

Oxidative stress drives CD8⁺ T-cell skin trafficking in patients with vitiligo through CXCL16 upregulation by activating the unfolded protein response in keratinocytes

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Background: In patients with vitiligo, an increased reactive oxygen species (ROS) level has been proved to be a key player during disease initiation and progression in melanocytes. Nevertheless, little is known about the effects of ROS on other cells involved in the aberrant microenvironment, such as keratinocytes and the following immune events. CXCL16 is constitutively expressed in keratinocytes and was recently found to mediate homing of CD8⁺ T cells in human skin.

Objective: We sought to explicate the effect of oxidative stress on human keratinocytes and its capacity to drive CD8⁺ T-cell trafficking through CXCL16 regulation.

Methods: We first detected putative T-cell skin-homing chemokines and ROS in serum and lesions of patients with vitiligo. The production of candidate chemokines was detected by using quantitative real-time PCR and ELISA in keratinocytes exposed to H₂O₂. Furthermore, the involved mediators were analyzed by using quantitative real-time PCR, Western blotting, ELISA, and immunofluorescence. Next, we tested the chemotactic migration of CD8⁺ T cells from patients with vitiligo mediated by the CXCL16-CXCR6 pair using the transwell assay.

Results: CXCL16 expression increased and showed a positive correlation with oxidative stress levels in serum and lesions of patients with vitiligo. The H₂O₂-induced CXCL16 expression was due to the activation of 2 unfolded protein response pathways: kinase RNA (PKR)-like ER kinase-eukaryotic initiation factor 2 α and inositol-requiring enzyme 1 α -X-box binding protein 1. CXCL16 produced by stressed keratinocytes induced migration of CXCR6⁺CD8⁺ T cells derived from patients with vitiligo. CXCR6⁺CD8⁺ T-cell skin infiltration is accompanied by melanocyte loss in lesions of patients with vitiligo.

Conclusion: Our study demonstrated that CXCL16-CXCR6 mediates CD8⁺ T-cell skin trafficking under oxidative stress in

patients with vitiligo. The CXCL16 expression in human keratinocytes induced by ROS is, at least in part, caused by unfolded protein response activation. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Oxidative stress, unfolded protein response, CXCL16, CXCR6, CD8⁺ T cells, skin migration

Vitiligo is an autoimmune skin disease characterized by progressive depigmentation caused by loss of melanocytes in the epidermis. Increased reactive oxygen species (ROS) levels were found in epidermis of patients with vitiligo.¹⁻⁴ ROS can lead to various alterations of the antioxidant system, melanin synthesis-related proteins, and membrane lipids in melanocytes, leading to homeostasis disruption and survival compromise of melanocytes.⁵⁻⁹ Moreover, stressed melanocytes undergo apoptosis and can also secrete exosomes that contain melanocyte-specific antigens and immunostimulatory signals, initiating the autoimmune reaction toward melanocytes, which contributes to disease progression.¹⁰⁻¹⁵

In addition to melanocytes, keratinocytes can also be targeted by oxidative stress in the epidermis.^{16,17} We previously found that overexpression of miR-25 in stressed keratinocytes interferes with the production and secretion of stem cell factor or basic fibroblast growth factor from keratinocytes, thus facilitating melanocyte loss in patients with vitiligo.¹⁸ As the predominant component of the epidermis, when impaired, keratinocytes give rise to an aberrant epidermal microenvironment, which in turn affects immune cell infiltration.^{19,20} Among lesional infiltrating T lymphocytes in patients with vitiligo, CD8⁺ T cells were identified as the major population.²¹ Further studies confirmed that these T cells are activated in a cytotoxic manner, are melanocyte specific, and locate at the dermal-epidermal junction close to melanocytes.²²⁻²⁴ Notably, T-cell infiltration has been observed in margins of active lesions,²¹ and van den Boorn et al²⁵ have showed that perilesional melanocyte-specific CD8⁺ T cells from patients with vitiligo could infiltrate autologous normal skin explants and kill melanocytes, which indicates the involvement of CD8⁺ T cells in disease progression. Nevertheless, how CD8⁺ T cells migrate into lesions from patients with vitiligo during oxidative stress is still not fully understood.

Several chemokines have been found to be responsible for CD8⁺ T-cell skin trafficking.^{26,27} Studies on a transgenic mouse model of vitiligo revealed that CD8⁺ T-cell skin accumulation depends on CXCR3, with CCR5 limited and CCR4 dispensable.²⁸ Rashighi et al found that levels of the chemokines CXCL9, CXCL10 and CXCL11, all of which are ligands of CXCR3, were significantly upregulated in vitiligo skin.²⁹ Functional

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Supported by the National Natural Science Foundation of China (nos. 81472863, 81130032, 81402599, and 81472893).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 23, 2016; revised August 17, 2016; accepted for publication October 3, 2016.

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0091-6749

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<http://dx.doi.org/10.1016/j.jaci.2016.10.013>

Abbreviations used

DAPI: 4'-6-Diamidino-2-phenylindole
 eIF2 α : Eukaryotic initiation factor 2 α
 ER: Endoplasmic reticulum
 HSPA5: Heat shock 70-kDa protein 5
 IRE1 α : Inositol-requiring enzyme 1 α
 NF- κ B: Nuclear factor κ B
 PERK: Kinase RNA (PKR)-like ER kinase
 ROS: Reactive oxygen species
 shRNA: Short hairpin RNA
 siRNA: Small interfering RNA
 UPR: Unfolded protein response
 XBP1: X-box binding protein 1

studies in mouse models of vitiligo confirmed the IFN- γ -CXCL10-CXCR3 axis is critical for both the progression and maintenance of depigmentation.^{29,30} However, although this collective evidence suggests that CXCL10 principally drives disease, it does not preclude additional involvement of other chemokines in patients with vitiligo. Recently, the interaction of a novel chemokine pair, CXCL16-CXCR6, has been reported to mediate homing of CD8⁺ T cells in human skin.^{31,32} CXCL16, the unique ligand of CXCR6, is constitutively expressed on keratinocytes in healthy epidermis, and its secretion can be induced by cellular stress.^{33,34}

Thus we hypothesize that ROS-impaired keratinocytes mediate CD8⁺ T-cell infiltration in lesions of patients with vitiligo through a CXCL16-dependent mechanism.

