of AD through an increase in keratinocyte apoptosis¹⁶. In this study, expression of IL-32 γ was increased in TNF- α /IFN- γ -treated HaCaT cells. Serum levels of IL-32 γ were also elevated in AD patients. Thus, IL-32 γ appears to be involved in inflammatory responses. However, the anti-inflammatory role of IL-32 γ overexpression was proved through experimental AD models. PA-induced AD model demonstrated that the increased expression of IL-32 γ and administration of rhIL-32 γ significantly decreased progression of AD accompanied by reduction of AD-related cytokines and inflammatory cell recruitment. In an *in vitro* study, IL-32 γ overexpression reduced the expression of inflammatory cytokines in response to TNF- α and IFN- γ in HaCaT cells. Therefore, the increase in IL-32 γ is intended to safeguard the inflammatory response rather than to increase it in the AD skin lesion.

Various cytokines are associated with AD pathogenesis. Many studies indicated that Th2 and Th2 promoting cytokines mainly contribute in AD pathogenesis. Recent studies indicated that IL-31 enhances brain-derived natriuretic peptide, and thus plays a central role for itching, release and synthesis of cytokine and chemokine in skin cells⁵². IL-31 overexpressing Tg mice closely resemble AD skin phenotype⁵³. Skin-specific IL-33 expressing Tg mice also closely resemble atopic dermatitis by increased Th2 cytokines and chemokines and activation of ILC2 cells⁵⁴. IL-33 level was higher in skin tissues from AD patients compared with controls⁵⁵. Keratinocytes expressing IL-33 could activate and recruit type 2 cells, such as skin-resident ILC2 cells, mast cells, eosinophils and Th2 cells, including allergic inflammation^{5, 56, 57}. These cells also release IL-31 in skin tissues, and IL-31 enhances inflammatory cytokine production and mediates pruritis⁵⁸. Therefore, newly discovered AD cytokines, IL-31 and IL-33, are important in AD pathogenesis. However, IL-32 γ adequately blocked IL-31 and IL-33 expression and inflammatory cell infiltration in PA- and MC903-induced AD tissues. Our results showed that serum levels of IL-32 γ and IL-33 were elevated in AD patients, but serum level of IL-31 was not significantly changed compared to healthy controls even though previous studies indicated that IL-31 was increased in tissues and serum of AD patients^{52, 59, 60}. ROC analysis with AD patient samples indicated serum IL-32 γ was the most significantly distinguished between AD patients and controls, but serum levels of IL-33 were slightly

distinguished. Additionally, previous study indicated that IL-32 β was the predominant isoform of IL-32 and was dominantly increased by various stimulators⁴³. The serum analysis indicated that IL-32 β level was 6-fold higher than IL-32 γ in healthy control, but IL-32 β level was not much different between healthy controls and AD patients. Moreover, ROC analysis indicated that the AD diagnosis cut-off value, sensitivity, specificity and AUC of IL-32 β were much less than those of IL-32 γ . We also investigated the effect of rhIL-32 α , the effect on the PA-induced AD was found, but the effect was much less than IL-32 γ (data not shown). Thus, unlike to IL-31 and IL-33, IL-32 γ as a predominant IL-32 could prevent the development of AD through reduction of cytokine production and AD-related immune cell infiltration.

MicroRNAs are involved in the regulation of the normal development of the immune system and in the pathogenesis of chronic inflammatory disorders⁶¹. Recently, various miRNAs were found to be dysregulated in AD patients, implying their general role in skin inflammation⁴². It is reported that miR-124 and miR-146a suppress AD pathogenesis through inhibition of proinflammatory cytokine expression in keratinocytes^{29, 30}. Further, miR-302e diminishes allergic inflammation in HMC-1 human mast cells²⁵. Serum and urine levels of miR-205 are significantly upregulated in children with AD compared with the healthy control group⁴². In the present study, we found that miR-205 played a critical role in inflammatory responses and AD pathogenesis. Moreover, previous our study found that IL-32 α suppresses endothelial inflammation via inhibition of miR-205 expression³¹. IL-32 γ suppressed the AD-like skin inflammation in PA and MC903-induced mice by inhibition of miR-205. We also observed that rhIL-32 γ administration alleviated PA-induced AD pathogenesis by inhibition of miR-205 expression as well as allergic inflammatory responses. We further found that IL-32 γ downregulated miR-205 expression by regulating the Dgcr8/Dicer1 axis-dependent miRNA biogenesis pathway. Our results revealed that miR-205 inhibitor suppressed the expression of pro-inflammatory cytokines, but miR-205 mimic increased the expression of pro-inflammatory cytokines in TNF- α /IFN- γ -treated HaCaT cells. Serum analysis also indicated that miR-205 was significantly increased in AD patients. Spearman's correlation analysis showed that serum levels of IL-32 γ and miR-205 were significantly correlated in AD patients. These results indicated that IL-32 γ reduced AD pathogenesis by inhibition of miR-205-mediated inflammatory responses.

