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

Yong Sun Lee, MSc,[a]* Sang-Bae Han, PhD,[a]* Hyeon Joo Ham, BSc,[a] Ju Ho Park, MSc,[a] Jong Sung Lee, BSc,[a] Dae Yeon Hwang, PhD,[b] Young Suk Jung, PhD,[c] Do Young Yoon, PhD,[d] and Jin Tae Hong, PhD[a] *Chungbuk, Miryang, Busan, and Seoul, Korea*

Background: IL-32 is a novel cytokine involved in many inflammatory diseases. However, the role of IL-32γ, an isotype of IL-32, in atopic dermatitis (AD) has not been reported.
Objective: We investigated the effects of IL-32γ on development of AD and its action mechanisms.
Methods: We used phthalic anhydride (PA) and an MC903-induced AD model using wild-type and IL-32γ transgenic mice. We conducted the therapy experiments by using recombinant IL-32γ protein in a reconstructed human skin model and PA-induced model. We conducted a receiver operating characteristic analysis of IL-32γ with new AD biomarkers, IL-31 and IL-33, in serum from patients with AD.
Results: Dermatitis severity and epidermal thickness were significantly reduced in PA- and MC903-induced IL-32γ transgenic mice compared with in wild-type mice. The concentration of AD-related cytokines was reduced in PA- and MC903-induced IL-32γ transgenic mice compared with in wild-type mice. Subsequent analysis showed that IL-32γ inhibits miR-205 expression in PA- and MC903-induced skin tissue samples and TNF-α/IFN-γ–treated HaCaT cells. IL-32γ reduced NF-κB activity in skin tissue samples from PA- and MC903-induced mice and TNF-α/IFN-γ–treated HaCaT cells. NF-κB inhibitor treatment with IL-32γ expression further suppressed expression of inflammatory mediators as well as miR-205 in TNF-α/IFN-γ–treated HaCaT cells. Furthermore, recombinant IL-32γ protein alleviated AD-like inflammation in *in vivo* and reconstructed human skin models. Spearman correlation analysis showed that serum levels of IL-32γ and miR-205 were significantly concordant in patients with AD.
Conclusion: Our results indicate that IL-32γ reduces AD through the inhibition of miR-205 expression via inactivation of NF-κB. (J Allergy Clin Immunol 2020;146:156-68.)

Key words: *Atopic dermatitis, IL-32γ, miR-205, NF-κB*

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by complex pathophysiology accompanied by an itchy and scaly rash.[1] AD is induced by an impairment of the skin barrier function, dysfunctional immune system, and genetic and environmental risk factors.[2] AD pathogenesis consists of acute and chronic phases.[3] The acute phase of AD is mediated by T_H2 cells, which produce various cytokines such as IL-4, IL-5, and IL-13 in lesioned skin of patients with AD.[4] These cytokines play a critical role in the promotion of IgE synthesis to initiate IgE-mediated allergic inflammation by activating mast cells and eosinophils.[4,5] In addition, T_H2 cytokines directly affect epidermal keratinocytes, which produce proinflammatory cytokines that induce an inflammatory response through infiltration of immune cells into the skin lesions. Keratinocytes in the skin are the crucial providers or initiators of AD.[6] Activated keratinocytes express various proinflammatory cytokines and chemokines such as thymic stromal lymphopoietin (TSLP), TNF-α, IL-1, IL-6, IL-25, IL-33, C-C motif chemokine ligand 17, and C-C motif chemokine ligand 22, which contribute to infiltration of inflammatory cells and allergic inflammation in AD pathogenesis.[3,7,8] After the acute phase, the chronic phase of AD is mediated by T_H1 cells. Infiltrated and accumulated eosinophils and macrophages actively secrete IL-12, which stimulates the accumulation and development of IFN-γ–expressing T_H1 cells in the lesion.[4] The increased level of IFN-γ in the lesion exacerbates AD by stimulating the homing of immune cells and inducing spongiosis of the skin epidermis.

IL-32γ was cloned as a gene induced by IL-18 and was formerly known as natural killer cell transcript 4.[9] IL-32 has 6 splice variants, IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, and IL-32ζ, but the functional differences between these isoforms remain unknown.[10,11] IL-32 modulates the generation of TNF-α, IL-1β, IL-6, and IL-10 in inflammatory diseases.[12] It has also been implicated in many inflammatory disorders, and

From the [a]College of Pharmacy and Medical Research Center, Chungbuk National University; [b]the Department of Biomaterial Science, College of Natural Resources and Life Science/Life and Industry Convergence Research Institute, Pusan National University, Miryang; [c]the College of Pharmacy, Pusan National University; and [d]the Department of Bioscience and Biotechnology, Konkuk University, Seoul.
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Corresponding authors: Jin Tae Hong, PhD, College of Pharmacy and Medical Research Center, Chungbuk National University, Osongsaengmyeong 1-ro, Osong-eup, Heungdeok-gu, Cheongju, Chungbuk, 28160, Republic of Korea. E-mail: jinthong@chungbuk.ac.kr. Or: Do Young Yoon, PhD, Department of Bioscience and Biotechnology, Konkuk University, Gwangjin-gu, Seoul 05029, Republic of Korea. E-mail: ydy4218@konkuk.ac.kr.

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Abbreviations used
AD: Atopic dermatitis
AUC: Area under the curve
BAY: Bay 11-7082
IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
ILC2: Type 2 innate lymphoid cell
miRNA: MicroRNA
NF-κB: Nuclear factor-kappa B
PA: Phthalic anhydride
RHS: Reconstructed human skin
ROC: Receiver operating characteristic
Treg: Regulatory T
TSLP: Thymic stromal lymphopoietin
WT: Wild-type

its expression has been correlated with disease severity in patients with Crohn disease, rheumatoid arthritis, colitis, inflammatory bowel disease, and chronic obstructive pulmonary disease.[13-15] It is reported that IL-32 contributes to AD pathogenesis by inducing keratinocyte apoptosis.[16] In addition, serum levels of IL-32 were increased in patients with AD compared with in controls.[16] Our previous study showed that IL-32γ inhibits the expression of TNF-α and IL-1β via inactivation of NF-kB signaling in colon cancer.[17] In addition, IL-32β reduces expression of TNF-α, IL-1β, and IL-6 in tumor and spleen tissue samples of a mouse xenograft model.[18] It has also been reported that IL-32γ acts as an anti-inflammatory cytokine that inhibits 12-*O*-tetradecanoylphorbol-13-acetate–induced skin inflammation.[19] However, the direct roles of IL-32γ in AD pathogenesis have not been studied.

It is known that microRNAs (miRNAs) are involved in the pathogenesis of many diseases, including AD.[20-22] miR-155 may contribute to development of AD through upregulation of T-cell activation.[23] It has also been reported that miR-29b promotes keratinocyte apoptosis via suppression of the antiapoptotic protein BCL2L2 in AD.[24] Recent studies have revealed that miR-302e attenuates release of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and TSLP in the human mast cell line HMC-1.[25] Previous studies have demonstrated that NF-κB plays a critical role in development of AD.[26] NF-κB inhibitors adequately inhibit allergic inflammation, including inflammatory cell infiltration and AD-related cytokine release in an NC/Nga mouse AD model.[27,28] In addition, miR-146a inhibits NF-κB signaling to prevent inflammatory responses in keratinocytes and AD.[29] Yang et al reported that miR-124 suppresses chronic skin inflammation in AD via inhibition of NF-κB signaling in keratinocytes.[30] Further, miR-205 is upregulated in serum and urine of patients with AD compared with in healthy controls and IL-32α suppresses miR-205 biogenesis to inhibit endothelial cell inflammation.[21,31] miR-205 is increased as a result of activation of NF-κB signaling in lung cancer,[32] and it is regulated by NF-κB signaling in breast cancer.[33]

In this study, to investigate whether IL-32γ inhibits AD pathogenesis via suppression of miR-205 through inactivation of NF-κB, we compared AD development in wild-type (WT) and IL-32γ transgenic mice by 2 types of chemical-induced models and analyzed the relationship between IL-32γ level and patients with AD. Our data demonstrate the significant suppressive effect of IL-32γ in development of AD.

