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Advanced Glycation End Products-Induced Activation of Keratinocytes: A Mechanism Underlying Cutaneous Immune Response in Psoriasis

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Keywords
Advanced glycation end products · Keratinocyte · Interleukin-36 alpha · Signal transducer and activator of transcription 1/3 · Psoriasis

Abstract
Psoriasis is a common inflammatory skin disease, in which epidermal keratinocytes play a vital role in its pathogenesis by acting both as the responder and as the accelerator to the cutaneous psoriatic immune response. Advanced glycation end products (AGEs) are a class of proinflammatory metabolites that are commonly accumulating in cardiometabolic disorders. Recent studies have also observed the increased level of AGEs in the serum and skin of psoriasis patients, but the role of AGEs in psoriatic inflammation has not been well investigated. In the present study, we initially detected abnormal accumulation of AGEs in epidermal keratinocytes of psoriatic lesions collected from psoriasis patients. Furthermore, AGEs promoted the proliferation of keratinocytes via upregulated Keratin 17 (K17)-mediated $p27^{KIP1}$ inhibition followed by accelerated cell cycle progression. More importantly, AGEs facilitated the production of interleukin-36 alpha (IL-36α) in keratinocytes, which could enhance T helper 17 (Th17) immune response. In addition, the induction of both K17 and IL-36α by AGEs in keratinocytes was dependent on the activation of signal transducer and activator of transcription 1/3 (STAT1/3) signaling pathways. At last, the effects of AGEs on keratinocytes were mediated by the receptor for AGEs (RAGE). Taken together, these findings support that AGEs potentiate the innate immune function of keratinocytes, which contributes to the formation of psoriatic inflammation. Our study implicates AGEs as a potential pathogenic link between psoriasis and cardiometabolic comorbidities.

Introduction

Psoriasis is a common immune-mediated inflammatory skin disease that is clinically characterized by sharply marginated erythematous papules or plaques covered by adherent silvery scales [1]. It is widely accepted that the disorder of adaptive immunity led by T cells plays a central role in the pathogenesis of psoriasis [2, 3]. Skin-infiltrated T cells, especially T helper (Th) cells, produce a series of cytokines such as interferon-gamma (IFN-γ), interleukin-17 (IL-17), and interleukin-22 (IL-22) that cause the hyperproliferation of epidermal keratinocytes, which is a key event in the formation of psoriasis [4]. Intriguingly, as a kind of epithelial cell with strong innate

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immune potency, keratinocytes are not only "victims" but also "criminals" of psoriatic inflammation [5]. Activated keratinocytes are the main generator of chemokines like C-X-C motif chemokine ligand 10 (CXCL10) and C-C motif chemokine ligand 20 (CCL20) that mediate the cutaneous infiltration of T cells as well as the source of certain cytokines like interleukin-36 (IL-36) family members that are crucial contributors to the psoriatic cytokine circuit [1, 6, 7]. Nevertheless, the mechanism underlying the activation of innate immune function of keratinocytes in psoriasis is still not fully clarified.

It has been noted that psoriasis is not merely a simple dermatosis but is associated with a variety of comorbidities, especially cardiometabolic diseases including cardiovascular diseases as well as obesity, hypertension, dyslipidemia, and diabetes that are also the components of metabolic syndrome [1, 8]. Indeed, cardiometabolic comorbidities have been recognized as vital parameters to be considered during the management of psoriasis [9, 10]. Based on these clinical findings, a series of laboratory studies have indicated that overlapped inflammatory response led by immune cells like T cells and multiple proinflammatory cytokines gives rise to the concurrence of psoriasis and cardiometabolic disorders [11–13]. Moreover, it is worth noting that metabolic system has been shown to influence immunity. For instance, adipose tissue secretes a group of proteins called adipokines including leptin, visfatin, and resistin that could promote the immune response in psoriasis [11, 14]. In addition, hypertrophic adipocytes could even directly produce cytokines like interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α), which are referred to as adipocytokines and link the pathogenesis of obesity and psoriasis [15]. Therefore, metabolic abnormality could play a vital role in psoriatic inflammation, which is well worth further investigation.

Advanced glycation end products (AGEs) are a group of heterogeneous metabolites generated via irreversible non-enzymatic interactions that are called Maillard reactions between reducing sugars and proteins, lipids, or nucleic acids [16, 17]. By binding to receptor for advanced glycation end products (RAGE) that is a pattern recognition receptor (PRR) widely expressed on cell surface and a crucial driver of innate immune response [16, 18], AGEs activate multiple proinflammatory signaling pathways and further lead to the production of various immune mediators. Excessive formation and accumulation of AGEs have been indicated to contribute to the development of a variety of metabolic disorders, including diabetes, atherosclerosis, and cardiovascular diseases [16, 19, 20]. Notably, a number of studies have reported that both the serum and the cutaneous level of AGEs is elevated in psoriasis [21, 22], especially in the patients with severe psoriasis [23]. Based on our previous finding that RAGE is overexpressed in lesional keratinocytes of psoriasis [24], we hypothesized that increased AGEs could activate the innate immune function of keratinocytes and promote the cutaneous immune response in psoriasis.

To testify this hypothesis, the present study focused on investigating the proinflammatory effects of AGEs on keratinocytes and the influence of AGE-primed keratinocytes on T-cell response, trying to figure out the role of AGEs in the immune pathogenesis of psoriasis and provide a viewpoint on the crosstalk between metabolic abnormality and psoriatic inflammation.

Materials and Methods

Patients and Clinical Samples

Serum samples were collected from 30 patients with plaque psoriasis whose disease severity was assessed via psoriasis area and severity index (PASI) scoring and 30 age- and sex-matched healthy donors. Detailed and summarized demographic and clinical information of these donors are described in online suppl. Tables 1, 2 and 3 (for all online suppl. material, see https://doi.org/10.1159/000534639). Lesional and peri-lesional skin samples were collected from 5 patients with plaque psoriasis who accepted biopsy at the Department of Dermatology, Xijing Hospital. Normal skin samples were collected from 5 healthy volunteers who underwent cosmetic surgery at Department of Plastic Surgery, Xijing Hospital. Peripheral blood mononuclear cells (PBMCs) were collected from 5 patients with plaque psoriasis. All the patients claimed no other autoimmune diseases and had not been receiving any systemic treatment within 1 month prior to sample collection. Informed consent was obtained from all the patients and healthy donors.

Keratinocyte Culture and Treatment

Normal human keratinocytes (NHKs) were extracted from the prepuces donated by healthy individuals who accepted circumcision. All the donors provided informed consent. NHKs were cultured in serum-free keratinocyte growth medium (MEPI500CA, Gibco, USA) supplemented with human keratinocyte growth supplement (S0015, Gibco, USA), and the second- or third-passage NHKs were used in the study. BSA (2221-BSA, BioVision, USA) or BSA-AGEs (2221, BioVision, USA) were used to treat NHKs at different concentrations as indicated. Small interfering RNA (siRNA) targeting STAT1 (STAT1 siRNA) (sc-44123, Santa Cruz Biotechnology, USA), STAT3 (STAT3 siRNA) (sc-29493, Santa Cruz Biotechnology, USA), or RAGE (RAGE siRNA) (sc-36374, Santa Cruz Biotechnology, USA) were transfected into NHKs by using Lipofectamine 3000 (L3000015, Invitrogen, USA) according to the manufacturer's recommendations.

PBMC Isolation and Treatment

PBMCs were isolated using density gradient centrifugation with lymphocyte separation solution (7111011, Dakewe, China) according to the manufacturer's instructions. Briefly, the same volumes of whole blood obtained from psoriasis patients, sterile phosphate buffer solution (PBS), and lymphocyte separation solution were mixed together and centrifuged at 2,000 rpm for 20 min, with the acceleration level set as 1. The intermediate layer was transferred to a new sterile centrifuge tube with equal volume of sterile PBS added, and centrifuged at 1,300 rpm for 10 min, with the acceleration level set as 3. The supernatant was discarded and red blood cell lysis solution was added and then centrifuged at 1,300 rpm for 10 min, with the acceleration level set as 3. Finally, the supernatant was discarded, and the pellet was PBMCs. Isolated PBMCs were incubated by the supernatants of differently pretreated NHKs with the addition of 2 pg/mL IL-36α neutralizing antibody (IL-36α N) (MAB10781, R&D Systems, USA) or 100 ng/mL IL-36R antagonist (IL-36R A) (HY-P71965, MedChemExpress, USA) into the incubation system or stimulated by 1 μg/mL recombinant human IL-36α (rhIL-36α) (1078-IL-025/CF, R&D Systems, USA).

Enzyme-Linked Immunosorbent Assay

Human AGEs Quantitative Enzyme-Linked Immunosorbent Assay (ELISA) Kit (ml062867, Enzyme-linked Biotechnology, China) was used to detect the level of AGEs in serum samples according to kit instructions. Human IL-36α Quantitative ELISA Kit (EHC057a, NeoBioscience, China), human IL-36β Quantitative ELISA Kit (EHC058b, NeoBioscience, China), human IL-36γ Quantitative ELISA Kit (EHC056g, NeoBioscience, China), and human IL-17A Quantitative ELISA Kit (E-EL-H5812c, Elabscience, China) were used to analyze serum or cell culture supernatant samples according to the manufacturer's instructions. Absorbance was measured at 450 nm by Model 680 Microplate Reader (Bio-Rad, United States).