NF- κ B is an important factor in regulation of pro-inflammatory cytokines and chemokines in AD²⁸. Reduced NF- κ B activation in CD4 T cells decreases AD-like skin inflammation in NC/Nga mice AD model⁶². Topical application of NF- κ B inhibitor effectively suppresses allergic inflammation such as inflammatory cytokine production and infiltration of inflammatory cells in NC/Nga mice AD model²⁸. We found that IL-32 γ overexpression decreased NF- κ B activity in PA- and MC903-induced skin tissues and TNF- α /IFN- γ -induced HaCaT cells. IL-32 γ overexpression could block the translocation of NF- κ B transcription factor through suppression of I κ B α phosphorylation. NF- κ B signaling is associated with various miRNAs⁶³. miR-146a suppressed chronic skin inflammation by inhibition of NF- κ B signaling through downregulation of caspase recruitment domain-containing protein 10, NF- κ B upstream factor. miR-124 inhibited p65 expression by direct binding to reduce inflammatory responses in human primary keratinocytes³⁰. Previous studies have shown that NF- κ B signaling regulates miR-205 expression that contain NF- κ B binding site³². Web-based miRNA-gene network analysis showed that RELA (p65) regulates miR-205. In *in vivo* and *in vitro* studies revealed IL-32 γ suppressed NF- κ B signaling in PA-induced skin tissues and TNF- α /IFN- γ -induced HaCaT cells. Combination of IL-32 γ and NF- κ B inhibitor, BAY, also synergistically reduced TNF- α /IFN- γ -induced inflammatory cytokine expression by further inhibition of miR-205 expression. These findings suggest that IL-32 γ -mediated NF- κ B inactivation contributed to prevent AD pathogenesis through suppression of miR-205 expression.

Taken together, we demonstrated that unlike IL-31 and IL-33, IL-32 γ suppressed atopic dermatitis via inhibition of miR-205 expression through inactivation of NF- κ B pathway.

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AUTHOR CONTRIBUTIONS

Y.S.L. and S.B.H. conducted most of the experiments, performed data analysis, generated most of the experimental mice and were the primary writer of the manuscript. H.J.H. assisted *in vivo* and *in vitro* experiments. J.H.P assisted all animal experiments. J.S.L. assisted data analysis. D.Y.H. and Y.S.J. provided advice throughout the project. D.Y.Y. and J.T.H. supervised the entire project and had a major role in experimental design, data interpretation, and writing the manuscript.

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FIGURE LEGENDS

FIG 1. IL-32 γ inhibits PA-induced atopic dermatitis. (A) Gene (IL-32)-disease network was analyzed based on the GWAS/OMIM/DEG records ($p<10^{-6}$). (B) The morphological and histological changes in mice after 4-week treatment as described in the Materials and Methods. The photographs are representative of each group of mice. Scale bar, 50 μ m. (C) Bar graphs indicate clinical score and epidermal thickness. $n=7$. (D) mRNA expression and protein level of IL-32 γ in skin tissues. $n=5$. ** $p<0.01$ and *** $p<0.001$.