METHODS

Cell culture and transfection

HaCaT cells were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2. HaCaT cells were transfected with pcDNA3.1(+)-6xMyc-IL-32γ vector or control vector by using the Lipofectamine 3000 transfection reagent in Opti-MEM, according to the manufacturer's specifications (Invitrogen, Waltham, Mass). For miR-205 transfection, HaCaT cells were transfected with miR-205 mimic, miR-205 inhibitor, or its controls (Qiagen GmbH, Hilden, Germany) by using Lipofectamine RNAiMAX reagent in Opti-MEM, according to the manufacturer's (Invitrogen) specifications. Transfected cells were stimulated with TNF-α/IFN-γ (20 ng/mL; PeproTech Inc, Rocky Hill, NJ) for the 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 of the Laboratory Animal Research Center at Chungbuk National University, Korea (ethics 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 4-mouse cages with automatic temperature control (21°C-25°C) at relative humidity levels of 45% to 65% with a 12-hour light-dark cycle. Food and water were provided *ad libitum*. WT (C57BL/6) mice were purchased from DBL (Eumsung, Korea). Generation of hIL-32γ transgenic mice has been described in a previous study.[17] In brief, a 705-bp fragment of the hIL-32γ gene was subcloned into the *Eco*RI sites of the pCAGGS expression vector. IL-32γ insertion was confirmed by amplification of genomic DNA isolated from the tails of transgenic mice by using the 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 the tails of transgenic mice, and PCR analysis was performed for IL-32γ gene expression. WT mice do not express IL-32γ. IL-32γ transgenic mice have no overt phenotype compared with WT mice. The IL-32γ transgenic mice were viable and fertile, and they 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-like skin, PA or MC903 (a vitamin D_3 analog) were used. The PA-induced AD model was developed as previously described.[34] A 5% PA solution or acetone and olive oil vehicle (acetone and olive oil in a 4:1 ratio) was applied to the dorsal skin 3 times per week for 4 weeks. The MC903-induced AD model was developed as previously described.[35] An MC903 solution (45 μM) or ethanol was applied to dorsal skin for 17 days (5 days of treatment, a 2-day interruption, 5 days of treatment, a 2-day interruption, and 2 days of 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 RHS model

A reconstructed human skin (RHS) model (Neoderm-ED) was purchased from TEGO Science Inc (Seoul, Korea). The RHS model contained epidermis and dermis. The RHS model generated AD-like inflammation via use of an inflammatory cocktail according to the previously described method with some modifications.[36-38] In brief, an inflammatory cocktail (an AD cocktail consisting of 30 ng/mL of IL-4, 30 ng/mL of IL-13, and 3.5 ng/mL of TNF-α [PeproTech Inc] with or without supplementation with recombinant human IL-32γ [100 ng/mL; YbdY Biotech, Seoul, Korea]) was added to the culture medium for 6 days. The culture medium was changed every 48 hours.

Immunohistochemistry

Skin tissue samples and RHS tissue samples were removed, fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then

sectioned into 5-μm-thick slices. Hematoxylin and eosin staining and immunohistochemistry were performed as described previously.[39] The slides were incubated with the following specific primary antibodies: F4/80 (Santa Cruz Biotechnology, Dallas, Tex), arginase-1 (Cell Signaling, Beverly, Mass), CD86 (GeneTex, Taiwan), Ly6G (Invitrogen), and phosphorylated p65 and FOXP3 (Abcam, Cambridge, Mass). Toluidine blue was used for mast cell staining. Detailed information is presented in Table E1 (in the Online Repository available at www.jacionline.org).

ELISA assay

Cytokines and IgE levels were measured by ELISA kits provided by KOMA Biotech (Seoul, Korea), R&D Systems (Minneapolis, Minn), and Abcam following the manufacturer's protocol. Human serum samples were analyzed by using IL-31, IL-32β, IL-32γ, and IL-33 ELISA kits purchased from myBioSource (IL-32β and IL-32γ; San Diego, Calif) and KOMA Biotech (IL-31 and IL-33) following the manufacturer's instructions. Detailed information is presented in Table E1.

Western blot analysis

Western blot analysis was performed as described previously.[19] The membranes were incubated with the following specific primary antibodies: IL-32 (BioLegend, San Diego, Calif); p50, histone H1, and β-actin (Santa Cruz Biotechnology), inducible nitric oxide synthase, cyclooxygenase-2 and p65 (Abcam), and phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and IκBα (Cell Signaling). Detailed information is presented in Table E1.

Quantitative real-time PCR

Total RNA from skin tissue samples and RHS tissue samples and cells was extracted by using a RiboEx RNA Extraction Kit (GeneAll Biotechnology, Seoul, Korea), and cDNA was synthesized by using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, Calif). Quantitative real-time PCR was performed by using specific primers in a StepOnePlus PCR System (Applied Biosystems) (see Table E2 in the Online Repository available at www.jacionline.org). The values obtained for the target gene expression were normalized to 18S and quantified relative to the expression in control samples. For the miRNA experiment, miRNA of skin tissue samples and cells was extracted by using an miRNeasy Mini Kit (Qiagen GmbH) and miRNA of serum was extracted by using an miRNeasy Serum/Plasma Kit (Qiagen). cDNA was generated with the miScript II RT Kit (Qiagen). Premature and mature miR-205 primers were purchased from Qiagen. Rnu6B was used as an internal control for the skin tissue samples and cells. miR-39 (*Caenorhabditis elegans*) was used as an internal control for the serum miRNA analysis.

Luciferase assay

HaCaT cells were transiently cotransfected with IL-32γ plasmid vector and luciferase-expressing NF-κB plasmid vector (Promega, Madison, Wis) for 24 hours. The transfected cells were treated with TNF-α/lFN-γ (20 ng/mL) for the indicated time points. Luciferase activity was measured by using a luciferase assay kit (Promega) following the manufacturer's instructions.

Human samples

Human serum samples from patients with AD and healthy controls (20 samples from each group) were obtained from Chungbuk National University Hospital Biobank and Korea Institute of Radiological & Medical Sciences 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 (institutional review board approval No. CBNU-201902-BR-786-01).

Gene-disease/miRNA-gene network analyses

The gene-disease network of IL-32 was analyzed by using the Disease-Connect web server (www.disease-connect.org),[40] which analyzed the gene-disease network on the basis of various sources, genome-wide association studies, the catalog 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 by using the ONCO.IO database tool (https://onco.io), which is based on the manually curated annotation of experimentally verified molecular interactions described in the literature.

Statistical analysis

All experiments were conducted in triplicate and repeated at least 3 times with similar results. Statistical analysis was performed with GraphPad Prism 4 software (GraphPad Software, San Diego, Calif). Group differences were analyzed by 1-way ANOVA followed by the Tukey multiple comparison test. The Spearman test was used to analyze the correlation between IL-31, IL-32γ, and IL-33 and miR-205 in patient samples. All values were presented as means plus or minus SDs. Significance was set at a *P* value less than .05 for all tests.

RESULTS

IL-32γ suppresses experimentally induced AD pathogenesis

IL-32 has been known to have a proinflammatory or anti-inflammatory role in various diseases.[13] Using gene-disease relation analysis, we found that IL-32 is closely associated with many skin diseases, including AD (Fig 1, *A*), To explore the relationship between IL-32γ and AD, we evaluated the role of IL-32γ on experimentally induced AD pathogenesis by treatment with PA. PA-treated WT mice showed symptoms indicative of erythema, edema, and erosion in their dorsal skin. These changes were impeded in PA-induced IL-32γ transgenic mice (Fig 1, *B*). To investigate the suppressive effect of IL-32γ on AD, histologic analysis of the dorsal skin was performed (Fig 1, *B*). Clinical score and epidermal thickness were also significantly reduced in the PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (Fig 1, *C*). We determined mRNA expression and protein level of IL-32γ in PA-induced skin tissue samples. The results showed that the mRNA expression and protein level of IL-32γ were increased in PA-induced skin tissue samples from IL-32γ transgenic mice (Fig 1, *D*). To confirm the role of IL-32γ, we further developed an experimental model of AD induced by the vitamin D_3 analog MC903. AD symptoms were reduced in MC903-induced IL-32γ transgenic mice compared with in MC903-induced WT mice (see Fig E1, *A* in the Online Repository available at www.jacionline.org). Morphologic and histologic analyses of AD lesions from skin showed reduction of hyperkeratosis, parakeratosis, and acanthosis, along with reduction of the infiltration of dermal inflammatory cells in MC903-induced IL-32γ transgenic mice compared with in 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-32γ transgenic mice compared with in MC903-induced WT mice. These results thus showed that IL-32γ could suppress experimental AD pathogenesis.