METHODS**Patients and samples**

Skin tissue and peripheral blood were collected from patients given a diagnosis of vitiligo according to clinical and histologic manifestation in the Department of Dermatology, Xijing Hospital of the Fourth Military Medical University. All subjects included in the study displayed initiation of depigmentation within 3 months and had not received any systemic or topical therapy for at least 3 months before sample collection. Blood serum samples and skin tissues (obtained from the perilesional skin) were collected from 16 patients with vitiligo. Control serum and skin samples were obtained from 16 age- and sex-matched healthy control subjects during plastic surgery. In addition, we collected nonlesional skin from 5 of the abovementioned patients with vitiligo. Furthermore, 27 patients with vitiligo in the progressive stage were enrolled and treated with intramuscular injections of 1 mL of Diprosan (containing 2 mg of betamethasone sodium phosphate and 5 mg of betamethasone dipropionate) for once and then narrow-band UVB (the initial dose was 0.3 J/cm², with increments of 0.1 J/cm² in every dose up to a maximum of 3 J/cm²) was administered once every other day by using a Waldmann UV 1000 L (Waldmann, Waldmann UV 1000 L whole-body phototherapy chamber containing) whole-body phototherapy chamber containing narrow-band UVB (Phillips TL01, Amsterdam, The Netherlands) tubes, and topical tacrolimus 0.1% was applied twice daily on all spreading lesions. Peripheral blood was collected before therapy and 2 months later; at that time, 80% of patients reported no spreading, new lesions, or both. All subjects consented by written and informed agreement for inclusion in this study. All samples were deidentified before use in experiments. The investigational protocol was approved by the Ethics Committee of the Fourth Military Medical University, according to the Declaration of Helsinki principles.

Cell cultures

Primary keratinocytes were generated from healthy subjects and cultured in keratinocyte-SFM (Gibco, Carlsbad, Calif). Cells were grown to 80%

confluency and used in 3 to 5 passages. A human keratinocyte cell line (HaCaT) was cultured in Modified Medium RPMI 1640 with 10% FBS and L-glutamine (Invitrogen, Carlsbad, Calif). H₂O₂ (Sigma, St Louis, Mo) was added at different concentrations (125, 250, and 500 μ mol/L). For experiments, supernatants were harvested for ELISA, and the remaining cells were processed for RNA and/or protein extraction at the times indicated.

Antibodies and reagents

The primary antibodies used for Western blots against CXCL16 (GTX116706) were purchased from GeneTex (Irvine, Calif). Antibodies against eukaryotic initiation factor 2 α (eIF2 α ; ab5369), phospho-eIF2 α S51 (ab32157), CD8 α (ab108343), CXCR6 (ab8023), and MelanA (ab51061) were purchased from Abcam (Cambridge, United Kingdom). Antibodies against heat shock 70-kDa protein 5 (HSPA5; C50B12), kinase RNA (PKR)-like ER kinase (PERK; D11A8), phospho-PERK (Thr980) (16F8), inositol-requiring enzyme 1 α (IRE1 α ; 14C10), spliced X-box binding protein 1 (XBP1; D2C1F), nuclear factor κ B (NF- κ B) p65 (L8F6), and phospho-NF- κ B p65 (S536 [93H1]) were purchased from Cell Signaling Technology (Danvers, Mass). Antibodies used against XBP1 (24168-1-AP) and phospho-IRE1 α (NB100-2323) were from Proteintech (Chicago, Ill). The antibody against NF- κ B p65 (D14E12) used for chromatin immunoprecipitation was from Cell Signaling Technology.

ELISA

Human CXCL9, CXCL10, CXCL11, and CXCL16 Quantikine ELISA kits and CCL1, CCL5, CCL17, CCL22, CCL27, and CCL28 DuoSet ELISA kits were used to analyze patient's serum or cell supernatant samples, according to the manufacturer's instructions (R&D Systems, Minneapolis, Minn).

Determination of H₂O₂ concentration

The H₂O₂ concentration was determined by using the H₂O₂ assay kit (Beyotime Biotechnology, Shanghai, China). H₂O₂ can oxidize ferrous ions (Fe²⁺) to ferric ions (Fe³⁺) that can react with xylenol orange in a colorimetric way, which makes the quantification of H₂O₂ levels possible with a spectrometer. The detection limit can be as low as 1 μ mol/L H₂O₂ in the sensitive fluorometric assay. For tissue samples, a suction blister was applied to harvest fresh epidermal samples, which were then washed in cold PBS (pH 6.0), homogenized with a homogenizer sitting in lysis buffer (at a ratio of 100 mL per 5 mg), and centrifuged. Before testing, epidermal supernatants and serum samples were diluted for the indicated times in PBS. All the operations were carried out on ice and protected from light. Finally, test tubes containing 50- μ L samples and 100- μ L test solutions were placed at room temperature for 30 minutes and measured immediately with a spectrometer at a wavelength of 560 nm. The concentration of H₂O₂ released was calculated from a standard concentration curve in triplicate experiments.

Quantitative real-time PCR

Total RNA was extracted with TRIzol (Takara, Ohtsu, Japan), and cDNA was synthesized from 1 μ g of total RNA by using oligo-dT and SuperScript II compared with transcriptase (Tiangen Biotech, Beijing, China). Quantitative real-time PCR (qRT-PCR) was conducted with the SYBR Premix Ex Taq II (Takara, Ohtsu, Japan) and the iQ5 PCR Detection System (Bio-Rad Laboratories, Hercules, Calif). The primers used in this study are listed in Table E1 in this article's Online Repository at www.jacionline.org. Relative gene expression levels were normalized to human β -actin levels and calculated by using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method.

Western blot analysis

Thirty micrograms of total protein was subjected to electrophoresis on a 10% SDS-PAGE (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Mass). The blotted membranes were blocked in a solution of 5% fat-free dried milk diluted in Tris-buffered saline and incubated with indicating primary antibodies and then with

horseradish peroxidase-conjugated secondary antibodies, washed, and detected by using a chemiluminescence detection kit (KPL, Gaithersburg, Md).