FIG 2. IL-32 γ suppresses PA-induced skin inflammation. WT and IL-32 γ Tg mice were treated with 5% PA for 4 weeks. (A) The concentration of Th2-related cytokines, IL-4, IL-5, IL-13, IL-31, IL-33 and TSLP, in skin tissues. $n=6$. (B) The concentration of Th1 and Th17-related cytokines, TNF- α , IL-1 β , IL-6 and IL-17, in skin tissues. $n=6$. (C and D) The serum concentration of IL-4, IL-13 and TNF- α and IgE. $n=6$. (E) The concentration of IL-10 in skin tissues. $n=6$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

FIG 3. IL-32 γ inhibits miR-205 expression. (A) Expression of premature and mature-miR-205 in PA-induced skin tissues was determined by qPCR. $n=6$. (B) Expression of mature-miR-205 in TNF- α /IFN- γ -treated HaCaT cells was determined by qPCR. $n=4$. (C) mRNA expression of Drosha, Dgcr8 and Dicer1 in PA-induced skin tissues was determined by qPCR. $n=3$. (D) HaCaT cells were transfected with

miR-205 inhibitor or mimic (40 μ M). After 24 h, cells were treated with TNF- α and IFN- γ (20 ng/mL) for 24 h. The mRNA expression of TNF- α , IL-1 β , IL-6, TSLP and IL-32 γ was determined by qPCR. $n=3$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

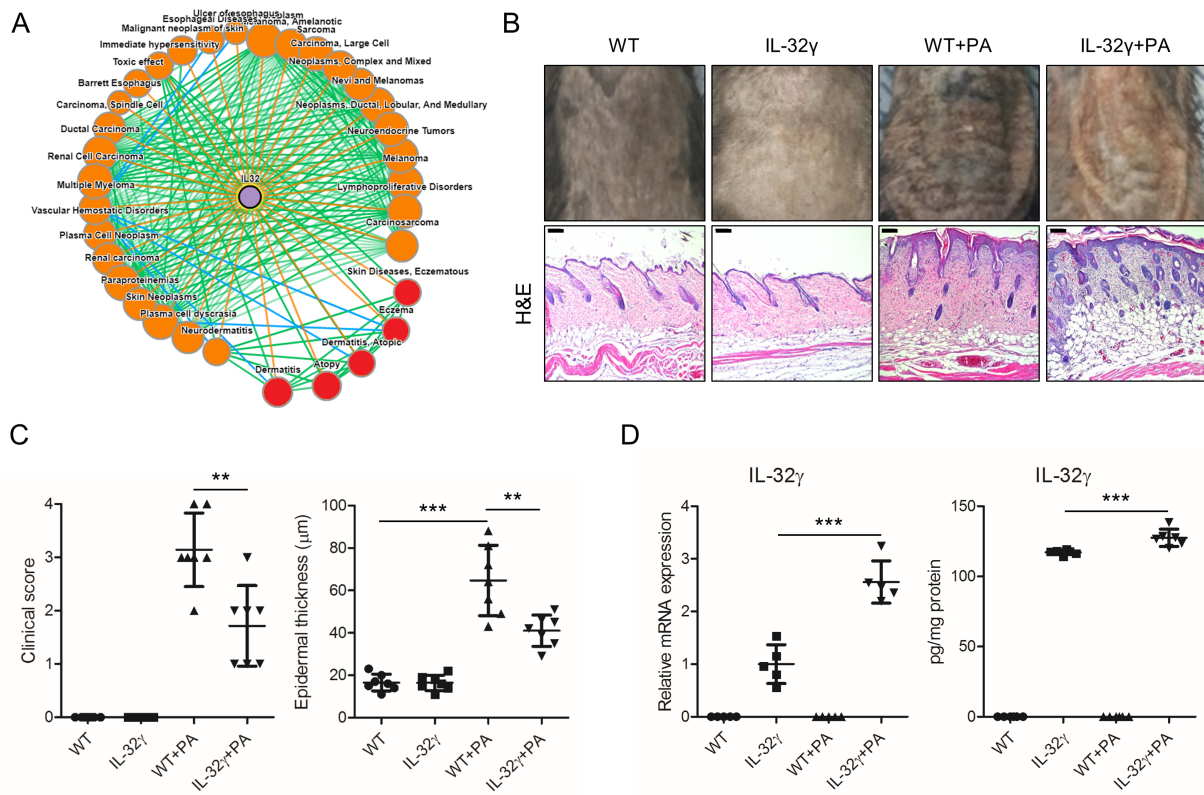
FIG 4. IL-32 γ suppresses NF- κ B activation. (A) Expression of phosphorylated I κ B α in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions in PA-induced skin tissues by Western blot analysis. (B) Expression of phosphorylated p65 (p-p65) in PA-induced skin tissues by IHC analysis. Scale bar, 50 μ m. (C and D) HaCaT cells were transfected with IL-32 γ plasmid vector. After 24 h, cells were treated with TNF- α /IFN- γ for 24 h. Expression of phosphorylated I κ B α in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions by Western blot analysis (C). NF- κ B transcriptional activity was measured by luciferase assay using transfection of luciferase-expressing NF- κ B plasmid vector (D). $n=4$. (E and F) HaCaT cells were transfected with IL-32 γ plasmid vector. After 24 h, cells were treated with TNF- α /IFN- γ for 24 h. The expressions of miR-205 (E) and pro-inflammatory cytokines, TNF- α , IL-1 β , IL-6 and TSLP (F), were analyzed by qPCR. $n=3$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