Immunofluorescence Analysis

Deparaffinized 5-μm skin sections were subjected to heat-mediated antigen retrieval with Tris-EDTA buffer (pH 9.0), followed by blocking with 5% normal goat serum for 1 h, except that the slides for detection of AGEs were blocked with 1% BSA/10% goat serum/0.3 M glycine in 0.1% PBST following manufacturer's instructions. Cultured cells on coverslips were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min, followed by blocking with 5% normal goat serum for 30 min. Samples were then incubated with primary antibodies (AGEs Rabbit mAb, 1:200, ab23722, Abcam, UK; IL-36α Rabbit mAb, 1:200, ab269271, Abcam, UK; p-STAT1 Rabbit mAb, 1:200, 9167, Cell Signaling Technology, USA; p-STAT3 Rabbit mAb, 1:200, 9145, Cell Signaling Technology, USA; and Rabbit mAb IgG isotype control, 1:200, ab172730, Abcam, UK) as isotype control at 4°C overnight and with corresponding secondary antibodies (Goat anti-Rabbit IgG Green 488, 1:200, ab150077, Abcam, UK; Goat anti-Rabbit IgG Red Cy3, 1:200, ab6939, Abcam, UK) at room temperature for 1 h away from light. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (1:1,000, ab104139, Abcam, UK) at room temperature for 10 min in the dark. Samples were washed three times with PBS after each step. The fluorescence was detected by laser confocal microscopy (#LSM510, Carl Zeiss AB, Germany). ImageJ 64 software was utilized to analyze the fluorescence intensity. The average fluorescence intensity of three randomly fields per sample was calculated and data were expressed in fold of control.

Cell Viability Assay

Cell viability was detected by the Cell Counting Kit-8 (CCK8) assay (C008, 7 Sea Biotech, China) and resazurin-conversion assay (QDY-002-C, RHINO BIO, China). According to the manufacturer's instructions, 4×10^3 cells were seeded in each well of a 96-well culture plate overnight. Cells were treated as indicated and then incubated with CCK8 reagents (10 μL diluted in 100 μL fresh medium) at 37°C for 1 h or with 20 μL resazurin dye reagent directly added into previous supernatants at 37°C for 8 h. Cell viability was indicated by absorbance detected at 450 nm by Model 680 Microplate Reader (Bio-Rad, USA).

Annexin V-FITC/Propidium Iodide Apoptosis Assay

Cell apoptosis was detected with the Annexin V-FITC/propidium iodide (PI) cell apoptosis kit (A005, 7 Seas Biotech, China) according to the manufacturer's instructions. In short, cells were collected and resuspended in 400 μL binding buffer. Then, 5 μL of Annexin V-FITC and 10 μL PI were added and the cells were incubated at room temperature for 15 min in the dark. The apoptosis rates were detected by flow cytometry (FC500, Beckman Coulter, Miami, FL, USA) and analyzed with Expo32 software (Beckman Coulter, USA).

5-Ethynyl-2′-Deoxyuridine (Edu) Proliferation Assay

EdU proliferation assay was detected using Cell-Light EdU Apollo® 567 In Vitro Kit (C10310-1, RiboBio, China). 100 μL medium with 50 μM EdU was added to each well and incubated for 2 h. Subsequently, cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. Cells were cultured with 2 mg/mL glycine for 5 min followed by washing with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min. 100 μL Apollo® staining reaction solution was added to each well and incubated at room temperature for 30 min away from light followed by permeabilized with 0.5% Triton X-100 in PBS and washing with methanol. Cell nuclei were counterstained with Hoechst reagent 33342. Images were taken and analyzed using High-Content Imaging Pathway 855 (BD, Franklin Lakes, NJ, USA). EdU-positive cells were calculated by (EdU add-in cells/Hoechst stained cells) × 100%.

Cell Cycle Analysis

Cells were synchronized by keratinocyte growth supplement starvation for 24 h before treated following manufacturer's instructions. Then cells were harvested by trypsinization and collected by centrifugation. After washing with PBS, the cells were fixed by adding 1 mL prechilled 70% ethanol and placed at 4°C overnight. Removing ethanol by centrifugation, the cells were then washed with PBS and stained with a solution containing RNase A and propidium iodide (C001, 7Sea biotech, China) at 37°C for 30 min away from light. Cell cycle distribution was detected by flow cytometry using a BD FACS instrument (BD Biosciences, USA).

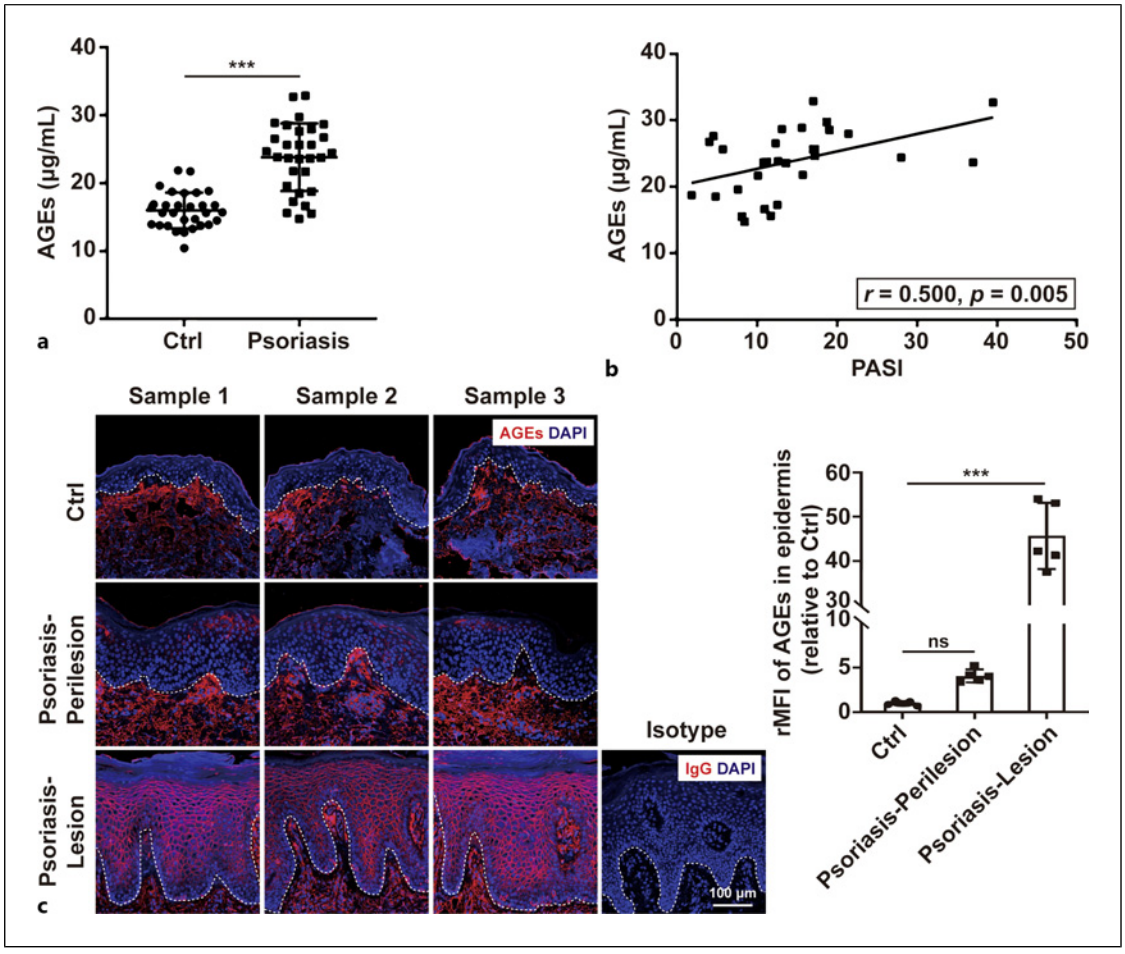
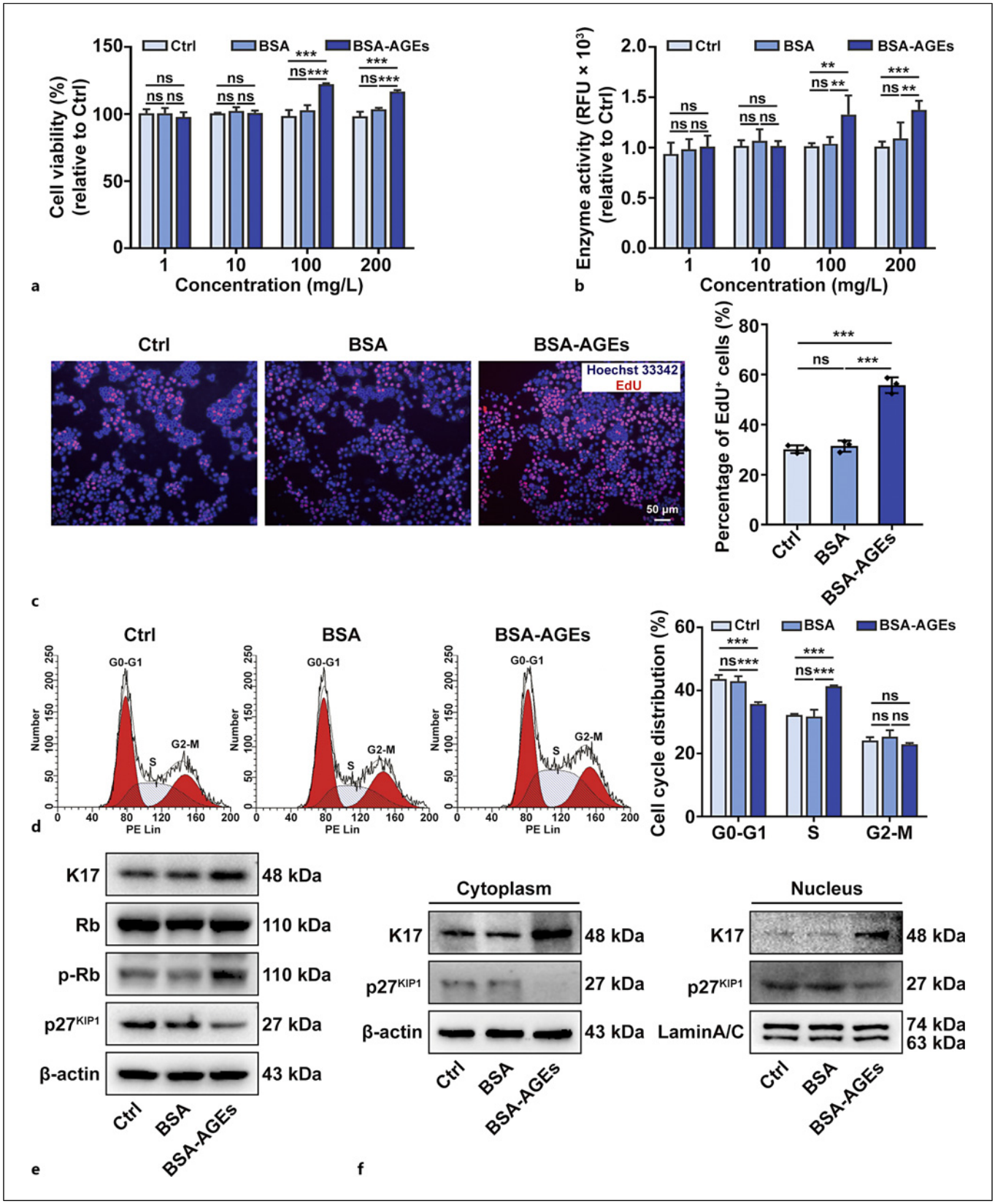