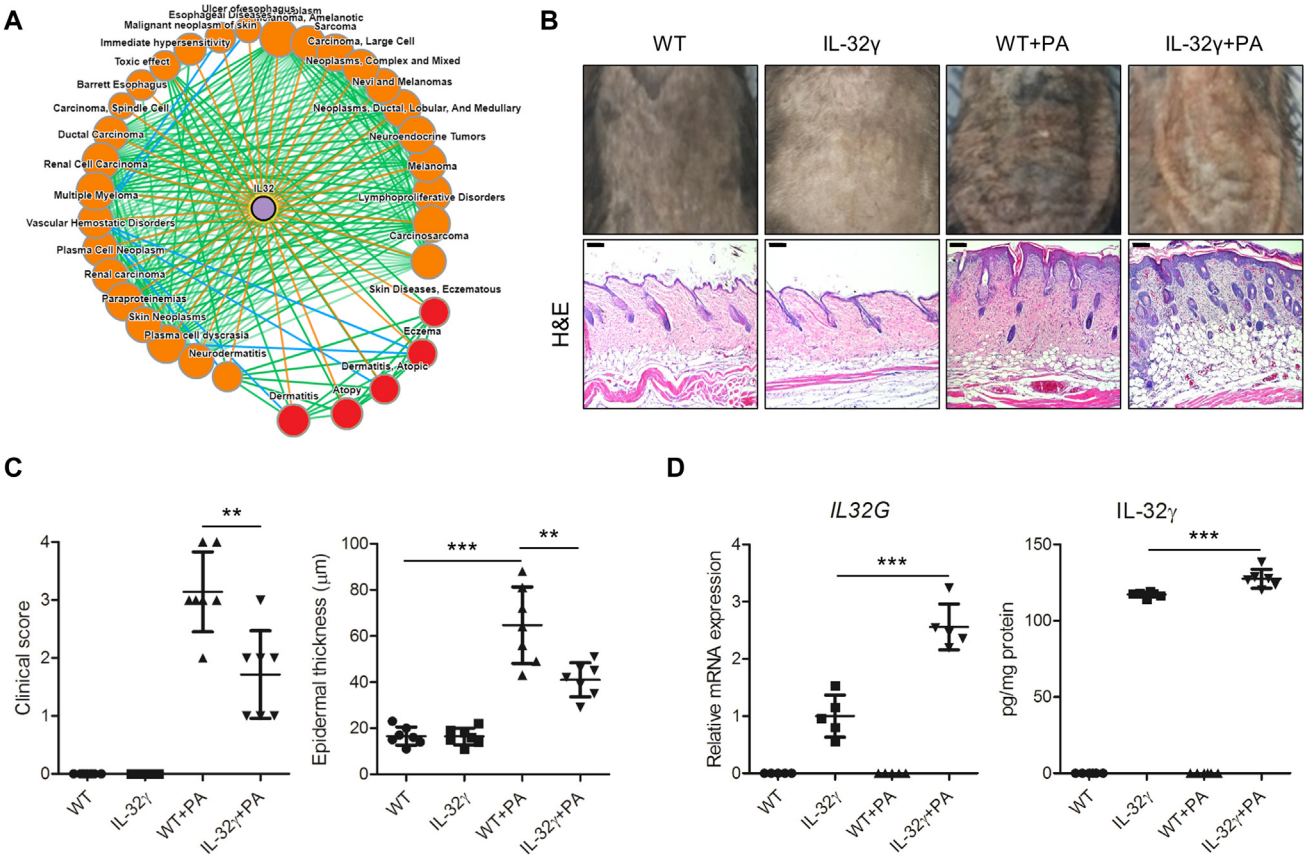

FIG 1. IL-32γ inhibits PA-induced AD. **A,** Gene (IL-32)-disease network was analyzed on the basis of genome-wide association studies/Online Mendelian Inheritance in Man/Database of Essential Genes records ($P < 10^{-6}$). **B,** The morphologic and histologic changes in mice after 4 weeks of treatment as described in the Materials and Methods. The photographs are representative of each group of mice. Scale bar = 50 μm. **C,** Bar graphs indicate clinical score and epidermal thickness (n = 7). **D,** mRNA expression and protein level of IL-32γ in skin tissue samples (n = 5). $^{**}P < .01$; $^{***}P < .001$. *H&E,* Hematoxylin and eosin.

IL-32γ reduces inflammatory responses

To determine whether IL-32γ could reduce allergic inflammation in PA-induced AD skin tissue samples, we primarily evaluated the changes of auricular lymph node weight. The lymph node weight of the PA-induced IL-32γ transgenic mice was significantly lower than that of the PA-induced WT mice (see Fig E2, *A* in the Online Repository available at www.jacionline.org). In addition, expression levels of the inflammation markers cyclooxygenase-2 and inducible nitric oxide synthase were significantly reduced in skin tissue samples of PA-induced IL-32γ transgenic mice compared with in the skin tissue of PA-induced WT mice (see Fig E2, *B*). Previous studies indicated that inflammatory cells mediate AD. We thus investigated the infiltration of inflammatory cells in AD skin tissue samples. We found a decreased infiltration of $F4/80^+$ macrophages and arginase-1^+ macrophages in PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (see Fig E3, *A* and *B* in the Online Repository available at www.jacionline.org). But $CD86^+$ macrophages were not changed in either unstimulated or PA-induced skin tissue samples (see Fig E3, *A* and *B*). Furthermore, there was less infiltration of $Ly6G^+$ neutrophils and mast cells in the skin tissue samples of PA-induced IL-32γ transgenic mice than in the skin tissue samples of PA-induced WT mice (see Fig E3, *A* and *B*). Previous research indicated that $FOXP3^+$ regulatory T (Treg) cells control AD by suppression of inflammatory cell recruitment in an ovalbumin-induced AD mouse model.[41] We thus analyzed whether IL-32γ controls skin inflammation by $FOXP3^+$ Treg cells. Increased numbers of $FOXP3^+$ cells were accumulated in the skin tissue samples of PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (see Fig E3, *A* and *B*). In addition, we further analyzed the mRNA levels of eosinophil and type 2 innate lymphoid cell (ILC2) markers. The results showed that the levels of expression of eosinophil (*Siglecf*, *Epx* and *Prg2*) and ILC2 (*Sca1*, *Cd25*, and *Klrg1*) markers were decreased in PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (see Fig E4, *A* and *B* in the Online Repository available at www.jacionline.org). T_H2-related cytokines are critical factors for AD pathogenesis.[7] We analyzed T_H2-related cytokines in PA-induced skin tissue samples. ELISA analyses showed that the levels of T_H2-related cytokines, including IL-4, IL-5, IL-13, IL-31, IL-33, and TSLP, were reduced in skin tissue samples from PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (Fig 2, *A*). Quantitative real-time PCR analyses also showed that mRNA levels of T_H2-related cytokines were significantly reduced in skin tissue samples from PA-induced IL-32γ transgenic mice compared with in PA-induced WT mice (see Fig E5, *A* in the Online Repository available at www.jacionline.org).