FIG 5. IL-32 γ reduces the inflammation in AD-like RHS model. RHS model were cultured for AD cocktail with/without rhIL-32 γ for 6 days. (A) Histological changes in AD-RHS model. A bar graph presents epidermal thickness. $n=6$. Scale bar, 50 μ m. (B) Phosphorylation of I κ B α in AD-RHS model. (C) Expression of mature-miR-205 in AD-RHS model. $n=4$. (D) mRNA expression of IL-32 γ , TNF- α , IL-1 β , IL-6 and TSLP in AD-RHS model. $n=4$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

FIG 6. rhIL-32 γ therapy alleviates experimental AD development. WT mice were treated with 5% PA for 4 weeks. From 3rd week, rhIL-32 γ (1 μ g/mouse) was subcutaneously injected to dorsal skin 3 h after PA treatment. (A) Morphological and histological changes in mice after rhIL-32 γ therapy. Bar graphs indicate clinical score, epidermal thickness and mast cell number. $n=6$. Scale bar, 50 μ m. (B) The protein level of TNF- α , IL-4, IL-13, IL-31, IL-33 and TSLP, in skin tissues. $n=5$. (C) Serum

concentration of IgE. $n=6$. (D) Expression of premature and mature-miR-205 in skin tissues. $n=4$. $*p<0.05$, $**p<0.01$ and $***p<0.001$. (E) Expression of phosphorylated I κ B α in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions by Western blot analysis in PA-induced WT mice with/without rhIL-32 γ therapy.

FIG 7. Serum analysis of IL-31, IL-32 γ , IL-33 and miR-205 in AD patients. (A) The serum level and ROC curve of IL-31, IL-32 γ , IL-33 and miR-205 in AD patients and healthy controls. $n=20$. (B) Spearman's correlation test between IL-31, IL-32 γ and IL-33 for miR-205. $*p<0.05$ and $***p<0.001$.