Fig. 1. The level of AGEs was significantly elevated in psoriasis patients. **a** Serum level of AGEs in patients with psoriasis (n = 30) and healthy controls (n = 30) was detected by ELISA. **b** Correlation between serum levels of AGEs and PASI scores was analyzed by Spearman rank correlation test in patients with psoriasis (n = 30). **c** Cutaneous level of AGEs (red) in lesional and peri-lesional skin samples from psoriasis patients (n = 5) and skin samples from healthy controls (n = 5) was detected by immunofluorescence. Representative samples were shown. Nuclei were counterstained with DAPI (blue). Isotype control was shown with a lesional sample. Bar graphs represent the mean ± SD of AGE fluorescence intensity. rMFI: relative mean fluorescence intensity. ***$p < 0.001$.

Western Blot Assay

The cells were washed three times with PBS and lysed with RIPA lysis buffer (P0013C, Beyotime, China) (containing 1% PMSF) at 4°C for 20 min. The supernatant was collected by centrifugation to extract total cellular protein. Nuclear and cytosolic proteins were extracted using nuclear and cytosolic protein extraction kit (P0027, Beyotime, China) according to the manufacturer's instructions. Protein concentrations were detected using the BCA Protein Assay Kit (23227, Thermo Fisher Scientific, USA). Equal amounts of proteins were loaded and separated by 10% SDS-PAGE (Bio-Rad, USA) and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with 5% nonfat milk for at room temperature 1 h and then incubated with primary antibodies against cytokeratin 17 (ab109725, Abcam, UK), retinoblastoma protein (Rb) (4H1) (9309, Cell Signaling Technology, USA), phospho-Rb (Ser807/811) (D20B12) (8516, Cell Signaling Technology, USA), $p27^{KIP1}$ (D69C12) (3686, Cell Signaling Technology, USA), STAT1 (D1K9Y) (14994, Cell Signaling Technology, USA), phospho-STAT1 (Tyr701) (58D6) (9167, Cell Signaling Technology, USA), STAT3 (D3Z2G) (12640, Cell Signaling Technology, USA), phospho-STAT3 (Tyr705) (D3A7) (9145, Cell Signaling Technology, USA), RAGE (ab37647, Abcam, UK), β-actin (8H10D10) (3700, Cell Signaling Technology, USA), lamin A/C (4C11) (4777, Cell Signaling Technology, USA) at 4°C overnight. After washed with TBST (TBS + 0.1% [v/v] Tween-20) three times, the membranes were incubated with corresponding secondary antibodies (Goat Anti-Rabbit IgG Antibody, Peroxidase Conjugated, AP132P, Sigma-Aldrich, USA; Goat Anti-Mouse IgG Antibody, Peroxidase Conjugated, AP124P, Sigma-Aldrich, USA) at room temperature for 1 h. The protein bands were detected with the Western Blotting Imaging System (Cat. 871BRO7308, Bio-Rad, USA).



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Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted with Trizol reagent (15596018, Invitrogen, USA) and then reversely transcribed to cDNA with PrimerScript RT reagent kit (AK4301, TaKaRa, Japan). The quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed using SYBR Premix Ex Taq II (AKA1008, TaKaRa, Japan) by the real-time PCR detection system (iQTM5, Bio-Rad, USA). The relative expression of mRNA was normalized to β-actin. Primer sequences are listed as follows: IL-36α (forward 5′-TCAGGATATCAATCATCGGGTG-3′ and reverse 5′-GGCAATAGTGACTGGAGACATA-3′), IL-36β (forward 5′-GAGCTGCTTCATGGGAACCCTTG-3′ and reverse 5′-CCTTCCTGGCATTCCTATGTTGGTC-3′), IL-36γ (forward 5′-AAGGTTGGAGAACAGCCCACATTG-3′ and reverse 5′-GGGTGGAGGTCCTACCAGTCTTG-3′), β-actin (forward 5′-GGCTACAGCTTCACCACCAC-3′ and reverse 5′-TGCGCTCAGGAGGAGC-3′).

Flow Cytometry

PBMCs were harvested and washed with phosphate-buffered saline (PBS). Then PBMCs were incubated with FITC anti-human CD4 antibody (357406, BioLegend, USA) for 30 min. After washing with PBS, PBMCs were fixed with 100 μL Diluent Fixation/Permeabilization Concentrate (00-5523-00-2511819, Invitrogen, USA) for 30 min. Following repeated PBS washes, 1 mL Permeabilization Buffer (00-5523-00-2511274, Invitrogen, USA) was added to incubate for 50 min. Subsequently, PBMCs were incubated with PE Anti-Human RORγt Antibody (12-6988-82, eBioscience, USA), PE Anti-Rat IgG2α, κ Isotype control (12-4321-80, eBioscience, USA), APC Anti-Human IL-17A Antibody (512334, BioLegend, USA) or APC Anti-Mouse IgG1, κ Isotype Control (400141, BioLegend, USA) for 30 min. The stained PBMCs were washed once with PBS and then resuspended in 400 μL PBS for flow cytometric analysis. All reactions were performed at room temperature away from light. The proportion of RORγt-positive or IL-17A-positive cells in CD4-positive T cells was finally analyzed by flow cytometry. The gating strategy is shown in online supplementary Figure 1.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism 7.0 software (GraphPad Software, USA). Demographic data were shown as median ± IQR or percentage. Mann-Whitney U test was used for continuous variables, and χ^2 test was used for categorical variables. Other data were shown as mean ± SD. Dual comparisons were performed with the unpaired 2-tailed Student's *t* test. One-way analysis of variance was used to analyze the experiments with multiple groups, and Tukey's multiple comparisons test was used to compare the mean of each group with the mean of every other group. Correlations between two variables were analyzed using the Spearman rank correlation test. p values of <0.05 were considered statistically significant. Each experiment was performed at least for three times.