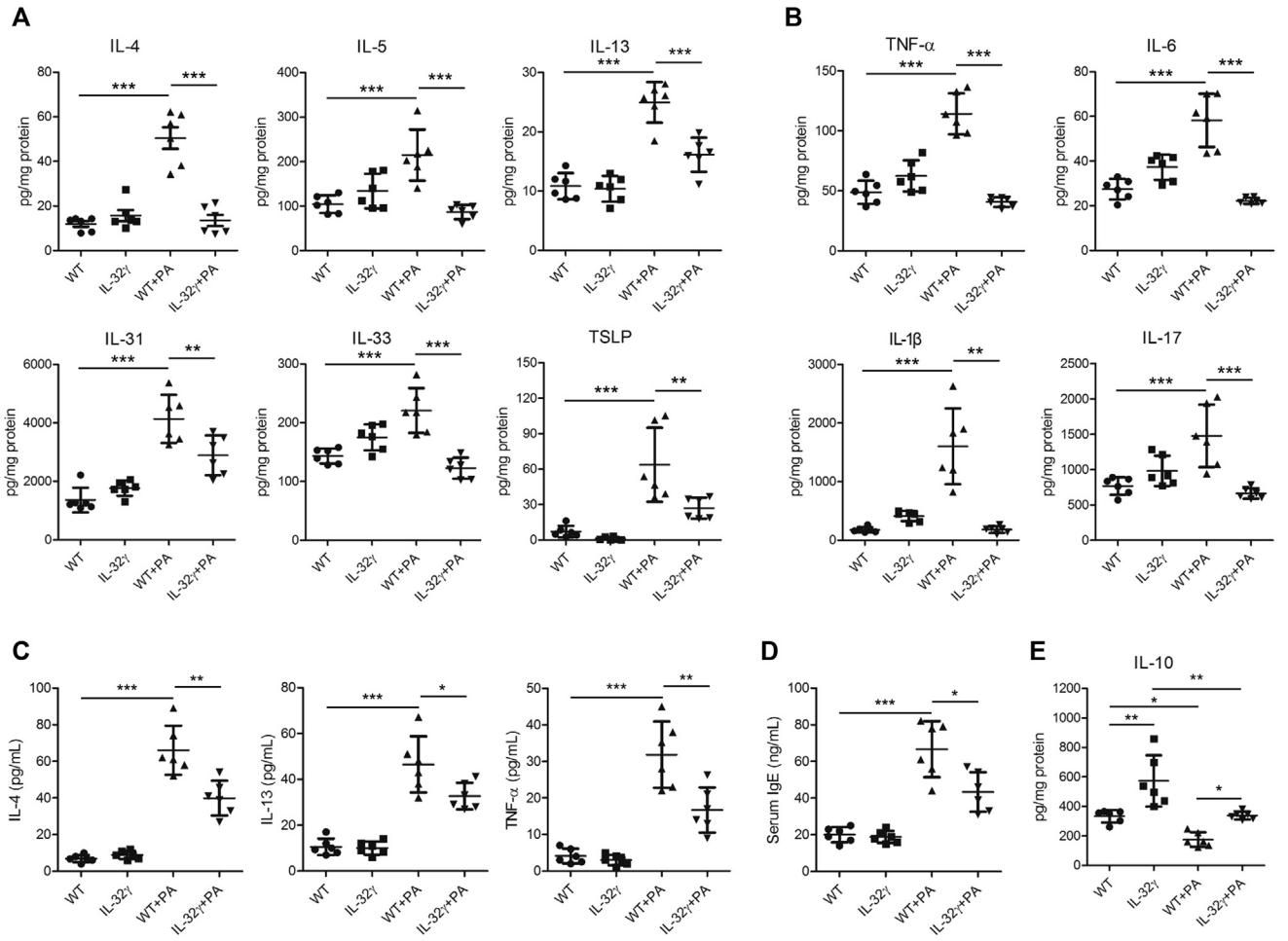

FIG 2. IL-32γ suppresses PA-induced skin inflammation. WT and IL-32γ transgenic mice were treated with 5% PA for 4 weeks. **A,** Concentrations of T_H2-related cytokines, IL-4, IL-5, IL-13, IL-31, IL-33, and TSLP in skin tissue samples (n = 6). **B,** Concentrations of T_H1- and T_H17-related cytokines, TNF-α, IL-1β, IL-6, and IL-17, in skin tissue samples (n = 6). **C** and **D,** Serum concentrations of IL-4, IL-13, and TNF-α (**C**) and IgE (**D**) (n = 6). **E,** Concentration of IL-10 in skin tissue samples (n = 6). **P* < .05; ***P* < .01; ****P* < .001.

The protein levels and mRNA expression of T_H1 and T_H17 cytokines (TNF-α, IL-6, IL-1β, and IL-17) were also markedly reduced in skin tissue samples from PA-induced IL-32γ transgenic mice compared with in 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-32γ transgenic mice compared with in PA-induced WT mice (Fig 2, *C* and *D*). As in the PA-induced model, the protein levels of TNF-α, IL-4, IL-13, IL-31, IL-33, and TSLP in skin tissue samples were also significantly reduced from MC903-induced IL-32γ transgenic mice compared with MC903-induced WT mice (see Fig E1, *C*). The mRNA levels of *Tnfa*, *Il4*, Il13, *Il31*, *Il33* and *Tslp* in skin tissue samples were also decreased from MC903-induced IL-32γ transgenic mice compared with MC903-induced WT mice (see Fig E5, *C*). Previous studies indicated that IL-32γ contributed to IL-10 production.[17,19] Thus, we analyzed the IL-10 levels in PA-induced skin tissue samples. The concentrations of IL-10 at the basal level and PA-induced condition were higher in the skin tissue samples from IL-32γ transgenic mice than in the skin tissue samples from WT mice. The concentration of IL-10 in PA-induced skin tissue samples was decreased in both WT and IL-32γ transgenic mice compared with unstimulated condition (Fig 2, *E*).

IL-32γ inhibits miR-205 expression

Our previous study revealed that IL-32α suppressed endothelial cell inflammation by inhibition of miR-205 expression.[31] Serum and urine levels of miR-205 are significantly upregulated in children with AD compared with in healthy controls.[42] We therefore analyzed whether IL-32γ could suppress AD through regulation of miR-205 expression. miR-205 expression was upregulated in PA- and MC903-induced skin tissue samples from WT mice compared with in nontreated WT mice, and it was significantly decreased in PA- and MC903-induced skin tissue samples from IL-32γ transgenic mice (Fig 3, *A* and see Fig E1, *E*). We further discovered that IL-32γ 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-32γ inhibited miR-205 expression by suppression of *Dgcr8* and *Dicer1*

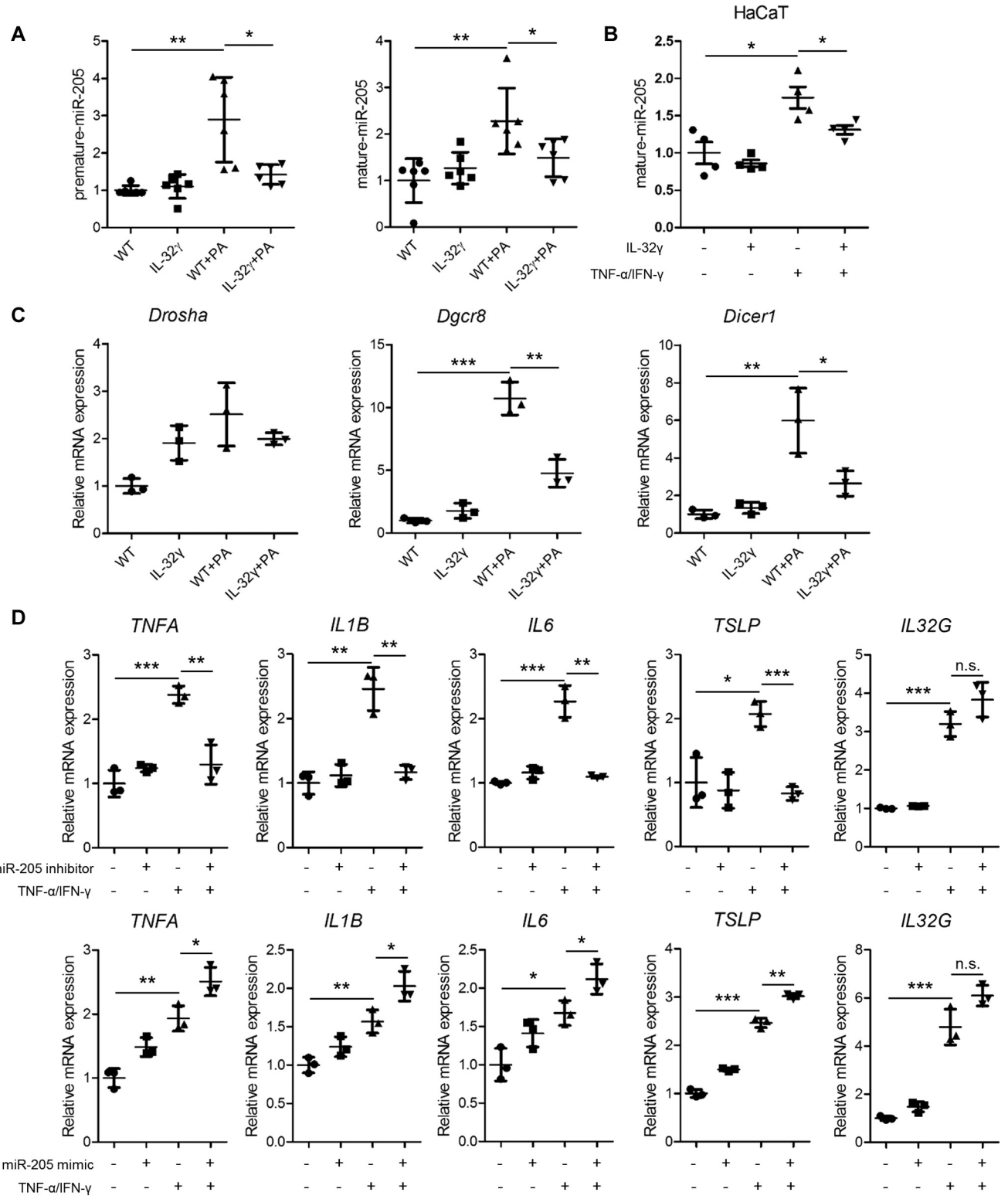

FIG 3. IL-32γ inhibits miR-205 expression. **A,** Expression of premature and mature miR-205 in PA-induced skin tissue samples was determined by quantitative real-time PCR (qPCR) (n = 6). **B,** Expression of mature-miR-205 in TNF-α/IFN-γ–treated HaCaT cells was determined by qPCR (n = 4). **C,** mRNA expression of *Drosha*, *Dgcr8,* and *Dicer1* in PA-induced skin tissue samples was determined by qPCR (n = 3). **D,** HaCaT cells were transfected with miR-205 inhibitor or mimic (40 μM). After 24 hours, cells were treated with TNF-α and IFN-γ (20 ng/mL) for 24 hours. mRNA expression of *TNFA*, *IL1B*, *IL6*, *TSLP*, and IL32G was determined by qPCR (n = 3). **P* < .05; ***P* < .01; ****P* < .001.