Immunofluorescence analysis

Immunofluorescence studies were performed on paraffin-embedded 5- μ m sections of skin samples from patients with vitiligo. After heat-mediated antigen retrieval with Tris-EDTA buffer (pH 9.0) was performed, the sections were incubated with primary antibodies for 16 hours at 4°C. Subsequently the secondary antibodies goat anti-mouse Cy3, goat anti-rabbit fluorescein isothiocyanate, or goat anti-rabbit Cy3 (at a dilution of 1:200; Abcam) were used for 45 minutes in the dark. After washing, slides were mounted with a 4',6-diamidino-2-phenylindole (DAPI; at a dilution of 1:2000; Abcam) for 10 minutes in the dark. Immunofluorescence staining was analyzed with an Olympus FV10 confocal laser scanning microscope 510 system (Carl Zeiss AB, Jena, Germany). Image J 64 software was used to count the numbers of CXCR6⁺CD8⁺ T cells (signal present with Cy3 overlap with fluorescein isothiocyanate) and MelanA-positive cells (signal present with Cy3 alone), the fluorescence intensity of phosphorylated eIF2 α (signal present with Cy3 alone), and the nuclear (signal present with Cy3 within the DAPI region of the cell) and cytoplasmic (signal present with Cy3 alone in the same cell) IRE1 α signal in skin samples.

Detection of XBP1 mRNA splicing

The method used for detection of unspliced and spliced XBP1 mRNA was as described previously by Jiang et al.³⁵ Briefly, compared with RT-PCR, products of XBP1 mRNA were obtained from total RNA extracted by using the primers 5'-CTGAAAAACAGAGTAGCAGCTCA-3' (forward) and 5'-TGGGTAGACCTCTGGGAGCTCCT-3' (reverse). Because a 26-bp fragment containing an *Apa*LI site is spliced on activation of XBP1 mRNA, the RT-PCR products were digested with *Apa*LI to distinguish the active spliced form from the inactive unspliced form. Subsequent electrophoresis revealed the inactive form as 2 cleaved fragments (278 and 195 bp) and the active form as a noncleaved fragment (447 bp).

Analyses with modulators of XBP1 splicing

Rapamycin³⁶ and salicylaldehyde,³⁷ the chemical inhibitors of XBP1 splicing, were used to detect any potential role of spliced XBP1 on H₂O₂-induced CXCL16 expression on keratinocytes. In these experiments cells were exposed to 100 nmol/L rapamycin or 100 μ mol/L salicylaldehyde 6 hours before exposure to H₂O₂.

Short hairpin RNA knockdown

The human PERK short hairpin RNA (shRNA), eIF2 α shRNA, IRE1 α shRNA, and control shRNA were kind gifts from Professor Zhang (School of Biomedical Sciences and Pharmacy, University of Newcastle), and were used to infect cells, as described by Luan et al.³⁸ After 48 hours of shRNA transfection, the cells were stimulated with 500 μ mol/L H₂O₂ and incubated for an additional 12 hours.

Small interfering RNA transfection

The small interfering RNA (siRNA) constructs for NF- κ B p65 (sc-29410) and the nontargeting control (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, Tex). After serum starvation, transfection of siRNA was carried out with Lipofectamine 3000 (Invitrogen), according to the manufacturer's guidelines. After 24 hours of siRNA transfection, the cells were stimulated with 500 μ mol/L H₂O₂ and incubated for an additional 12 hours.

Plasmid vectors and transfection

The pCMV-XBP1-GFP construct and pCMV-Control vectors were gifts from Professor Zhang (School of Biomedical Sciences and Pharmacy, University of Newcastle). Cells were transfected, as described by Luan et al.³⁸

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed by using the ChIP Assay Kit (Millipore, Temecula, Calif), according to the manufacturer's instructions. Briefly, HaCaT cells were cross-linked with DNA by using 1% formaldehyde at room temperature for 10 minutes. After washing, cells were lysed in SDS lysis buffer. DNA was sheared to small fragments of less than 500 bp in length by means of sonication. The recovered supernatant fraction was incubated with antibodies overnight on a rotor at 4°C. After washing, the precipitated NF- κ B p65 protein-DNA complexes were recovered by using protein G-Sepharose beads at 4°C for 16 hours. DNA was then purified with phenol/chloroform. A fraction was used as the PCR template (forward, 5'-ACCAGGGTTGGAGAAGACTAC-3'; reverse, 5'-GGGGCTAGGTCTACC GGG-3') to detect the presence of promoter sequences between -149 and -388 of CXCL16.

Migration assay

Primary keratinocytes and HaCaT cells were left either untreated or treated with H₂O₂ (500 μ mol/L). After 12 hours, culture supernatants were collected for the following chemotaxis assay. Blood-derived CD8⁺ T cells (1×10^5 cells cultured in 100 μ L of medium) from patients with vitiligo were sorted for the chemotaxis assay and separated from cell supernatants by using a 5.0- μ m polycarbonate membrane (Corning Life Sciences, Corning, NY). In addition, CXCL16 neutralization antibody (AF976; R&D Systems) and CXCL10 neutralization antibody (MAB266; R&D Systems) were used to block CXCL16 or CXCL10; CXCR6⁺CD8⁺ T cells and CXCR3⁺CD8⁺ T cells derived from patients with vitiligo were also used for the migration assay. Plates were left at 37°C for 3 hours. Migrated cells in the lower chamber were counted by means of flow cytometric analysis acquisition thereafter. Migration of CD8⁺ T cells was indicated by the percentage of input cells for each concentration of CXCL16 minus the percentage of cells migrating to the medium alone. Each experiment was performed in triplicate.

Statistical analyses

All statistical analyses were performed with GraphPad Prism software (3.0; GraphPad software, San Diego, Calif). Dual comparisons were made with the unpaired 2-tailed Student *t* test. Groups of 3 or more were analyzed by means of 1-way ANOVA with Dunnett posttests or 2-way ANOVA with Bonferroni posttests. Spearman correlation was used to correlate CXCL16 levels with H₂O₂ concentrations in patients with vitiligo. The results were considered significant at a *P* value of less than .05 and highly significant at *P* values of less than .01 and less than .001. Data represent means $\pm$ SDs for at least 3 independent experiments.