Results

The Level of AGEs Is Systemically Elevated in Psoriasis Patients

In order to evaluate the status of AGEs in psoriasis, we first collected serum samples from 30 psoriasis patients and 30 healthy controls and examined the level of AGEs via ELISA assay. It turned out serum level of AGEs was significantly increased in psoriasis patients compared with healthy controls (Fig. 1a, individual value shown in online suppl. Table 1). Moreover, the serum AGEs levels of the patients were positively correlated with PASI scores that represent the disease severity of psoriasis (Fig. 1b). Further immunofluorescence analysis showed constitutive existence of AGEs in the dermis of both healthy skin and psoriatic peri-lesional and lesional skin. However, prominent accumulation of AGEs in epidermal keratinocytes was only seen in psoriatic lesion rather than peri-lesional or healthy skin (Fig. 1c). Collectively, our findings confirm that the level of AGEs is systemically increased in psoriasis patients.

AGEs Promote the Proliferation of Keratinocytes via Upregulating K17 Expression

Given that AGEs are able to induce the proliferation of various types of cells [19, 25–27], we speculated that increased AGEs could contribute to the excessive proliferation of epidermal keratinocytes in psoriasis. To verify this, we first examined the growth of NHKs treated with BSA-AGEs via CCK8 assay and resazurin-reduction assay. As a result, both assays showed that BSA-AGEs at the concentration of 100 mg/L or 200 mg/L significantly promoted the growth of NHKs as indicated by higher cell viability and enzyme activity compared with control or BSA-treated cells (Fig. 2a and b). Based on these findings as

Fig. 2. AGEs promoted the proliferation of keratinocytes via upregulated K17-induced cell cycle progression. **a** Cell viability of NHKs treated with BSA or BSA-AGEs at the indicated concentrations for 48 h was determined by CCK8 assay. **b** Resazurin-conversion assay was used to determine the cell viability of NHKs treated with BSA or BSA-AGEs at the indicated concentrations for 48 h. **c** Cell proliferation of NHKs treated with BSA or BSA-AGEs for 24 h was analyzed by EdU assay. Bar graphs represent the mean ± SD of percentage of EdU^+ cells (red). Nuclei were counterstained with Hoechst 33342 (blue). **d** Cell cycle distribution of NHKs treated with BSA or BSA-AGEs for 24 h was measured using flow cytometry. Bar graphs represent the mean ± SD of percentage of cell cycle phases (G0–G1, S, G2-M). **e** Expression of K17, Rb, phosphorylated Rb, and $p27^{KIP1}$ in NHKs treated with BSA or BSA-AGEs for 24 h was detected by Western blot assay. **f** Expression of K17 and $p27^{KIP1}$ in cytoplasm and nucleus of NHKs treated with BSA or BSA-AGEs for 24 h was detected by Western blot assay. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

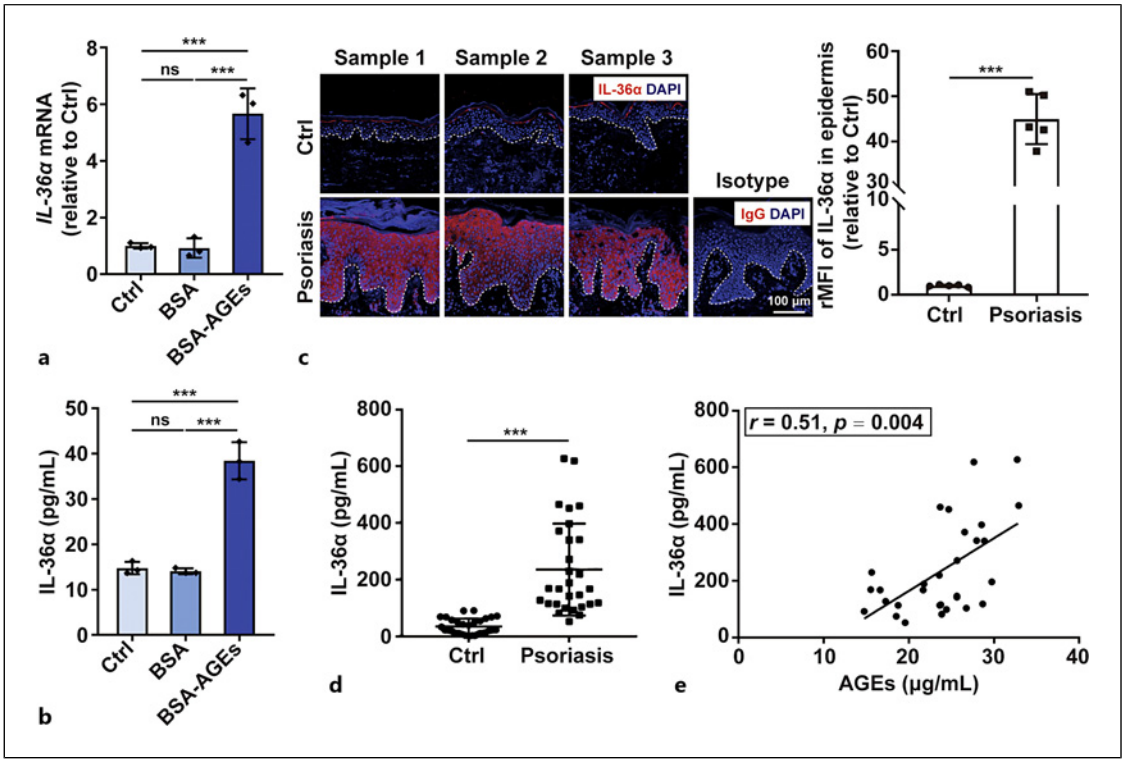


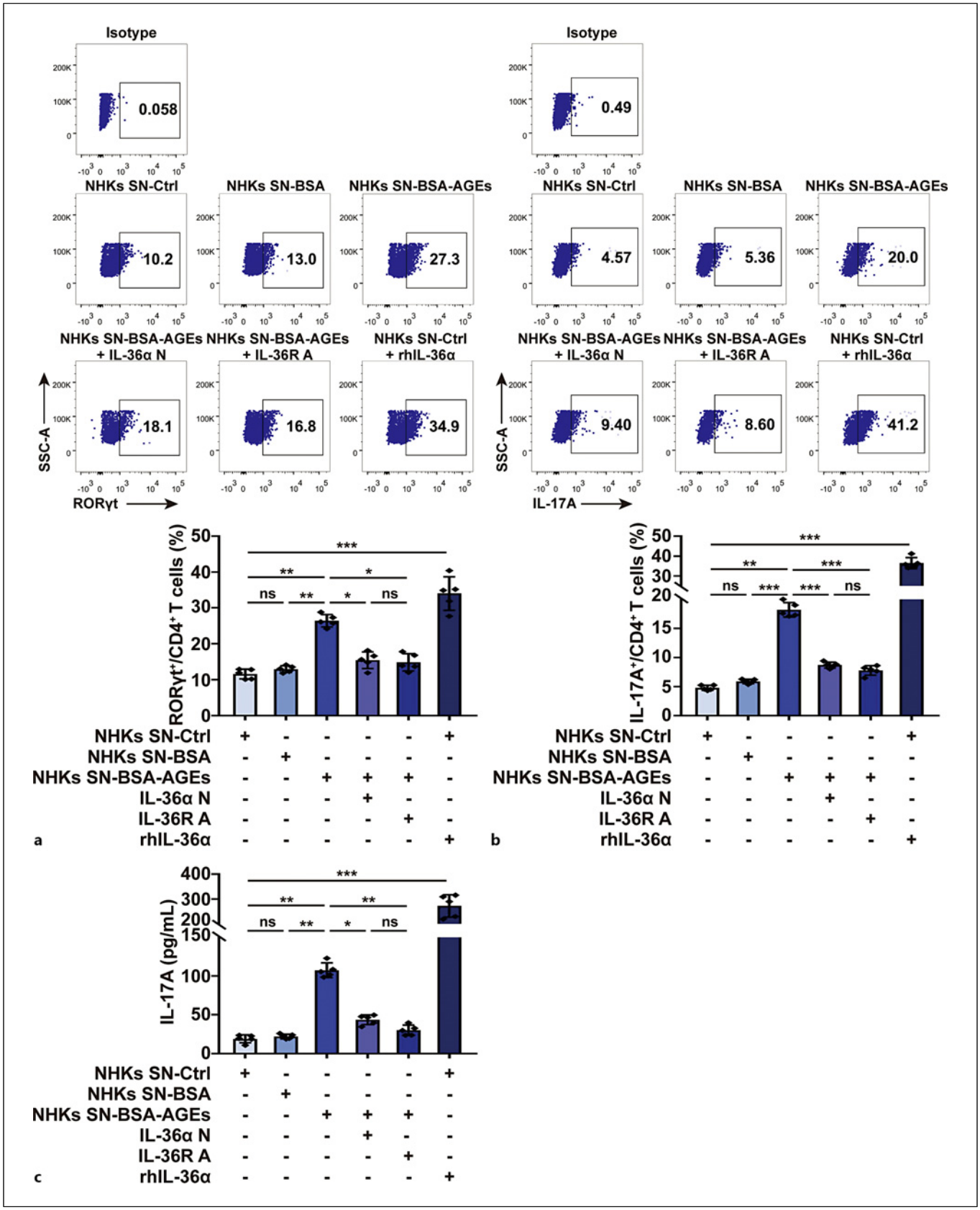
Fig. 3. AGEs facilitated the production of IL-36α from keratinocytes. **a** The mRNA level of IL-36α in NHKs treated with BSA or BSA-AGEs for 24 h was detected by qRT-PCR. **b** Secretion of IL-36α from NHKs treated with BSA or BSA-AGEs for 48 h was detected by ELISA. **c** Expression of IL-36α (red) in lesional samples from psoriasis patients (n = 5), and skin samples from healthy controls (n = 5) was detected by immunofluorescence. Representative samples were shown. Nuclei were counterstained with DAPI (blue). Isotype control was shown with a lesional sample. Bar graphs represent the mean ± SD of IL-36α fluorescence intensity. rMFI: relative mean fluorescence intensity. **d** Serum level of IL-36α in patients with psoriasis (n = 30) and healthy controls (n = 30) was detected by ELISA. **e** Correlation between serum levels of IL-36α and AGEs was analyzed by Spearman rank correlation test in patients with psoriasis (n = 30). *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant.