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 proinflammatory mediators. The results showed that inhibition of miR-205 significantly reduced the mRNA expression of *TNFA*, *IL1B*, *IL6*, and *TSLP* in TNF-α/IFN-γ–induced HaCaT cells (Fig 3, *D*). Moreover, miR-205 mimic treatment synergistically induced these proinflammatory cytokines in TNF-α/IFN-γ–induced HaCaT cells (Fig 3, *D*). However, TNF-α/IFN-γ–induced IL32G mRNA expression was not altered by the miR-205 mimic or the inhibitor themselves. These results suggest that IL-32γ inhibits AD-like skin inflammation via suppression of miR-205 expression.

IL-32γ suppresses activation of NF-κB in PA-induced skin tissue samples and keratinocytes

NF-κB signaling has been known to play a central role in inflammation, and inhibition of NF-κB activity suppresses AD pathogenesis.[26-28] Previous studies indicated that miR-205 is regulated by NF-κB 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* in the Online Repository available at www.jacionline.org). To determine whether IL-32γ inhibits AD pathogenesis via suppression of NF-κB–dependent miR-205 expression, we analyzed the NF-κB signaling by Western blot and IHC analysis in PA-induced skin tissue samples. IL-32γ inhibited nuclear translocation of p50 and p65 and activation of IκBα in PA-induced skin tissue samples (Fig 4, *A* and *B*). MC903-induced NF-κB activation was also suppressed in IL-32γ transgenic mice (see Fig E7 in the Online Repository available at www.jacionline.org). We found that phosphorylation of IκBα and nuclear translocation of p50 and p65 were inhibited in TNF-α/IFN-γ–induced IL-32γ–overexpressing HaCaT cells compared with TNF-α/IFN-γ–induced HaCaT cells (Fig 4, *C*). We examined the effect of IL-32γ on TNF-α/IFN-γ–induced NF-κB transcription activity. IL-32γ expression resulted in the inhibition of NF-κB luciferase activity induced by TNF-α/IFN-γ treatment (Fig 4, *D*). NF-κB 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-32γ showed a synergistic effect with BAY to inhibit inflammation and miR-205 expression (Fig 4, *E* and *F*). These data indicate that inhibition of NF-κB is implicated in IL-32γ–mediated inhibition of AD pathogenesis by suppression of miR-205 expression.

Knockdown of IL-32 increases skin inflammation in keratinocytes

We showed that IL-32γ has anti-inflammatory effect on AD-like skin inflammation in an AD mouse model and in keratinocytes. We tested whether knockdown of IL-32 enhances skin inflammation in keratinocytes. The results showed that inhibition of IL-32 by small interfering RNA increased activation of NF-κB in TNF-α/IFN-γ–induced HaCaT cells (see Fig E8, *A* in the Online Repository available at www.jacionline.org). In addition, mRNA expression of the inflammatory cytokines *TNFA*, *IL-1B*, *IL6*, and *TSLP* was much higher in TNF-α/IFN-γ–induced IL-32 knockdown HaCaT cells compared with in 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 with in control cells (see Fig E8, *C*). Further analysis indicated that miR-205 mimic transfection in TNF-α/IFN-γ–treated IL-32γ–overexpressing HaCaT cells showed decreased miR-205 expression (see Fig E8, *D*).

IL-32γ attenuates the inflammation in the AD-like RHS model

To confirm the anti-inflammatory effect of IL-32γ on AD development, we used AD-like RHS (the AD-RHS model). We found that rhIL-32γ treatment significantly inhibited epidermal thickness in the AD-RHS model (Fig 5, *A*). AD cocktail–induced NF-κB activation was also reduced in rhIL-32γ–treated RHS tissue samples (Fig 5, *B*). Increased levels of expression of miR-205 and the AD-related cytokines, *TNFA*, *IL1B*, *IL6*, *TLSP*, and IL32G, were suppressed by rhIL-32γ treatment (Fig 5, *C* and *D*).

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

We examined the therapeutic effect of IL-32γ in the PA-induced experimental AD model. The administration of recombinant human IL-32γ protein (rhIL-32γ) reduced PA-mediated AD development (Fig 6, *A*). Histologic analysis showed that rhIL-32γ treatment reduced dermal infiltration of mast cells and epidermal thickness (Fig 6, *A*). The protein levels of AD-related cytokines, including TNF-α, TLSP, IL-4, IL-13, IL-31, and IL-33, in skin tissue samples and serum IgE levels were significantly reduced by rhIL-32γ treatment (Fig 6, *B* and *C*). Moreover, treatment with rhIL-32γ reduced the mRNA levels of AD-related cytokines compared with those in PA-induced AD skin tissue samples (see Fig E5, *D*). We also found that administration of rhIL-32γ inhibited miR-205 expression and NF-κB activity in PA-induced mice (Fig 6, *D* and *E*).

Clinical correlation of IL-32γ and miR-205 in human AD

In this study, the expression of IL-32γ resulted in inhibition of AD pathogenesis by suppression of miR-205 expression through the regulation of NF-κB signaling. Using ELISA, we analyzed the levels of IL-32γ, miR-205, the recently discovered AD biomarkers IL-31 and IL-33 in the serum of patients with AD. The serum levels of IL-32γ, IL-33, and miR-205 were significantly increased in patients with AD compared with the levels in healthy controls (Fig 7, *A*). However, the serum level of IL-31 was not different between patients with AD and healthy controls (Fig 7, *A*). The serum levels of IL-32γ were 85.07 plus or minus 21.14 pg/mL in healthy controls and 116.14 plus or minus 32.62 pg/mL in patients with AD. The serum levels of IL-33 were 1056.856 plus or minus 1244.015 pg/mL in healthy controls and 2080.446 plus or minus 1485.41 pg/mL in patients with AD. The expression levels of miR-205 were 0.00029 plus or minus 0.000133 in healthy controls and 0.000457 plus or minus 0.000265 in patients with AD. We further performed a receiver operating characteristic (ROC) analysis for the diagnostic