RESULTS

Increased CXCL16 levels are positively correlated with H₂O₂ accumulation in samples from patients with progressive vitiligo and decrease after therapy

First, we detected the expression of putative T-cell skin-homing chemokines, including CXCL9, CXCL10, CXCL11 (ligands of CXCR3), CXCL16 (ligand of CXCR6), CCL5 (ligand of CCR5), CCL1 (ligand of CCR8), CCL17, CCL22 (ligands of CCR4), CCL27, and CCL28 (ligands of CCR10) in 16 patients with vitiligo and healthy control subjects. Levels of CXCL10 (see Fig E1, A, in this article's Online Repository at www.jacionline.org) and CXCL16 (Fig 1, A) were increased in serum from patients with vitiligo, whereas the expression of other chemokines showed no significant change (see Fig E1, A). To determine whether the increased production of chemokines is due to oxidative stress, we detected the H₂O₂ levels in parallel (Fig 1, B). Interestingly, in all chemokines analyzed, CXCL16 (Fig 1, C) and CXCL10 (see Fig E1, B) showed a positive correlation, whereas other chemokines did not.

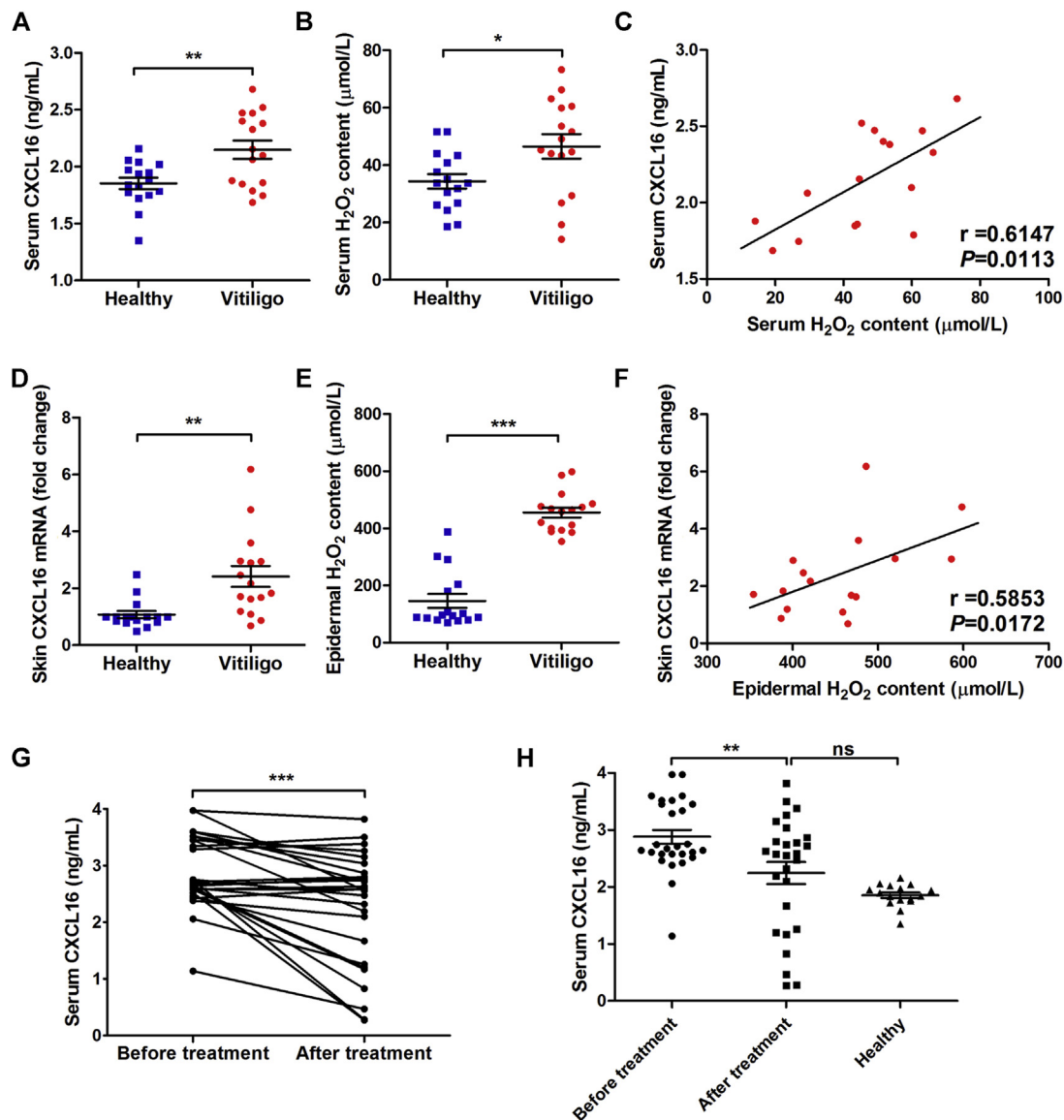


FIG 1. Increased CXCL16 levels are positively correlated with H₂O₂ accumulation in patients with vitiligo and decrease after therapy. **A-C**, Serum CXCL16 levels (Fig 1, A), H₂O₂ levels (Fig 1, B), and their correlations (Fig 1, C; $n = 16$). **D-F**, Skin CXCL16 mRNA (Fig 1, D), epidermal H₂O₂ accumulation (Fig 1, E), and their correlation (Fig 1, F) in lesions of patients with vitiligo ($n = 16$). **G** and **H**, Serum CXCL16 expression before and after treatment (Fig 1, G, $n = 27$; Fig 1, H, $n = 16$). ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Next, we found the mRNA expression of CXCL9, CXCL10, CXCL11, CXCR3, CCL5, and CXCL16 and its receptor, CXCR6, were upregulated in 8 perilesions from patients with vitiligo (see Fig E1, C). Interestingly, neither increased CXCL16 nor increased CXCR6 levels has been reported in skin from patients with vitiligo. In accordance with its serum level, the increased CXCL16 level was significantly correlated with epidermal H₂O₂ accumulation (Fig 1, D-F).

Next, we compared the serum CXCL16 level in 27 peripheral blood samples from patients with vitiligo before and after standard treatment. In all 27 cases enrolled, there was a good treatment response, as evidenced by repigmentation in lesional sites. As shown in Fig 1, G and H, 2 months after treatment, CXCL16 levels in peripheral blood showed a clear decrease.