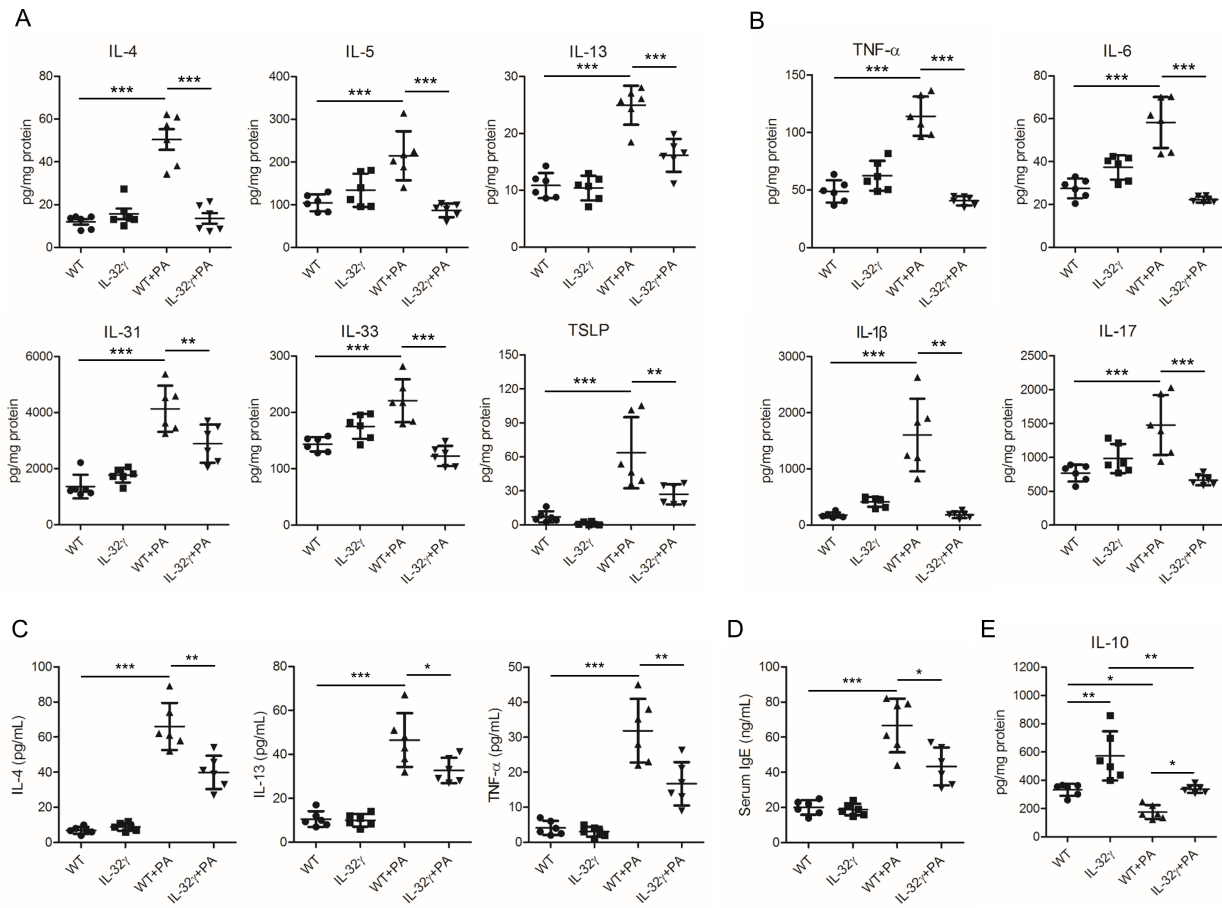


Figure 3.