well as previous reports [28, 29], BSA-AGEs at 100 mg/L were selected to stimulate NHKs thereafter. Subsequent flow cytometry analysis showed no influence of BSA-AGEs on the apoptosis rate of NHKs (online suppl. Fig. 2), whereas the EdU assay displayed more Edu$^+$ cells in the NHKs treated with BSA-AGEs (Fig. 2c), indicating that the enhanced growth of NHKs treated with BSA-AGEs was due to the proliferation of the cells.

Previous studies have reported that keratin 17 (K17), a key cytoskeleton protein for the proliferation of keratinocytes in psoriasis, accelerates cell cycle progression via mediating nuclear export and degradation of p27^{KIP1} that is a crucial regulator of G1-to-S phase transition in cell cycle [30]. We therefore went on to investigate whether this K17-led mechanism was involved in AGE-induced keratinocyte proliferation. As shown in our flow cytometry analysis, BSA-AGEs decreased the percentage of cells at G1 phase accompanied with significantly more cells into S phase, thus implicating a facilitated cell cycle progression in NHKs treated with BSA-AGEs (Fig. 2d). Further Western blot assay confirmed that BSA-AGEs upregulated the expression of K17 as well as the phosphorylated level of Rb that is a sign of cell cycle progression and downregulated the expression of p27^{KIP1} in NHKs (Fig. 2e). In addition, the elevated expression of K17 and the reduced expression of p27^{KIP1} caused by BSA-AGEs consistently emerged at both nuclear and cytoplasmic level in NHKs (Fig. 2f). Taken together, our results indicate that AGEs promote the proliferation of keratinocytes via K17-mediated p27^{KIP1} inhibition followed by accelerated cell cycle progression.

AGEs Facilitate the Production of IL-36α from Keratinocytes

Next, the proinflammatory effects of AGEs on keratinocytes were initially examined by evaluating the expression of IL-36 family members since they are important pathogenic cytokines mainly produced by



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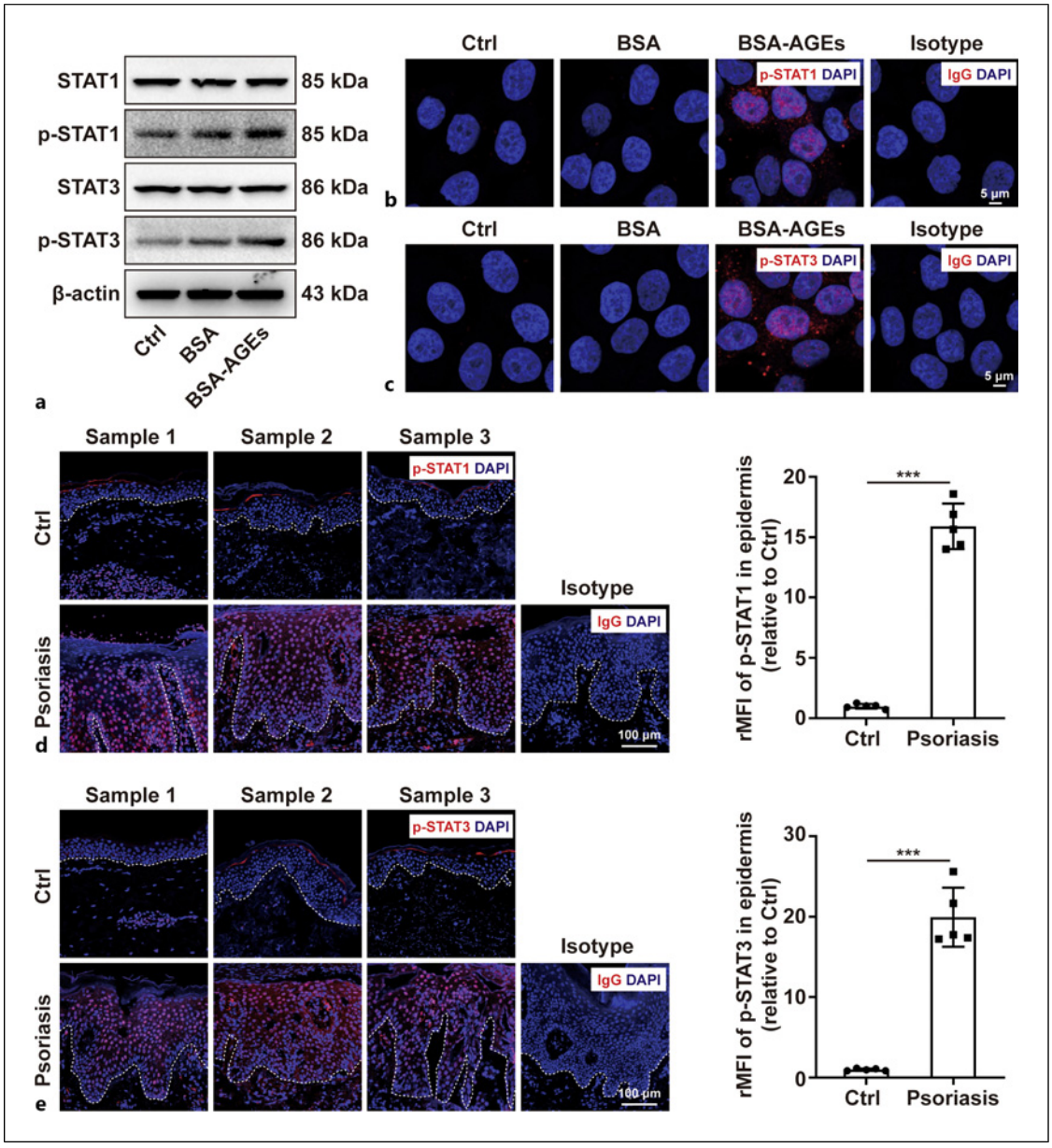

Fig. 5. AGEs activate STAT1/3 signaling pathways in keratinocytes. **a** Protein expression of STAT1, p-STAT1, STAT3, and p-STAT3 in NHKs treated with BSA or BSA-AGEs for 24 h were detected by Western blot assay. Immunofluorescence analysis of p-STAT1 (**b**) or p-STAT3 (**c**) in NHKs treated with BSA or BSA-AGEs for 24 h. Immunofluorescence analysis of p-STAT1 (red) (**d**) or p-STAT3 (red) (**e**) in lesional skin of patients with psoriasis ($n = 5$) and healthy controls ($n = 5$). Representative samples were shown. Nuclei were counterstained with DAPI (blue). Isotype control was shown with a lesional sample. Bar graphs represent the mean ± SD of p-STAT1 or p-STAT3 fluorescence intensity. rMFI: relative mean fluorescence intensity. ***$p < 0.001$.

Fig. 4. AGE-primed keratinocytes potentiate Th17 cell response via secreting IL-36α. The frequency of RORγt$^+$T (**a**) or IL-17A$^+$T (**b**) cells among CD4$^+$T cells was detected by flow cytometry analysis on PBMCs from psoriasis patients ($n = 5$) incubated for 48 h by the supernatants from NHKs, with the addition of IL-36α N, IL-36R A, or rhIL-36α into the incubation system, respectively. **c** The secretion of IL-17A from PBMCs of psoriasis patients ($n = 5$) treated with the supernatants from NHKs for 48 h with the addition of IL-36α N, IL-36 A, or rhIL-36α into the incubation system, respectively, was detected by ELISA. SN, supernatant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