H₂O₂ induces CXCL16 expression in human keratinocytes

Next, we examined the mRNA expression of chemokines in keratinocytes on exposure to different concentrations of H₂O₂ for 12 hours using qRT-PCR. In primary human keratinocytes the results showed that H₂O₂ significantly promoted the mRNA expression of CXCL16 in a dose-dependent manner, whereas CXCL9, CXCL10, and CXCL11 levels were slightly upregulated in keratinocytes treated with 500 μmol/L H₂O₂ (Fig 2, A). Although CXCL10 and CXCL16 are both key players in CD8⁺ T-cell skin accumulation,^{30,31} in primary human keratinocytes treated with H₂O₂, only CXCL16 secretion was significantly upregulated in a time- and dose-dependent manner (Fig 2, B). Similar results were detected in the human keratinocyte cell line HaCaT (see Fig E2 in this article's Online Repository at www.jacionline.org).

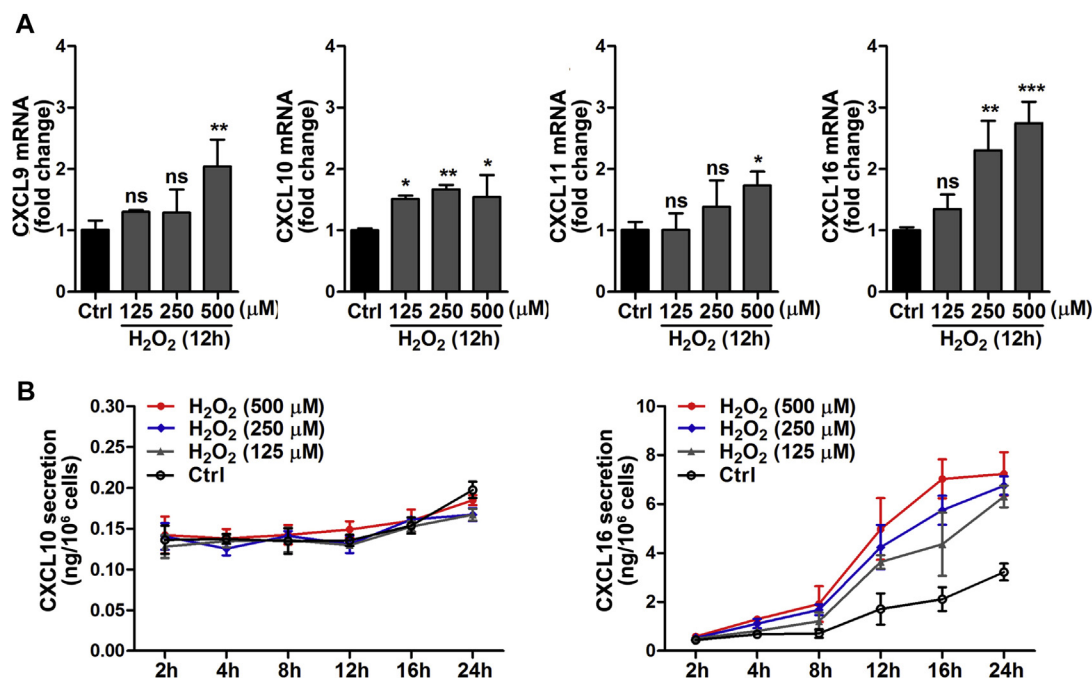


FIG 2. H_2O_2 induces CXCL16 expression in human keratinocytes. **A**, Expression of the chemokines CXCL9, CXCL10, CXCL11, and CXCL16 determined by using qRT-PCR in primary human keratinocytes treated with indicated concentrations of H_2O_2 for 12 hours ($n = 3$). **B**, Secretion of CXCL10 and CXCL16 by using ELISA in primary human keratinocytes exposed to H_2O_2 at the indicated time points ($n = 3$). ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Activation of the PERK-eIF2 α branch of unfolded protein response is responsible for CXCL16 expression in stressed keratinocytes

The unfolded protein response (UPR) is one of the cellular responses triggered by oxidative stress. One important effect of the UPR is driving tissue inflammation and proinflammatory cytokine and chemokine production.³⁹ Toosi et al¹⁰ demonstrated that the UPR is responsible for production of the immune mediators IL-6 and IL-8 in melanocytes after vitiligo-inducing phenol treatment. Recently, it was also found to be of central biological importance in keratinocytes.⁴⁰ In this regard we hypothesized that the UPR can mediate CXCL16 expression in keratinocytes regulated by ROS. Therefore we determined activation of the UPR in HaCaT cells treated with H_2O_2 (500 μ M/L) at the indicated time points. A significant induction of HSPA5 indicated UPR activation (Fig 3, A). The UPR is mediated by 3 endoplasmic reticulum (ER) membrane-associated proteins, namely PERK, IRE1 α , and activating transcription factor 6.⁴¹ Thus we tested all 3 molecules and found increased phosphorylation of PERK and eIF2 α within 3 hours after H_2O_2 treatment concomitant with increased expression of CXCL16 (Fig 3, A). Nevertheless, activating transcription factor 6 was not affected by H_2O_2 treatment (data not shown). In addition, immunofluorescence staining further confirmed increased phosphorylation and nuclear translocation of eIF2 α in HaCaT cells (Fig 3, B), and the intensity of phosphorylated eIF2 α in perilesional skin of patients with vitiligo was about twice as much as it was in healthy control subjects (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). On the other hand, knocking down either PERK (Fig 3, C) or eIF2 α (Fig 3, D) attenuated CXCL16 expression induced by H_2O_2 , indicating H_2O_2 -induced CXCL16 expression

is regulated, at least in part, by the PERK-eIF2 α branch of the UPR in keratinocytes.

To gain insight into the mechanism of PERK-eIF2 α pathway-mediated CXCL16 expression, we screened candidate transcription factors in the JASPAR database (<http://jaspar.genereg.net>) and found the -2000 to +1 region of the *CXCL16* promoter has 6 predicted binding sites for the transcription factor NF- κ B p65 (see Fig E4 in this article's Online Repository at www.jacionline.org), which is a target factor activated by PERK-mediated phosphorylation of eIF2 α under ER stress.⁴² Consistent with this, the H_2O_2 -induced phosphorylation of NF- κ B p65 (see Fig E5 in this article's Online Repository at www.jacionline.org) was inhibited by knockdown of either PERK or eIF2 α in HaCaT cells (see Fig E6 in this article's Online Repository at www.jacionline.org). Importantly, knockdown of NF- κ B p65 attenuates H_2O_2 -induced CXCL16 protein expression (Fig 3, E). Chromatin immunoprecipitation assays confirmed fragment -149 to -388 of the *CXCL16* promoter as the binding site of NF- κ B p65 (Fig 3, F). These data suggested that activation of NF- κ B p65 by the PERK-eIF2 α arm of the UPR participates in H_2O_2 -induced CXCL16 production in human keratinocytes.