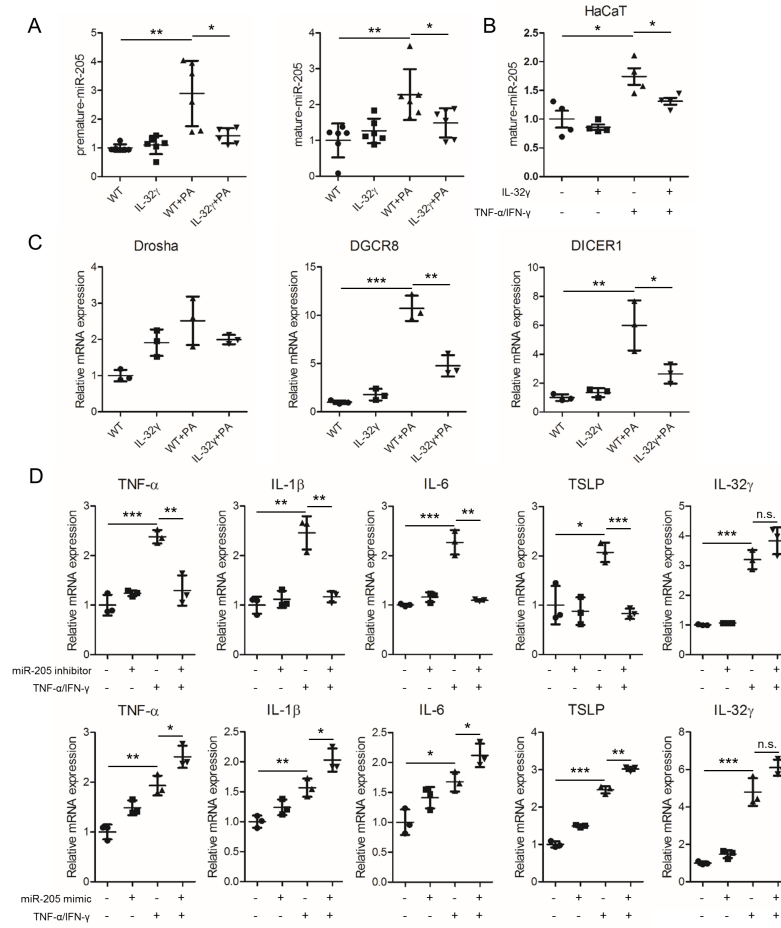


Figure 4.

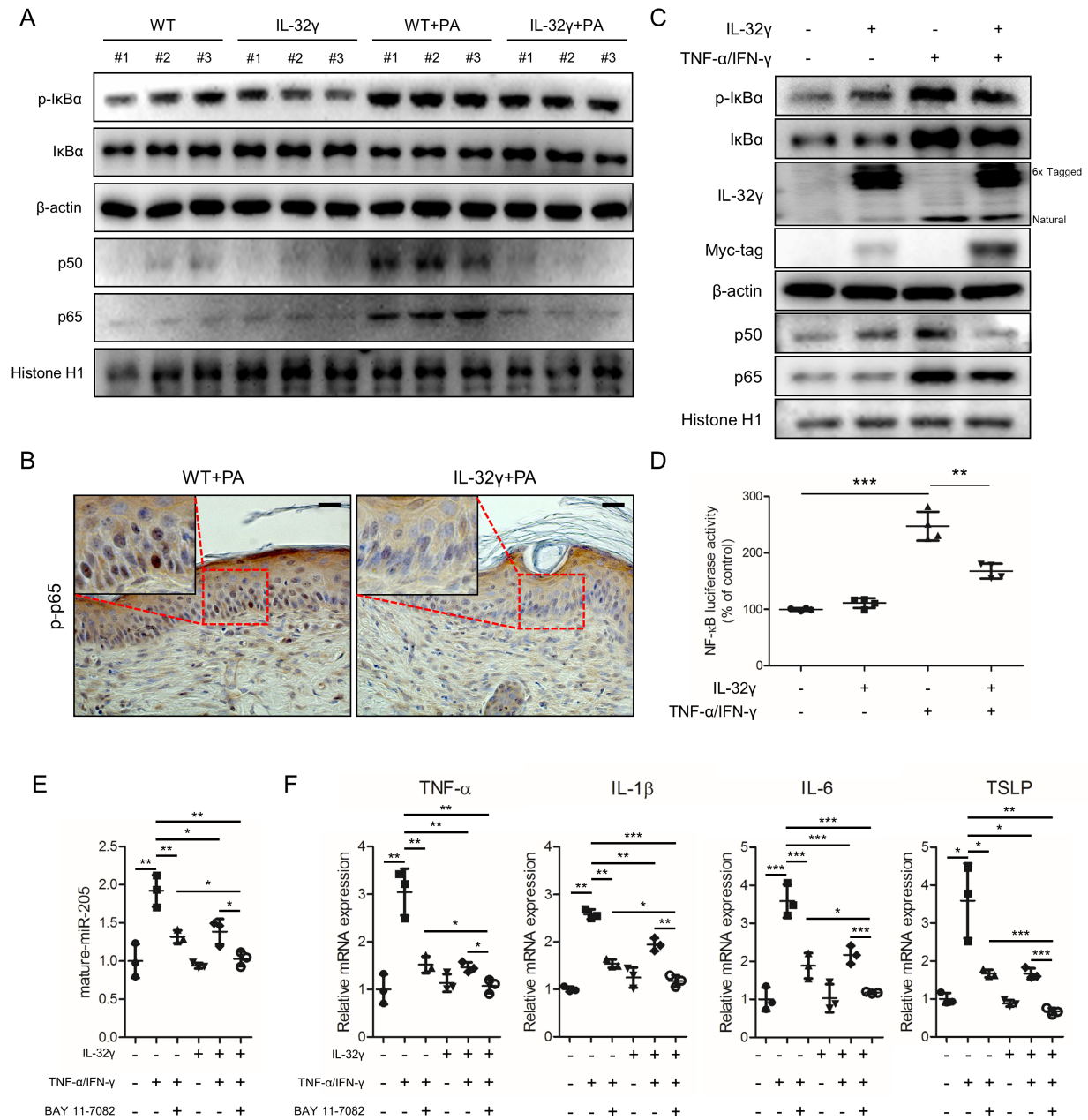


Figure 5.