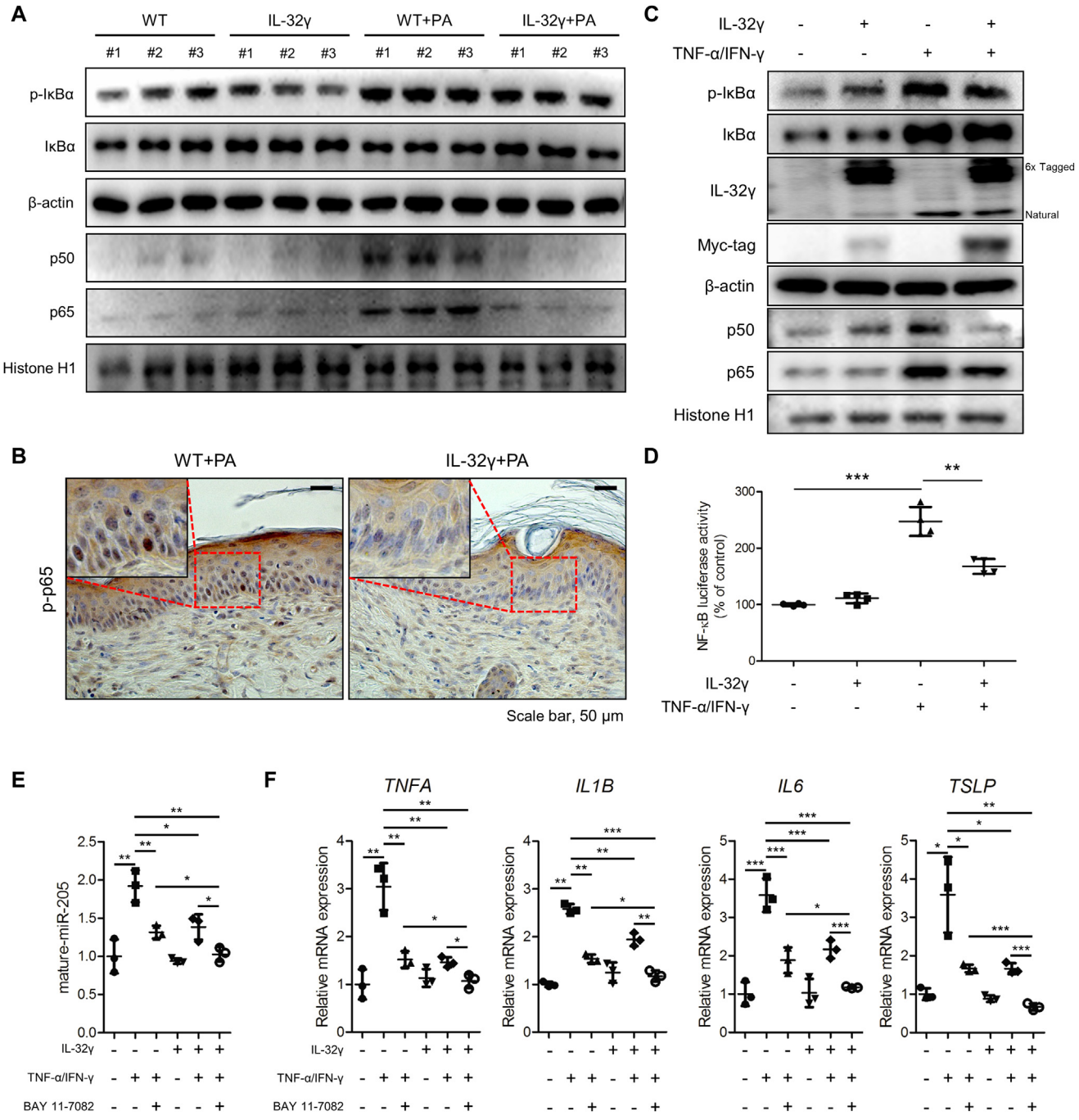

FIG 4. IL-32γ suppresses NF-κB activation. **A,** Expression of phosphorylated IκBα in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions in PA-induced skin tissue samples by Western blot analysis. **B,** Expression of phosphorylated p65 (p-p65) in PA-induced skin tissue samples by immunohistochemistry analysis. Scale bar = 50 μm. **C** and **D,** HaCaT cells were transfected with IL-32γ plasmid vector. After 24 hours, cells were treated with TNF-α/IFN-γ for 24 hours. Expression of phosphorylated IκBα in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions by Western blot analysis (**C**). NF-κB transcriptional activity was measured by luciferase assay by using transfection of luciferase-expressing NF-κB plasmid vector (**D**) (n = 4). **E** and **F,** HaCaT cells were transfected with IL-32γ plasmid vector. After 24 hours, cells were treated with TNF-α/IFN-γ for 24 hours. The levels of expression of miR-205 (**E**) and the proinflammatory cytokines *TNFA*, *IL1B*, *IL6*, and *TSLP* (**F**) were analyzed by quantitative real-time PCR (n = 3). **P* < .05; ***P* < .01; ****P* < .001.

reference. The AD diagnosis cutoff 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 cutoff value, sensitivity, specificity, and AUC of IL-32γ were 88.45, 85%, 85%, and 0.8850, respectively (Fig 7, *A*). The AD diagnosis cutoff value, sensitivity, specificity and AUC of IL-33 were 1524, 65%, 65%, and 0.7275, respectively (Fig 7, *A*). The AD diagnosis cutoff value, sensitivity, specificity and AUC of miR-205 were

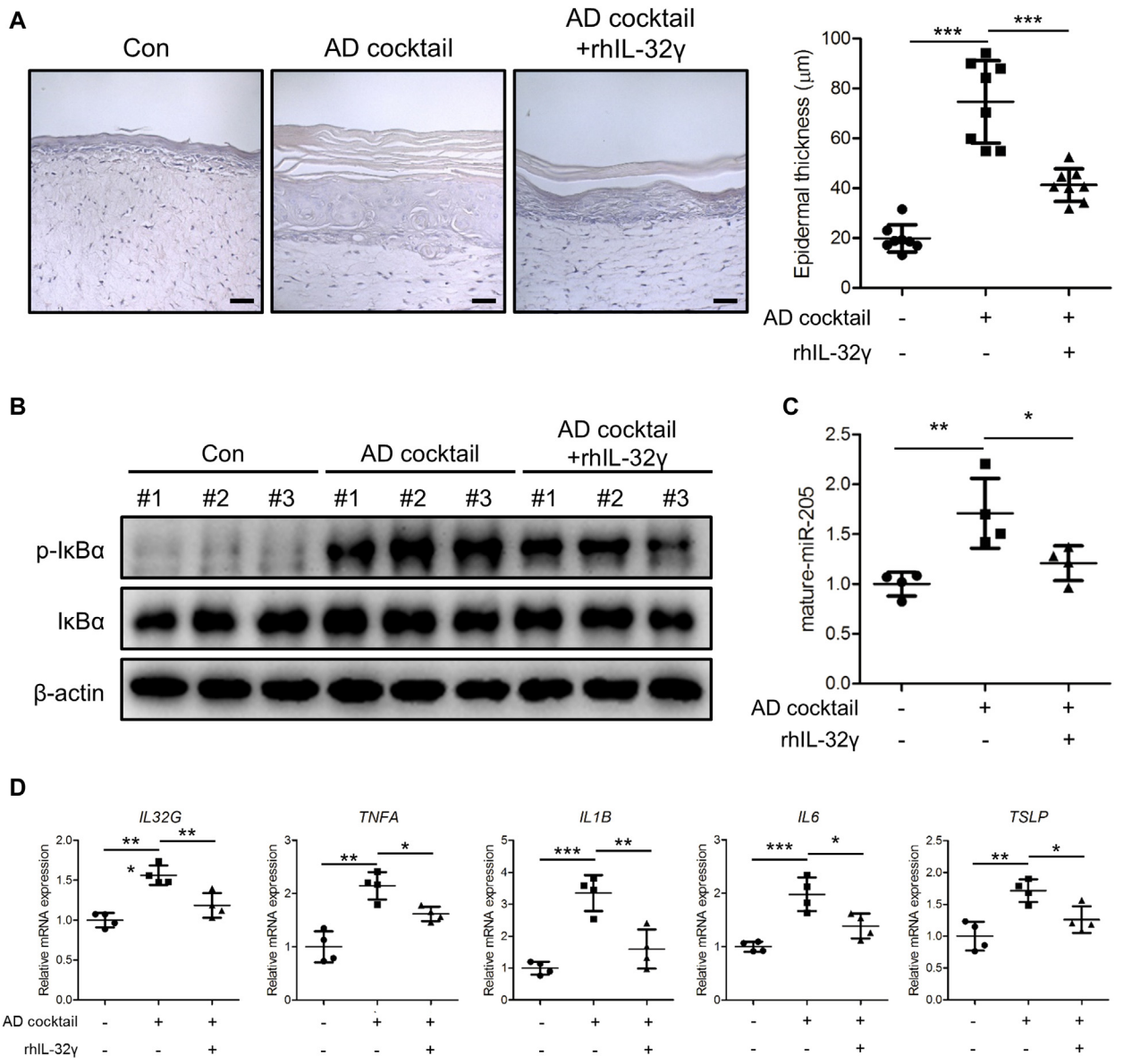

FIG 5. IL-32γ reduces the inflammation in AD-like RHS models. RHS model inserts were cultured for AD cocktail with or without rhIL-32γ for 6 days. **A,** Histologic changes in the AD-RHS model. Bar graph presents epidermal thickness (n = 6). Scale bar = 50 μm. **B,** Phosphorylation of IκBα in the AD-RHS model. **C,** Expression of mature miR-205 in the AD-RHS model (n = 4). **D,** mRNA expression of IL32G, *TNFA*, *IL1B*, *IL6*, and *TSLP* in the AD-RHS model (n = 4). **P* < .05; ***P* < .01; ****P* < .001. *Con*, Control.

0.000344, 60%, 60%, and 0.72, respectively (Fig 7, *A*). Thus, ROC analysis indicated that IL-32γ is the most significant and a good diagnostic reference for AD. The Spearman correlation test was used to evaluate the correlation between and IL-31, IL-32γ, and IL-33 for miR-205 in patients with AD. We found that the serum level of miR-205 correlated with the serum level of IL-32γ ($r = 0.5383$; $P = .0143$; Fig 7, *B*). However, the serum levels of IL-31 and IL-33 were not correlated with the serum level of miR-205 ($r = 0.1579$ and $P = .5061$ for IL-31-miR-205; $r = 0.2586$ and $P = .2709$ for IL-33-miR-205; Fig 7, *B*). A previous study indicated that IL-32β is the predominant isoform under inflammatory conditions.[43] Thus, we further analyzed the IL-32β level in patients with AD. The results showed that serum IL-32β level was no different between patients with AD and healthy controls (521.26 ± 182.75 pg/mL in healthy controls and 446.04 ± 213.94 pg/mL in patients with AD; see Fig E9 in the Online Repository available at www.jacionline.org). Moreover, ROC analysis indicated that the AD diagnosis cutoff value, sensitivity, specificity, and AUC of IL-32β were 430, 65%, 65%, and 0.6725, respectively. These values are much lower than those for IL-32β (see Fig E9).