Activation of the IRE1 α -XBP1 branch of the UPR also plays an important role in CXCL16 expression in stressed keratinocytes

In addition to PERK, IRE1 α -XBP1 is another important pathway of the UPR. Therefore we tested also the involvement of IRE1 α -XBP1 in CXCL16 induction by ROS in human keratinocytes. As shown in Fig 4, A, phosphorylation of IRE1 α significantly increased from 6 hours of H_2O_2 treatment

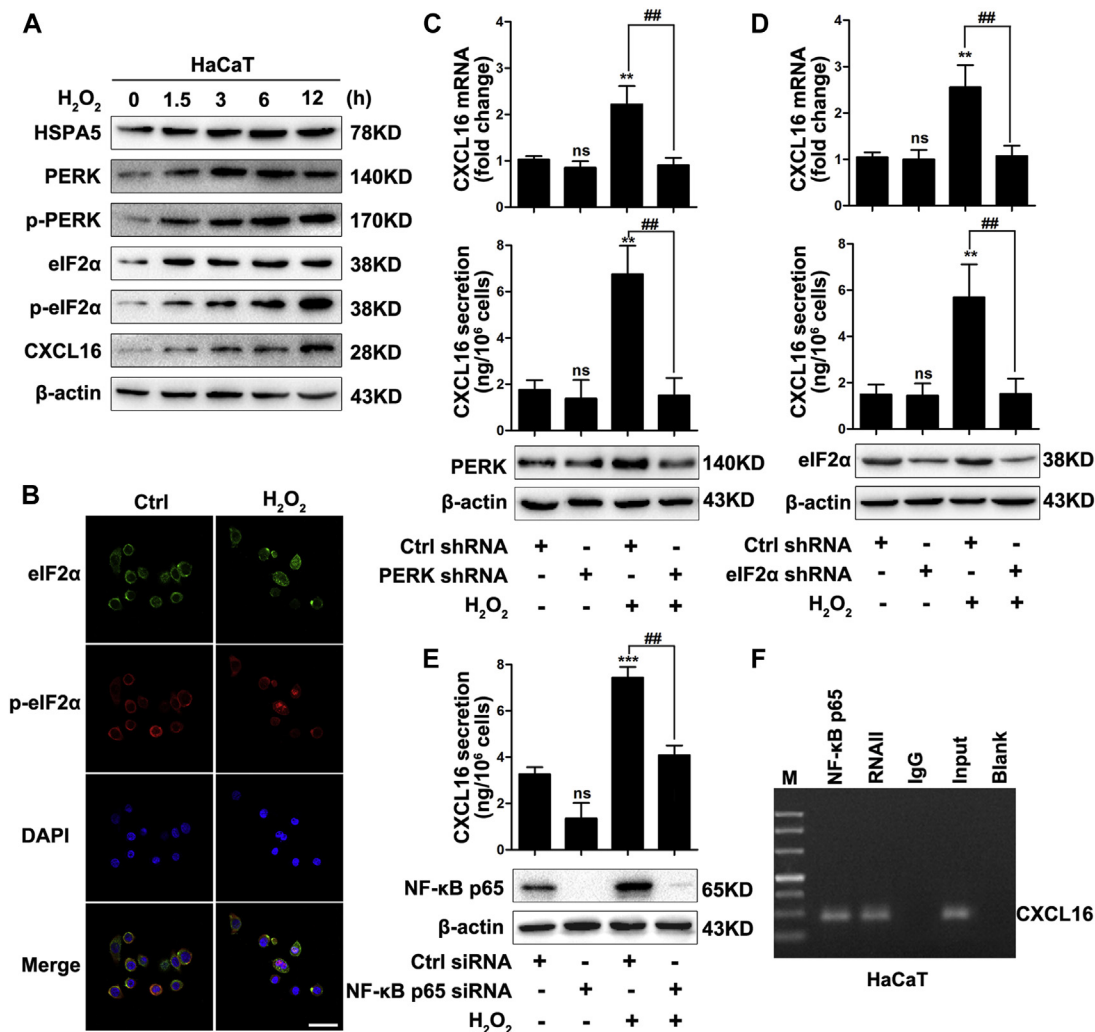


FIG 3. Activation of the PERK-eIF2α branch of the UPR is responsible for CXCL16 expression in stressed keratinocytes. **A**, CXCL16, HSPA5, and PERK-eIF2α expression in H₂O₂-treated HaCaT cells. **B**, Phosphorylated eIF2α in H₂O₂-treated HaCaT cells. Scale bars = 50 μm. **C-E**, CXCL16 expression from H₂O₂-treated HaCaT cells with knock down of PERK (Fig 3, C), eIF2α (Fig 3, D), or NF-κB p65 (Fig 3, E). **F**, Chromatin immunoprecipitation assay of NF-κB p65 to CXCL16 promoter. Statistical analysis was performed relative to control shRNA groups without H₂O₂ treatment (*) or those with H₂O₂ treatment (#). ns, Not significant. ***P* < .01 and ****P* < .001. ##*P* < .01.

concomitant with increased splicing of XBP1 (Fig 4, A). Immunofluorescence revealed increased nuclear fraction of phosphorylated IRE1α and spliced XBP1 in H₂O₂-treated HaCaT cells (Fig 4, B), and we found an increased ratio (nuclear/cytoplasm) of phosphorylated IRE1α signal, suggesting a higher nuclear translocation of phosphorylated IRE1α in perilesions in patients with vitiligo (see Fig E7 in this article's Online Repository at www.jacionline.org).