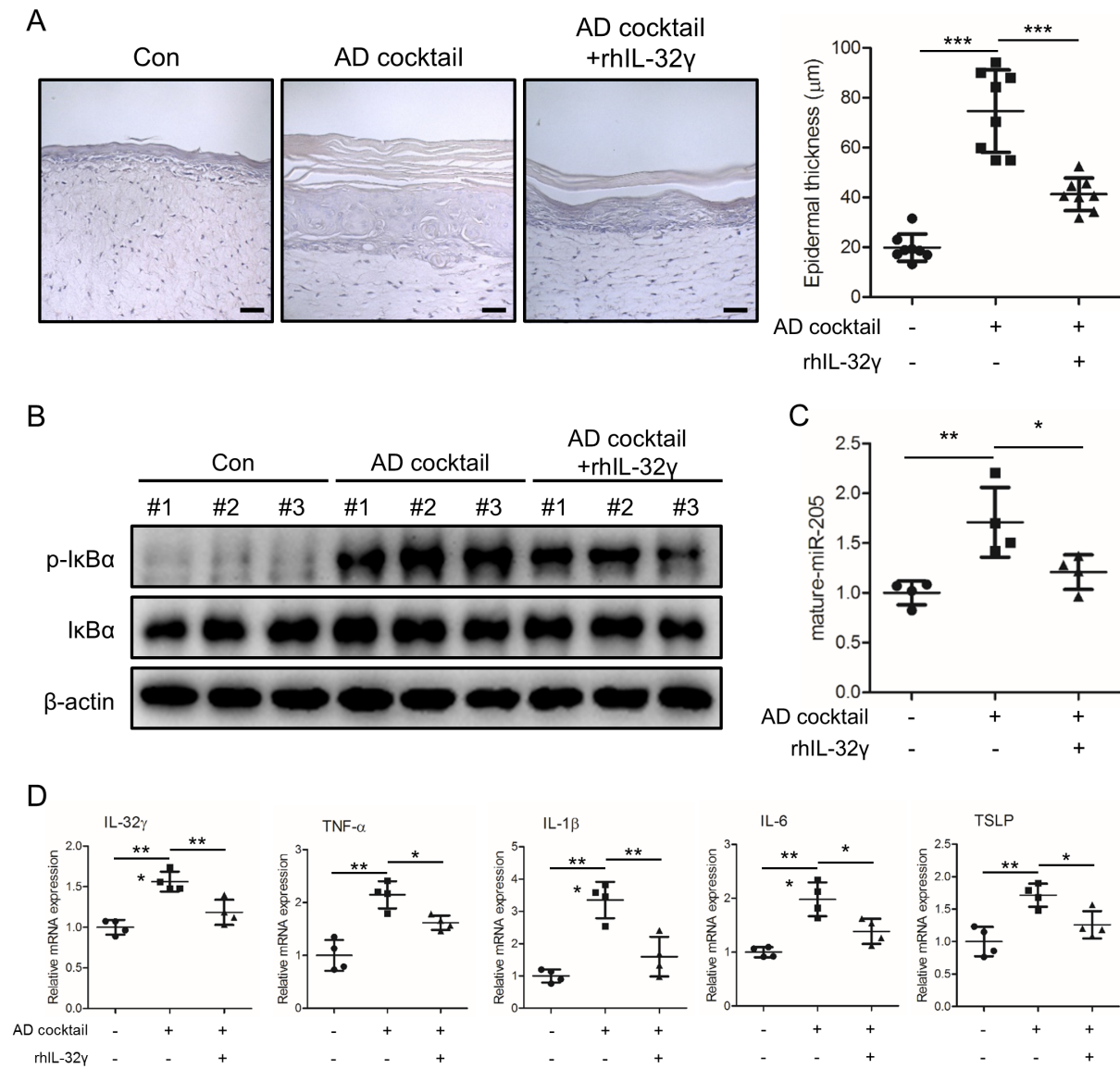


Figure 6.