DISCUSSION

AD is characterized by inflammation and numerous inflammatory cell infiltrations, mainly comprising T_H2 cells, macrophages, neutrophils, mast cells, and eosinophil infiltrates in AD skin lesions, and an increase of epidermal and dermal thickness.[44]

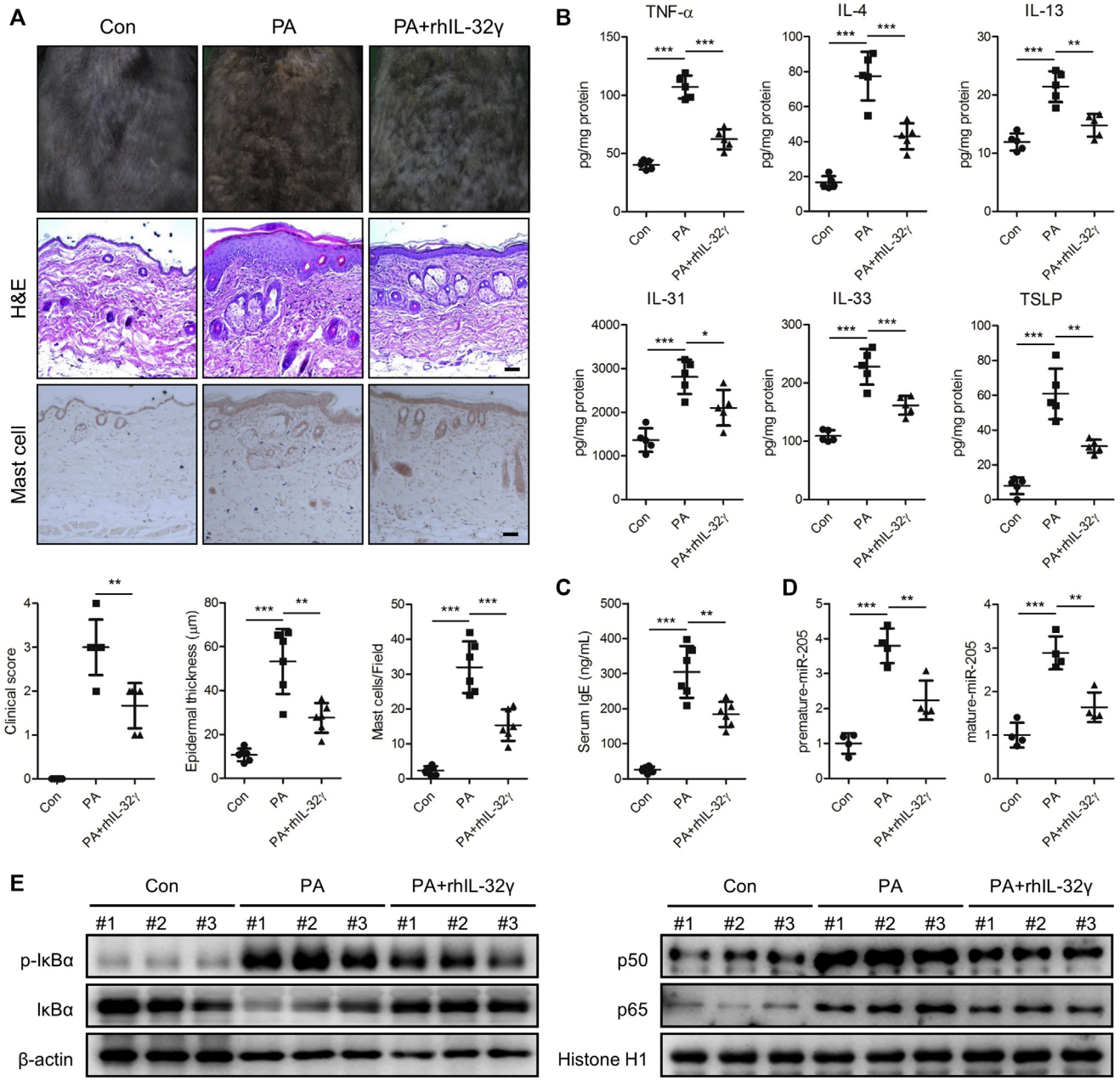

FIG 6. rhIL-32γ therapy alleviates experimental AD development. WT mice were treated with 5% PA for 4 weeks. From the third week, rhIL-32γ (1 μg per mouse) was subcutaneously injected under dorsal skin 3 hours after PA treatment. **A,** Morphologic and histologic changes in the mice after rhIL-32γ therapy. Bar graphs indicate clinical score, epidermal thickness, and mast cell number. (n = 6). Scale bar = 50 μm. **B,** The protein levels of TNF-α, IL-4, IL-13, IL-31, IL-33, and TSLP in skin tissue samples (n = 5). **C,** Serum concentration of IgE (n = 6). **D**, Expression of premature and mature miR-205 in skin tissue samples (n = 4). **P* < .05; ***P* < .01; ****P* < .001. **E,** Expression of phosphorylated IκBα in cytosolic fractions and nuclear translocation of p50 and p65 in nuclear fractions by Western blot analysis in PA-induced WT mice with and without rhIL-32γ therapy. *Con*, Control; *H&E*, hematoxylin and eosin.

In this study, IL-32γ mitigated histologic changes, such as epidermal hyperplasia and infiltration of inflammatory cells. Immunohistochemistry analysis showed accumulation of various inflammatory cells in PA-induced skin tissue samples, but IL-32γ overexpression repressed inflammatory cell infiltration in skin tissue samples. Antigen-specific IgE contributes to the perpetuation of allergic inflammation, potentially through the promotion of IL-4–, IL-5–, and IL-13–secreting activated T_H2 cells. IL-32γ 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-32γ has an inhibitory effect on the development of AD by regulation of cytokine release and inflammatory cell infiltration.

IL-32, which was originally named natural killer cell transcript 4, is a recently evaluated inflammatory cytokine.[45] Its transcriptions are highly expressed in immune tissue samples.[46]