To confirm that the effects of H₂O₂ on HaCaT cells were due to XBP1 splicing, 2 inhibitors of XBP1 splicing, rapamycin³⁶ and salicylaldehyde,³⁷ were used before H₂O₂ treatment. As shown in Fig 4, C, both inhibitors abrogated XBP1 splicing and CXCL16 secretion induced by H₂O₂. Moreover, the increased CXCL16 expression induced by H₂O₂ were markedly reversed by IRE1α (Fig 4, D) or XBP1 (Fig 4, E) knockdown, whereas overexpression of XBP1 further promoted CXCL16 expression enhanced by H₂O₂ exposure (Fig 4, F). In addition, using the

JASPAR database (<http://jaspar.genereg.net>), we found that the -2000 to +1 region of the CXCL16 promoter had 2 predicted binding sites for XBP1 (see Fig E8 in this article's Online Repository at www.jacionline.org), which partially illustrated the regulation rule between CXCL16 and XBP1. These results demonstrated that activation of IRE1α-XBP1, another key pathway of the UPR, is also involved in H₂O₂-induced expression of CXCL16 in keratinocytes.

CXCL16 induced CXCR6⁺CD8⁺ T-cell infiltration in perilesional and nonlesional skin of patients with vitiligo

To date, CXCR6 is the only known receptor for CXCL16. To determine the CXCR6 expression in infiltrating CD8⁺ T cells in patients with vitiligo, we performed double-immunofluorescence staining of CXCR6 and CD8 in 5 samples of perilesional and

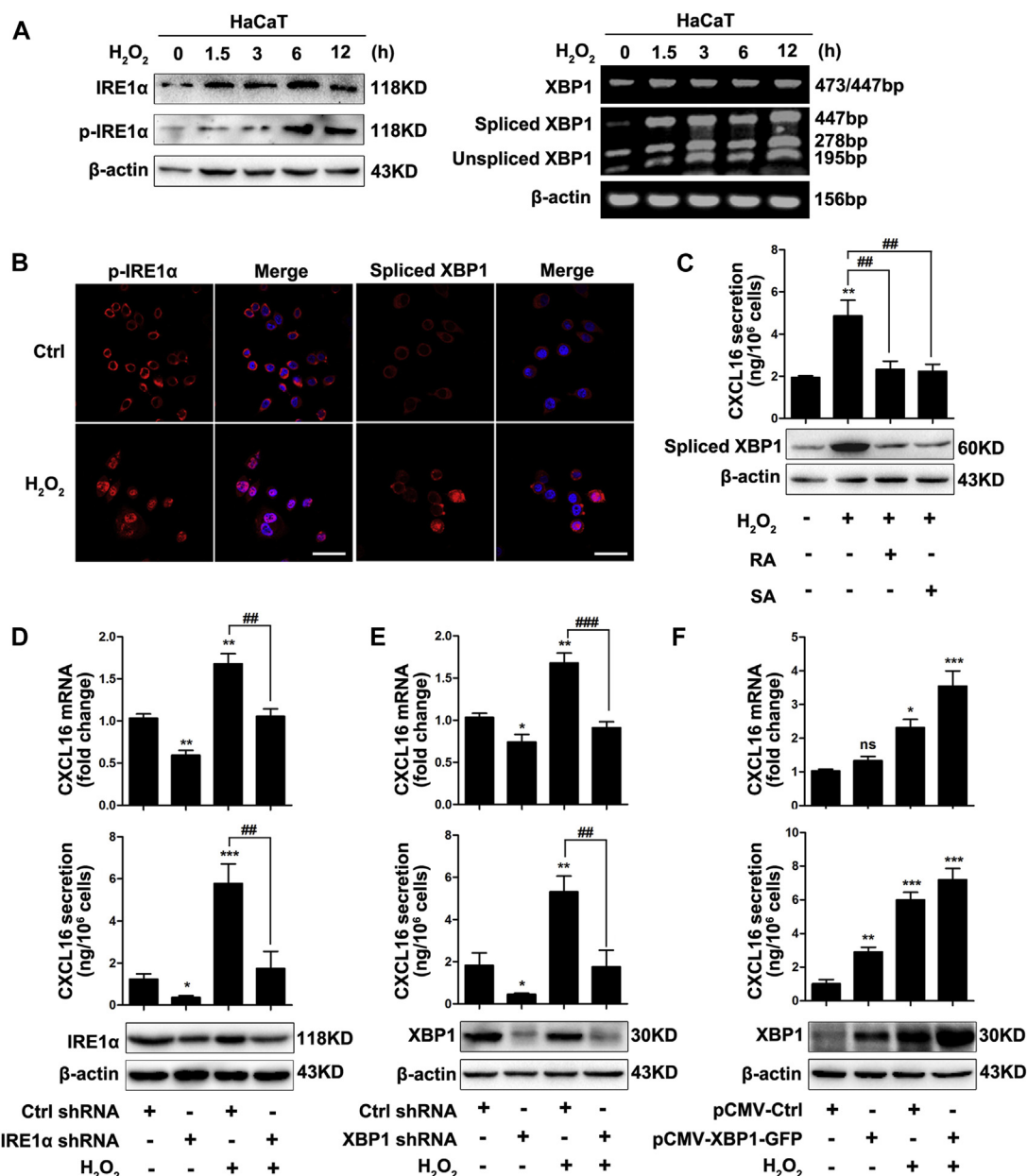


FIG 4. Activation of the IRE1α-XBP1 branch of the UPR also plays an important role in CXCL16 expression in stressed keratinocytes. **A**, IRE1α-XBP1 expression in H₂O₂-treated HaCaT cells. **B**, phosphorylated IRE1α and spliced XBP1 in H₂O₂-treated HaCaT cells. Scale bars = 50 μm. **C-F**, CXCL16 levels in H₂O₂-treated HaCaT cells pretreated with inhibitors of XBP1 splicing (Fig 4, C), knockdown of IRE1α (Fig 4, D), XBP1 (Fig 4, E), and XBP1 overexpression (Fig 4, F). Statistical analysis was performed relative to control shRNA groups without H₂O₂ treatment (*) or those with H₂O₂ treatment and pCMV control groups (#). ns, Not significant. **P* < .05, ***P* < .01, and ****P* < .001. ##*P* < .01 and ###*P* < .001.

nonlesional skin from patients with vitiligo and healthy control subjects. Notably, compared with nonlesional and healthy control skin, more CXCR6⁺CD8⁺ T cells were located at the basal epidermis and dermis of perilesions from patients with vitiligo (Fig 5, A and B). Interestingly, a sparse infiltrate of CXCR6⁺CD8⁺ T cells was also found in the nonlesional skin of patients with vitiligo and were located close to the dermal-epidermal junction, suggesting an aberrant microenvironment in the skin of patients with vitiligo without clinical visible depigmentation (Fig 5, A).