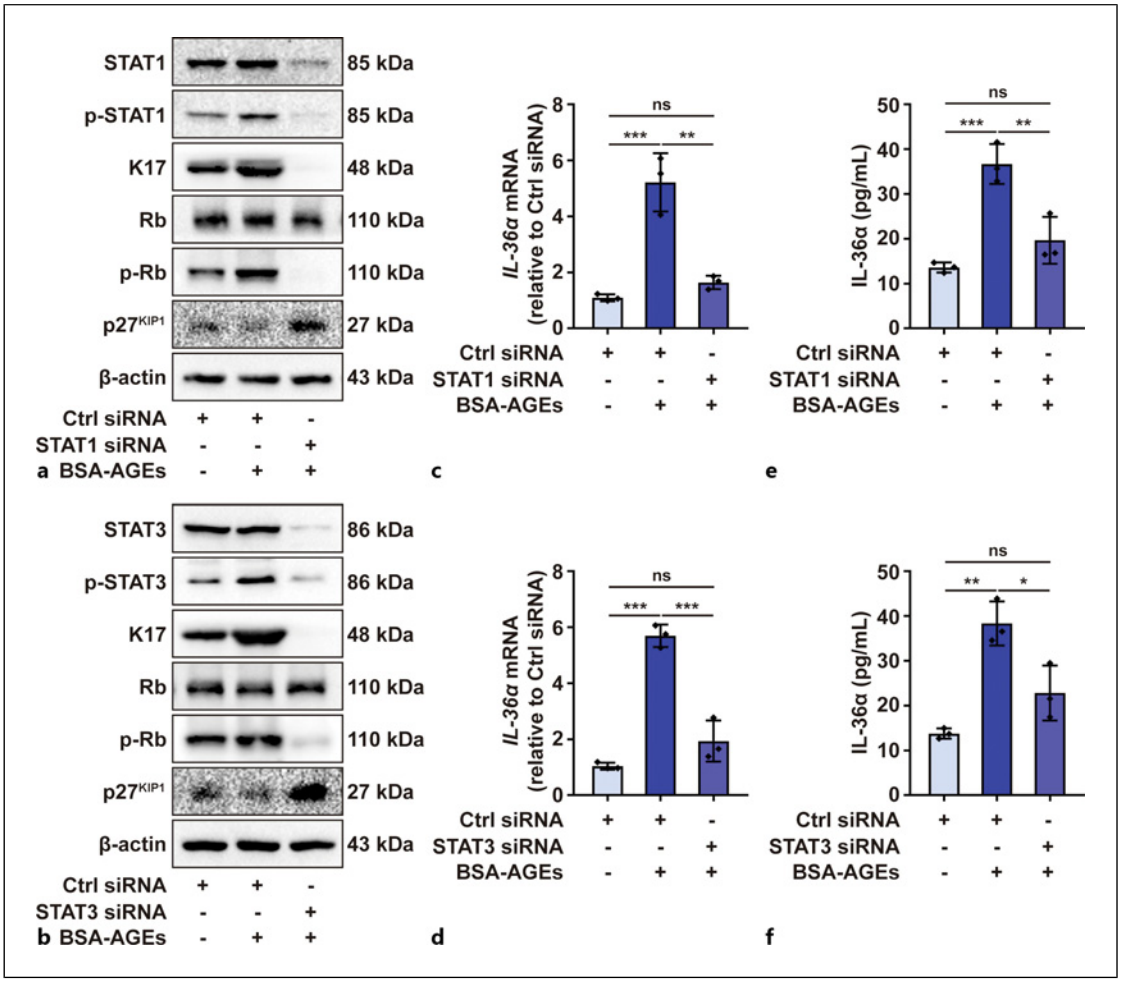

Fig. 6. STAT1/3 signaling pathways mediate AGE-induced expression of K17 and IL-36α in keratinocytes. Protein levels of STAT1, p-STAT1, STAT3, p-STAT3, K17, Rb, p-Rb, and $p27^{KIP1}$ in NHKs pre-transfected with STAT1 (**a**) or STAT3 (**b**) siRNA prior to BSA-AGEs stimulation for 24 h was determined by Western blot assay. The mRNA level of IL-36α in NHKs pre-transfected with STAT1 (**c**) or STAT3 (**d**) siRNA prior to BSA-AGEs stimulation for 24 h was determined by qRT-PCR. Secretion level of IL-36α from NHKs pre-transfected with STAT1 (**e**) or STAT3 (**f**) siRNA prior to BSA-AGEs stimulation for 48 h was determined by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: not significant.

keratinocytes in psoriasis [1, 31]. Our qRT-PCR and ELISA analysis showed that the mRNA expression and secretion level of IL-36α in NHKs were significantly increased with the treatment with BSA-AGEs (Fig. 3a, b), whereas the production of IL-36β and IL-36γ failed to be induced by BSA-AGEs in NHKs (online suppl. Fig. 3a, b). Subsequent immunofluorescence assay consistently observed prominent expression of IL-36α in the keratinocytes of psoriatic lesion (Fig. 3c). Moreover, serum levels of IL-36α were not only elevated in psoriasis patients but also positively correlated with serum level of AGEs in the patients (Fig. 3d, e). Accordingly, AGEs could be a strong activator of IL-36α production from keratinocytes in psoriasis.

AGE-Primed Keratinocytes Potentiate Th17 Cell Response via Secreting IL-36α

It is reported that IL-36α from skin-resident cells contributes to the activation of Th17 cells that are a key $CD4^+$T cell subgroup in psoriatic inflammation [32, 33]. Therefore, we went on to investigate whether AGE-primed keratinocytes could promote the activation of Th17 cells via releasing IL-36α. For this end, PBMCs donated by psoriasis patients were cultured with the supernatant harvested from NHKs with different treatments as indicated. Our flow cytometry analysis displayed that the percentage of Th17 cells marked by $ROR\gamma t^+$ in the $CD4^+$T cell subgroup was significantly increased not only by recombinant human IL-36α (rhIL-36α) as

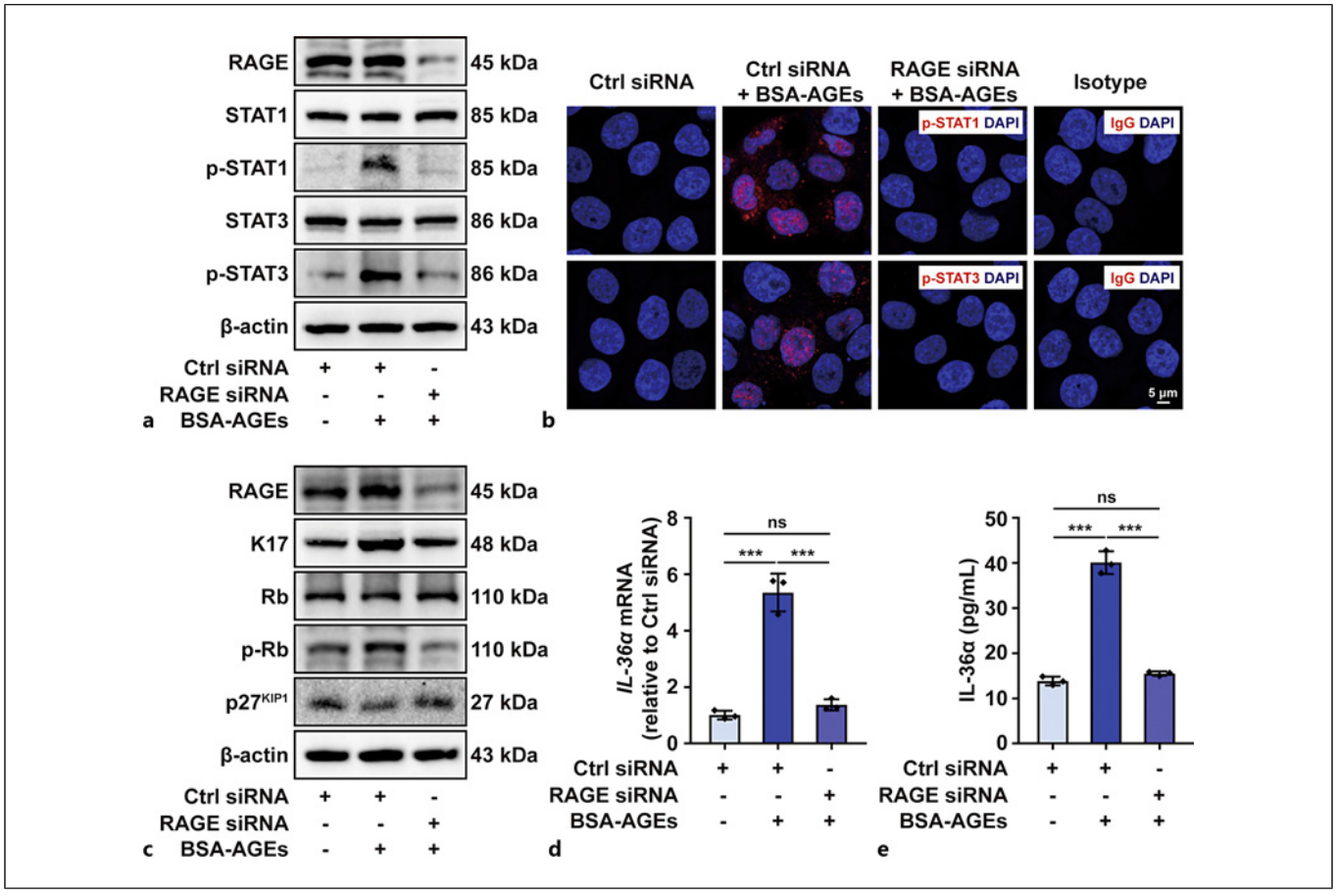

Fig. 7. AGEs exert pro-proliferative and proinflammatory effects on keratinocytes via RAGE. **a** Protein levels of RAGE, STAT1, p-STAT1, STAT3, p-STAT3 in NHKs pre-transfected with RAGE siRNA prior to BSA-AGEs stimulation for 24 h were determined by Western blot assay. **b** Immunofluorescence analysis of p-STAT1 and p-STAT3 in NHKs pre-transfected with RAGE siRNA prior to BSA-AGEs stimulation for 24 h. **c** Protein levels of RAGE, K17, Rb, p-Rb, and $p27^{KIP1}$ in NHKs pre-transfected with RAGE siRNA before BSA-AGEs stimulation for 24 h was determined by Western blot assay. **d** The mRNA level of IL-36α in NHKs pre-transfected with RAGE siRNA prior to BSA-AGEs stimulation for 24 h was determined by qRT-PCR. **e** Secretion level of IL-36α from NHKs pre-transfected with RAGE siRNA prior to BSA-AGEs stimulation for 48 h was determined by ELISA. ***$p < 0.001$, ns: not significant.