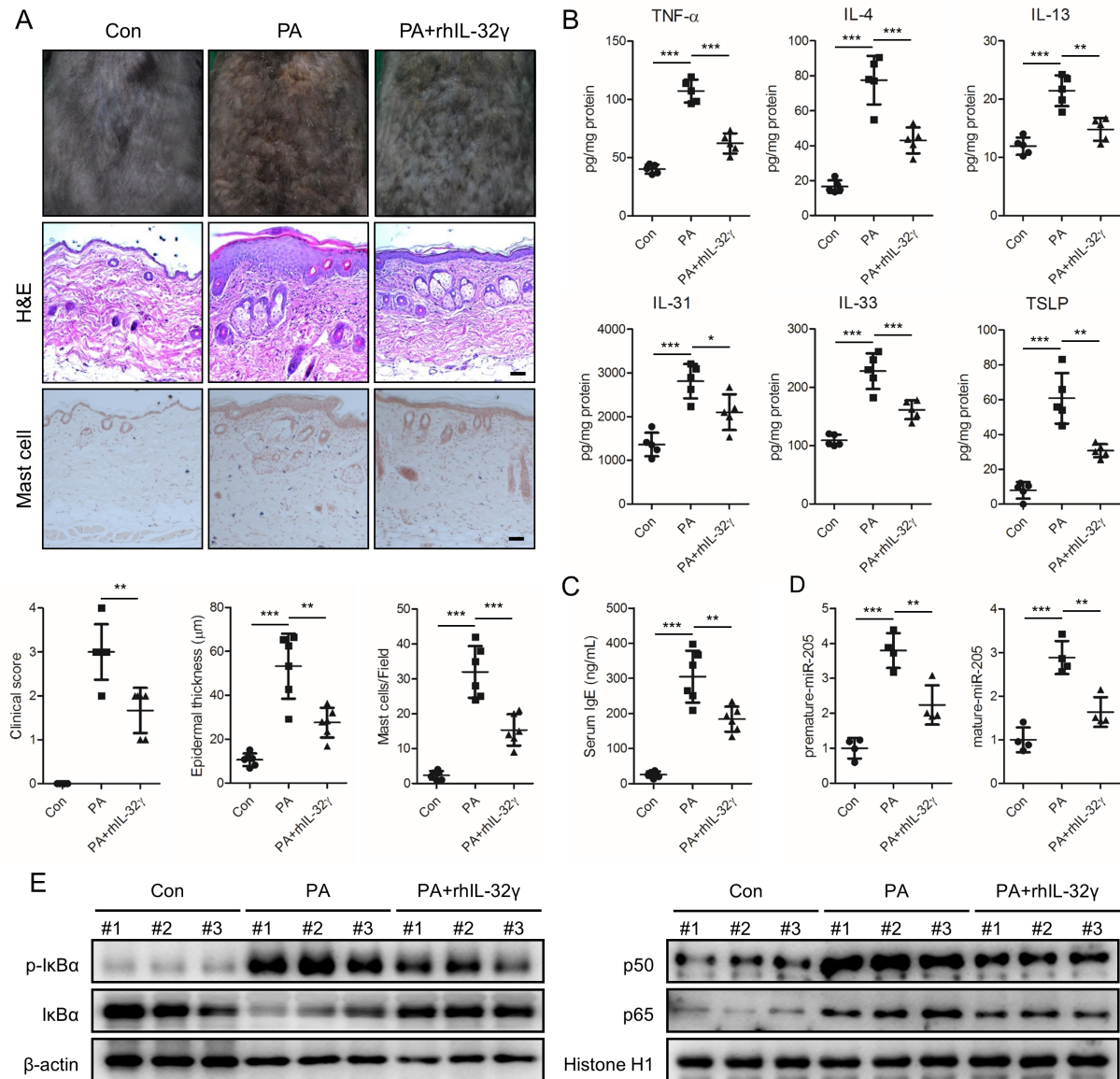


Figure 7.