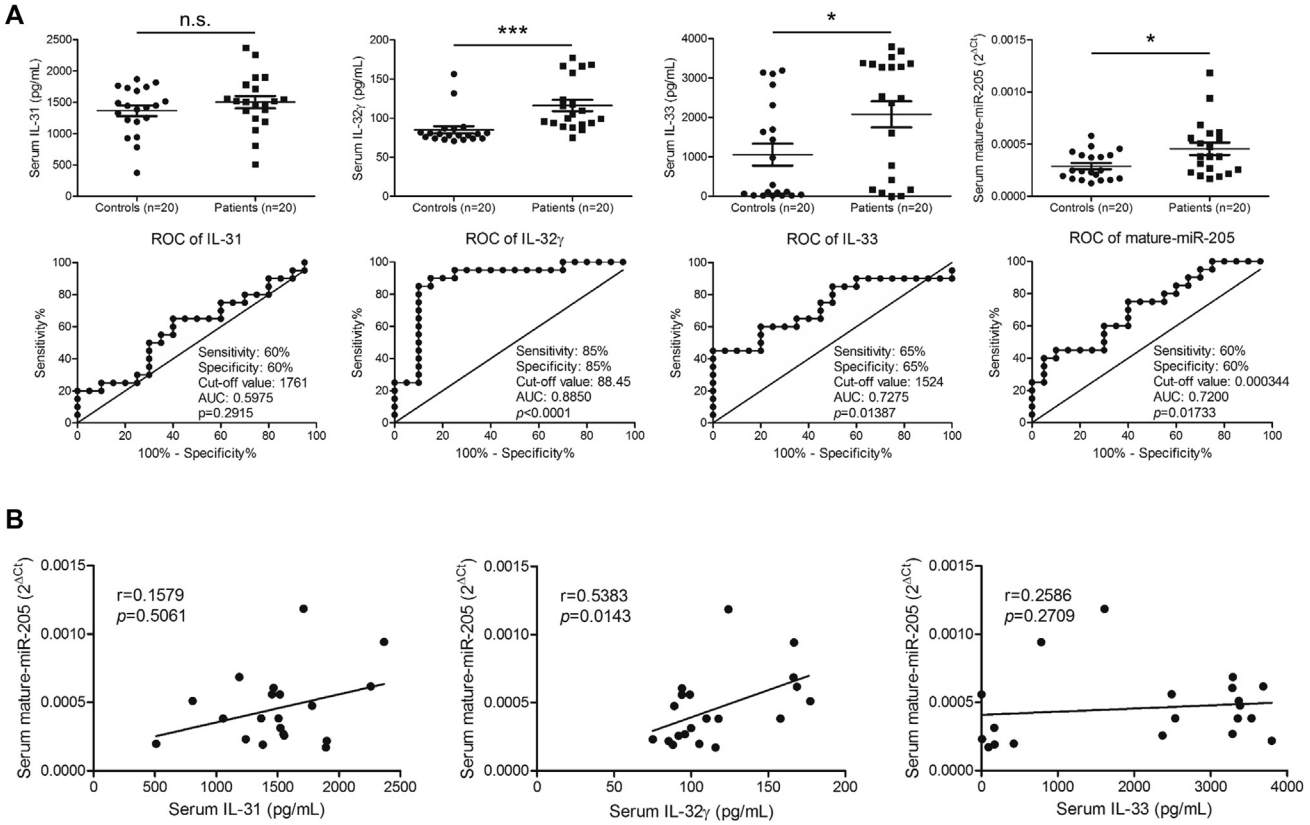

FIG 7. Serum analysis of IL-31, IL-32γ, IL-33, and miR-205 in patients with AD. **A,** The serum levels and ROC curves of IL-31, IL-32γ, IL-33, and miR-205 in patients with AD and healthy controls (n = 20). **B,** Spearman correlation test results between IL-31, IL-32γ, and IL-33 for miR-205. **P* < .05; ****P* < .001. *n.s.*, Not significant.

IL-32 has been reported to induce diverse proinflammatory cytokines such as TNF-α, IL-1α, IL-1β, and IL-6, especially in the early phase of the inflammatory process.[47] However, a previous study demonstrated that IL-32 isoforms have an anti-inflammatory role in various inflammatory diseases.[13] Recent studies revealed that IL-32γ overexpression inhibits the production of inflammatory cytokines in skin cancer, colon cancer, and liver injury.[17,19,48] In addition, IL-32γ overexpression increased IL-10 production under normal conditions and during inflammation.[14,19,49] IL-10 has an immunosuppressive effect on allergic inflammation that regulates T_H2-type cytokine production.[50,51] Thus, IL-32γ could in part suppress AD-like inflammation through an increase in IL-10 expression. On the other hand, previous studies revealed that IL-32 contributed to the pathogenesis of AD through an increase in keratinocyte apoptosis.[16] In this study, expression of IL-32γ was increased in TNF-α/lFN-γ–treated HaCaT cells. Serum levels of IL-32γ were also elevated in patients with AD. Thus, IL-32γ appears to be involved in inflammatory responses. However, the anti-inflammatory role of IL-32γ overexpression was proved through experimental AD models. The PA-induced AD model demonstrated that the increased expression of IL-32γ and administration of rhIL-32γ significantly decreased progression of AD by reduction of AD-related cytokines and inflammatory cell recruitment. In an *in vitro* study, IL-32γ overexpression reduced the expression of inflammatory cytokines in response to TNF-α and lFN-γ in HaCaT cells. Therefore, the increase in IL-32γ 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 have indicated that T_H2 and T_H2-promoting cytokines mainly contribute to AD pathogenesis. Recent studies have indicated that IL-31 enhances brain-derived natriuretic peptide which plays a central role for itching, release and synthesis of cytokine and chemokine in skin cells.[52] IL-31–overexpressing transgenic mice closely resemble the AD skin phenotype.[53] Skin-specific IL-33–expressing transgenic mice also closely resemble the AD skin phenotype by virtue of increased levels of T_H2 cytokines and chemokines and activation of ILC2 cells.[54] IL-33 level was higher in skin tissue samples from patients with AD compared with in samples from controls.[55] Keratinocytes expressing IL-33 could activate and recruit type 2 cells, such as skin-resident ILC2 cells, mast cells, eosinophils, and T_H2 cells, including allergic inflammation.[5,56,57] These cells also release IL-31 in skin tissue samples, and IL-31 enhances inflammatory cytokine production and mediates pruritus.[58] Therefore, the newly discovered AD cytokines IL-31 and IL-33 are important in AD pathogenesis. However, IL-32γ adequately blocked IL-31 and IL-33 expression and inflammatory cell infiltration in PA- and MC903-induced AD tissue samples. Our results show that serum levels of IL-32γ and IL-33 are elevated in patients with AD but serum level of IL-31 is not significantly changed compared with that in healthy controls, even though previous studies indicated that IL-31 was increased in tissue samples and

serum of patients with AD.[52,59,60] ROC analysis with samples from patients with AD indicated that serum IL-32γ level was distinguished most significantly between patients with AD and healthy controls, but serum levels of IL-33 were slightly distinguished. Additionally, a previous study indicated that IL-32β is the predominant isoform of IL-32 and is predominantly increased by various stimulators.[43] The serum analysis indicated that the IL-32β level was 6-fold higher than the IL-32γ level in healthy controls, but the IL-32β level was not much different between healthy controls and patients with AD. Moreover, ROC analysis indicated that the AD diagnosis cutoff value, sensitivity, specificity, and AUC of IL-32β are much lower than those of IL-32γ. We also investigated the effect of rhIL-32α; an effect on PA-induced AD was found, but the effect was much smaller than that of IL-32γ (data not shown). Thus, unlike IL-31 and IL-33, IL-32γ as a predominant isoform of IL-32 could prevent the development of AD through reduction of cytokine production and AD-related immune cell infiltration.

MicroRNAs are involved in regulation of the normal development of the immune system and in the pathogenesis of chronic inflammatory disorders.[61] Recently, various miRNAs were found to be dysregulated in patients with AD, implying their general role in skin inflammation.[42] It has been 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 cells.[25] Serum and urine levels of miR-205 are significantly upregulated in children with AD compared with in the healthy control group.[42] In the present study, we found that miR-205 played a critical role in inflammatory responses and AD pathogenesis. Moreover, our previous study found that IL-32α suppresses endothelial inflammation via inhibition of miR-205 expression.[31] IL-32γ suppressed the AD-like skin inflammation in PA- and MC903-induced mice by inhibition of miR-205. We also observed that administration of rhIL-32γ alleviated PA-induced AD pathogenesis by inhibition of miR-205 expression as well as allergic inflammatory responses. We further found that IL-32γ 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 proinflammatory cytokines whereas miR-205 mimic increased the expression of proinflammatory cytokines in TNF-α/lFN-γ–treated HaCaT cells. Serum analysis also indicated that the level of miR-205 is significantly increased in patients with AD. Spearman correlation analysis showed that serum levels of IL-32γ and miR-205 are significantly correlated in patients with AD. These results indicate that IL-32γ reduced AD pathogenesis by inhibition of miR-205–mediated inflammatory responses.

NF-κB is an important factor in regulation of proinflammatory cytokines and chemokines in AD.[28] Reduced NF-κB activation in CD4 T cells decreases AD-like skin inflammation in an NC/Nga mouse AD model.[62] Topical application of NF-κB inhibitor effectively suppresses allergic inflammation, such as inflammatory cytokine production and infiltration of inflammatory cells in NC/Nga mouse AD model.[28] We found that IL-32γ overexpression decreased NF-κB activity in PA- and MC903-induced skin tissue samples and TNF-α/IFN-γ–induced HaCaT cells. IL-32γ overexpression could block the translocation of NF-κB transcription factor through suppression of IκBα phosphorylation. NF-κB signaling is associated with various miRNAs.[63] miR-146a suppressed chronic skin inflammation by inhibition of NF-κB signaling through downregulation of caspase recruitment domain-containing protein 10, NF-κB upstream factor. miR-124 inhibited p65 expression by direct binding to reduce inflammatory responses in human primary keratinocytes.[30] Previous studies have shown that NF-κB signaling regulates miR-205 expression.[32] Web-based miRNA-gene network analysis showed that RELA (p65) regulates miR-205. *In vivo* and *in vitro* studies have revealed that IL-32γ suppresses NF-κB signaling in PA-induced skin tissue samples and TNF-α/IFN-γ–induced HaCaT cells. Combination of the IL-32γ and NF-κB 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-32γ–mediated NF-κB inactivation contributes to prevention of AD pathogenesis through suppression of miR-205 expression.

Taken together, these data demonstrate that unlike IL-31 and IL-33, IL-32γ suppressed AD via inhibition of miR-205 expression through inactivation of the NF-κB pathway.

Clinical implications: This study provides new insight regarding IL-32γ as a potential therapeutic molecule for treatment of AD.

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