expected but also by the treatment with the supernatant of BSA-AGE-stimulated NHKs, which, however, was offset by the addition of IL-36α N or IL-36R A into the supernatant (Fig. 4a). Meanwhile, the proportion of IL-$17A^+$T cells in the $CD4^+$T cell subgroup was also elevated after cultured with the supernatant of BSA-AGE-treated NHKs or rhIL-36α but showed no significant change upon the addition of IL-36α N or IL-36R A into the culture medium (Fig. 4b). Additional ELISA assay showed consistent findings that the supernatant of BSA-AGE-treated NHKs or rhIL-36α dramatically upregulated the secretion of IL-17A from PBMCs of psoriasis patients, whereas the co-treatment with IL-36α N or IL-36R A reversed the release of IL-17A from PBMCs brought by the supernatant of BSA-AGE-treated NHKs (Fig. 4c). To sum up, our results suggest that AGE-induced IL-36α secretion from keratinocytes promotes Th17 cell response in psoriasis.

STAT1 and STAT3 Signaling Pathways Mediate the Dual Effects of AGEs on Keratinocytes

It has been noted that both the proliferation and the expression of IL-36α in psoriatic keratinocytes could be induced by classical Th17 cytokines including IL-17 and IL-22 [30, 34, 35], which prompted us to investigate whether signal transducer and activator of transcription (STAT) 1 (STAT1) and STAT3 that are downstream signaling pathways of Th17 cytokines also mediated the proinflammatory and proliferative effects of AGEs on

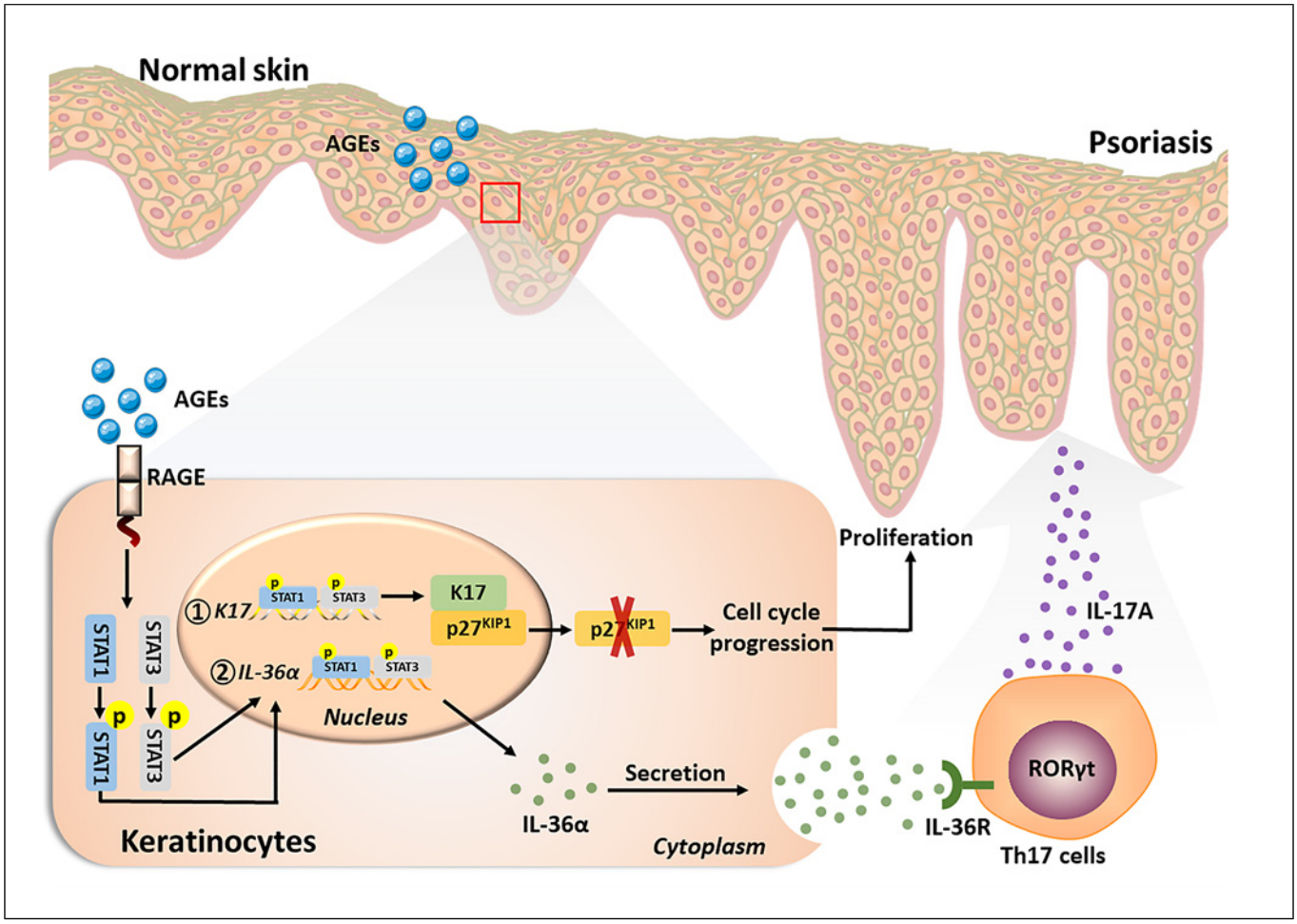

Fig. 8. Schematic representation of the role of AGEs in psoriatic inflammation. Accumulated AGEs in psoriatic epidermis bind to RAGEs on the membrane of keratinocytes and activate STAT1/3 signaling pathways. Subsequently, K17 is upregulated and thus causes nuclear export and degradation of $p27^{KIP1}$, which leads to the cell cycle progression and ultimately proliferation of keratinocytes. Meanwhile, IL-36α is overexpressed and secreted from keratinocytes and promotes Th17 cell response, which could contribute to the formation of cutaneous immune response in psoriasis.

keratinocytes. Our Western blot analysis revealed that BSA-AGEs obviously upregulated the phosphorylated level of STAT1 and STAT3 (p-STAT1 and p-STAT3) in NHKs (Fig. 5a). In parallel, the immunofluorescence assay in vitro showed enhanced nuclear expression of p-STAT1 and p-STAT3 in the NHKs treated with BSA-AGEs (Fig. 5b, c). Subsequent immunofluorescence assay in vivo confirmed strengthened phosphorylation of STAT1 and STAT3 in the keratinocytes of psoriasis lesion (Fig. 5d, e).

To further verify the intermediate role of STAT1/3 pathways in AGE-primed keratinocytes, siRNA was used to knock down STAT1/3 in NHKs followed by the treatment with BSA-AGEs. It turned out that along with the impaired expression of STAT1/3 and p-STAT1/3, the upregulation of K17 and phosphorylated Rb as well as the downregulation of $p27^{KIP1}$ induced by BSA-AGEs was counteracted in NHKs (Fig. 6a, b). Furthermore, the induction of mRNA expression of IL-36α in NHKs by BSA-AGEs was reduced following the knockdown of STAT1/3 (Fig. 6c, d). Final ELISA assay observed that the secretion of IL-36α from BSA-AGE-treated NHKs was likewise repressed by the knockdown of STAT1/3 (Fig. 6e, f). Taken together, these findings indicate that activated STAT1 and STAT3 signaling pathways account for AGE-induced proliferation and production of IL-36α in keratinocytes.

AGEs Are Sensed by Keratinocytes via RAGE

Finally, we examined whether the effects of AGEs on keratinocytes were delivered by RAGE based on our previous findings that RAGE is constitutively expressed

on the membrane of NHKs [24]. To this end, siRNA was again used to knock down RAGE prior to BSA-AGE treatment in NHKs. Western blot and immunofluorescence assay revealed that the expression of phosphorylated-STAT1/3 induced by BSA-AGEs were dramatically suppressed by knockdown of RAGE in NHKs (Fig. 7a, b). What is more, the upregulation of K17 and phosphorylated Rb as well as the downregulation of $p27^{KIP1}$ induced by BSA-AGEs were reversed by knockdown of RAGE (Fig. 7c). In addition, downregulation of RAGE decreased the mRNA and secretion levels of IL-36α in BSA-AGE-treated NHKs (Fig. 7d, e). Our results suggest that AGEs exert pro-proliferative and proinflammatory effects on keratinocytes via RAGE.

Discussion

The common concurrence of psoriasis and cardiometabolic disorders has been noted for decades. A recent systemic review and meta-analysis has indicated that the global prevalence of metabolic syndrome in patients with psoriasis impressively reaches 32%, with the patients from Latin America showing the highest prevalence of 47% [36]. Another meta-analysis has also confirmed an elevated risk in the incidence of CVD including both coronary artery disease and even myocardial infarction linked to psoriasis in both European and East Asian populations [37]. Although this clinical phenomenon could be well explained by enhanced systemic inflammation led by activated immune cells, especially T cells, as well as a series of cytokines (e.g., TNF-α and IL-17) in both psoriasis and cardiometabolic disorders [38–40], some recent studies have also noticed the role of metabolic abnormality in the immune-mediated pathogenesis of psoriasis [11, 15, 41]. As a special kind of metabolite that has proinflammatory effects, AGEs have been confirmed to be increased in both the serum and normal-appearing skin of psoriasis patients and decreased in the skin after effective treatment [22, 42–44]. Moreover, the cutaneous level of AGEs is positively correlated with carotid intima-media thickness and body mass index in psoriasis, indicating AGEs as a molecular link between psoriasis and cardiometabolic comorbidities [22].

Previous studies have reported increased AGEs in the skin of psoriasis patients by measuring skin autofluorescence (SAF) of patients using a non-invasive autofluorescence reader [22, 45]. This method could only detect SAF in normal appearing non-lesional skin of patients, since visible vessels, scars, or other skin abnormalities may distort the results [46]. Generally, SAF reflects the level of AGEs in the whole skin tissue, especially dermal collagen-linked AGEs, and also indicates systemic AGE level [46, 47], but epidermal AGEs that directly act on keratinocytes could not be evaluated independently by SAF. In the present study, we first observed increased serum levels of AGEs that were positively correlated with the disease severity in Chinese psoriasis patients, which is consistent with a previous report [23]. Moreover, our immunofluorescence assay showed a significant accumulation of AGEs in the lesional epidermis of psoriasis rather than peri-lesional epidermis or healthy epidermis, thus implicating a role of AGEs in the pathogenesis of psoriasis via acting on epidermal keratinocytes.

AGEs could be sourced from exogenous intake or endogenous production. Exogenous AGEs come from diet, cigarette smoke, ultraviolet radiation, air pollution, etc. Endogenous AGEs are produced in physiological metabolism of the skin or diseases related to inflammatory reactions or chronic metabolic disorders [48]. In the dermis, glucose and fructose could connect to amino acids in collagen and elastin, thus leading to the production of AGEs [49]. Consistently, our immunofluorescence assay showed substantial accumulation of AGEs in the dermis of both normal skin and psoriatic perilesional and lesional skin. However, abnormal epidermal accumulation of AGEs was only observed in psoriatic lesions, which could result from increased oxidative stress that leads to the formation of active aldehydes and their derivatives as well as subsequent production of AGEs [50, 51]. Additionally, previous studies have shown that inflammatory reaction induces a metabolic switch towards glycolysis in multiple innate immune cells like macrophages that could facilitate AGE formation [52]. Whether psoriatic keratinocytes are involved in this way of AGE production still needs further investigation, though enhanced glycolysis has been identified as a signature of psoriatic keratinocytes by several studies [53–55].

Abnormal proliferation of keratinocytes is a key event that directly causes the thickening of the skin in psoriasis [1]. Overexpressed K17 induced by cytokines, especially IL-17 and IL-22, is a crucial facilitator of the growth of keratinocytes [30, 56]. Furthermore, K17 has been shown to promote nuclear export and deactivation of $p27^{KIP1}$ which is a key negative regulator of G1-to-S phase transition in the cell cycle in cervical cancer [57]. Supplementary to these previous findings, the current study further showed that AGEs upregulated the expression of K17 and resulted in the nuclear export and degradation of $p27^{KIP1}$, leading to progressed cell cycle and ultimately the proliferation of keratinocytes. Multiple studies have

demonstrated the pro-proliferative effects of AGEs on different types of cells like mesangial cells and aortic smooth muscle cells [25–27]. However, some other studies have claimed the inhibitory effects of AGEs on cell proliferation or cell cycle progression in ovarian granulosa cells and intestinal epithelial cells [58, 59]. Therefore, the influence of AGEs on cell proliferation is probably cell type-dependent and could be completely different in different disease backgrounds.

The activation of innate immune function of keratinocytes has been recognized as a vital step in the formation of cutaneous inflammatory response in psoriasis [5]. On one hand, keratinocytes release damage-associated molecular pattern molecules like antimicrobial peptides like IL-37 and high mobility group box 1 (HMGB1) that trigger innate immune response via binding to PRRs expressed on dendritic cells and keratinocytes in paracrine or autocrine ways [24, 60, 61]. On the other hand, keratinocytes produce various chemokines that mediate the cutaneous infiltration of adaptive immune cells, especially T cells [62]. Recently, it has been noted that keratinocytes are also the main sources of certain cytokines like IL-36 family members [1, 63, 64]. In addition to previous findings that the expression of IL-36 family members in keratinocytes is induced by T cells-secreted cytokines such as IL-17 and IL-22 [65], our study observed that AGEs facilitated the production of IL-36α in keratinocytes. More than this, AGE-induced IL-36α release from keratinocytes promoted Th17 cell response as shown by our flow cytometry analysis on PBMCs incubated by the culture medium of AGE-primed keratinocytes. Consistent with our findings, the crosstalk between keratinocytes and T cell response via IL-36 has been indicated both in vitro and in vivo in imiquimod-induced psoriasiform mouse model [33, 66, 67]. Meanwhile, the role of IL-36α in enhancing Th17 response has also been demonstrated in other diseases including contact hypersensitivity and allergic rhinitis [32, 68]. Moreover, Takafumi Numata et al. [69] reported that IL-36α mediated the production of not only IL-17A but also IFN-γ, another vital cytokine indicated in Th1 response of psoriasis, in hapten-specific T cells. Therefore, our results support that AGEs are able to activate the innate immune function of keratinocytes and potentiate psoriatic immune response via IL-36α, though further studies are still needed to fully clarify the influence of IL-36α on the production of cytokines in T cells.

Various intracellular signaling pathways, including nuclear factor-κB signaling (NFκB) as the most common one, mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinases (JNK), have been reported to be activated following the binding of AGEs to RAGE on cell membrane [70]. In consideration of the effects of AGEs on the expression of K17 and IL-36α, both of which are regulated by STAT1/3 pathways downstream of IL-17 and IL-22 in psoriasis [34, 35, 71], the present study chose to focus on the two pathways and verified that AGE-induced overexpression of K17 and IL-36α was indeed dependent on the activation of STAT1/3 pathways as expected. In parallel with our findings, the activation of STAT1/3 pathways by AGEs has also been noted in other types of cells like macrophages, renal proximal tubular cells, and podocytes [72–74]. Given the role of STAT1/3 in transmitting the signaling of both the cytokines from T cells and other proinflammatory factors like AGEs in keratinocytes, it could be concluded that STAT1/3 pathways are the key processors that mediate the innate immune function of keratinocytes as well as the crosstalk between activated keratinocytes and adaptive T cell response in psoriasis. Notably, STAT1/3 signaling pathways mediate the production of quite a few inflammatory factors in psoriatic keratinocytes, especially various chemokines, e.g., CXCL9, CXCL10, CXCL11 [75, 76], as well as some other cytokines like IL-1β and IL-17C [77, 78]. Therefore, these chemokines and cytokines could be potentially induced by AGEs from keratinocytes, which is worth more studies to clarify.

In conclusion, our findings support that AGEs promote the proliferation of keratinocytes and potentiate the innate immune function of keratinocytes, which could be a key step in the formation of cutaneous psoriatic inflammation (Fig. 8). The present study indicates AGEs as a potential pathogenic link between psoriasis and cardiometabolic comorbidities. Further in vivo studies using psoriasiform mouse models are still needed to fully evaluate the role of AGEs in the development of psoriasis.

Statement of Ethics

The research protocol was designed and executed according to the principles of the Declaration of Helsinki and was approved by the Ethics Review Board of Fourth Military Medical University (approval number KY20193085). All participants were informed consent and signed a written consent form.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

Pan Kang, Weigang Zhang, and Chunying Li designed the study. Pan Kang, Jianru Chen, and Shiyu Wang performed the experiments. Pan Kang, Shaolong Zhang, and Shuli Li analyzed the data. Sen Guo, Pu Song, and Ling Liu wrote the manuscript. Gang Wang, Tianwen Gao, Weigang Zhang, and Chunying Li revised the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

Data are not publicly available due to ethical reasons. Further inquiries can be directed to the corresponding author.

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