To identify the effect of the CXCL16-CXCR6 axis on CD8⁺ T-cell migration, we performed migration assays *in vitro*. By using flow cytometry, the CD8⁺ T-cell population was sorted from patients with vitiligo. The transwell assay showed that culture supernatants of H₂O₂-treated HaCaT cells and primary keratinocytes induced migration of CD8⁺ T cells in patients with vitiligo (Fig 5, C). Both the neutralization antibody of CXCL10 and CXCL16 caused a decrease in CD8⁺ T-cell migration in patients with vitiligo that was only statistically significant in the latter. Notably, combination with CXCL10 antibody did not produce

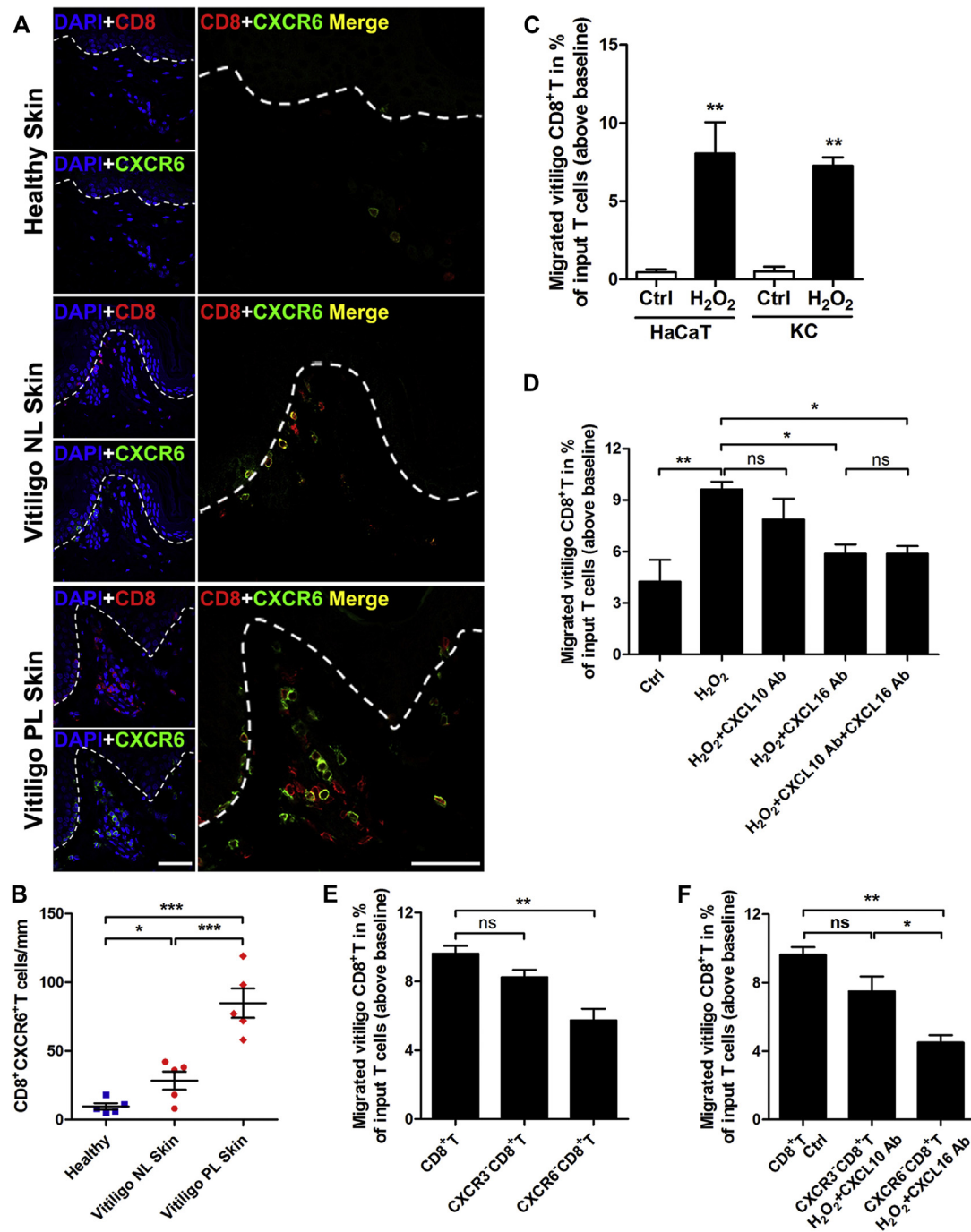


FIG 5. CXCL16 induced CXCR6⁺CD8⁺ T-cell infiltration in skin of patients with vitiligo. **A** and **B**, CXCR6⁺CD8⁺ T cells in skin of patients with vitiligo (Fig 5, A) and statistical results (Fig 5, B; n = 5). Scale bars = 50 μ m. **C**, CD8⁺ T-cell migration in patients with vitiligo in response to H₂O₂-treated HaCaT cells and primary keratinocytes (KC). **D-F**, Response of CD8⁺ T cells to H₂O₂-stimulated HaCaT cells with CXCL16/CXCL10 blockade (Fig 5, D), CXCR6/CXCR3 elimination (Fig 5, E), or CXCL16-CXCR6/CXCL10-CXCR3 signal disturbed (Fig 5, F; n = 3). ns, Not significant. **P* < .05, ***P* < .01, and ****P* < .001.

an additive blocking effect of CXCL16 antibody (Fig 5, D). Next, we detected migration of CXCR6⁺CD8⁺ and CXCR3⁺CD8⁺ T cells in response to the stressed keratinocytes. Consistently, results showed that CXCR6 elimination handicapped CD8⁺ T-cell migration toward the H₂O₂-stimulated keratinocytes, whereas CXCR3 elimination caused no significant change (Fig 5, E). Furthermore, we confirmed that blocking the chemokine

pair CXCL16-CXCR6 reduced the migration of CD8⁺ T cells in patients with vitiligo to more than 50%, yet the CXCL10-CXCR3 pair block showed no significant effect (Fig 5, F). Taken together, these results indicated that CXCL16, through interaction with its receptor, CXCR6, contributes to the trafficking of peripheral CD8⁺ T-cell skin infiltration from patients with vitiligo. More importantly, for the first time, these

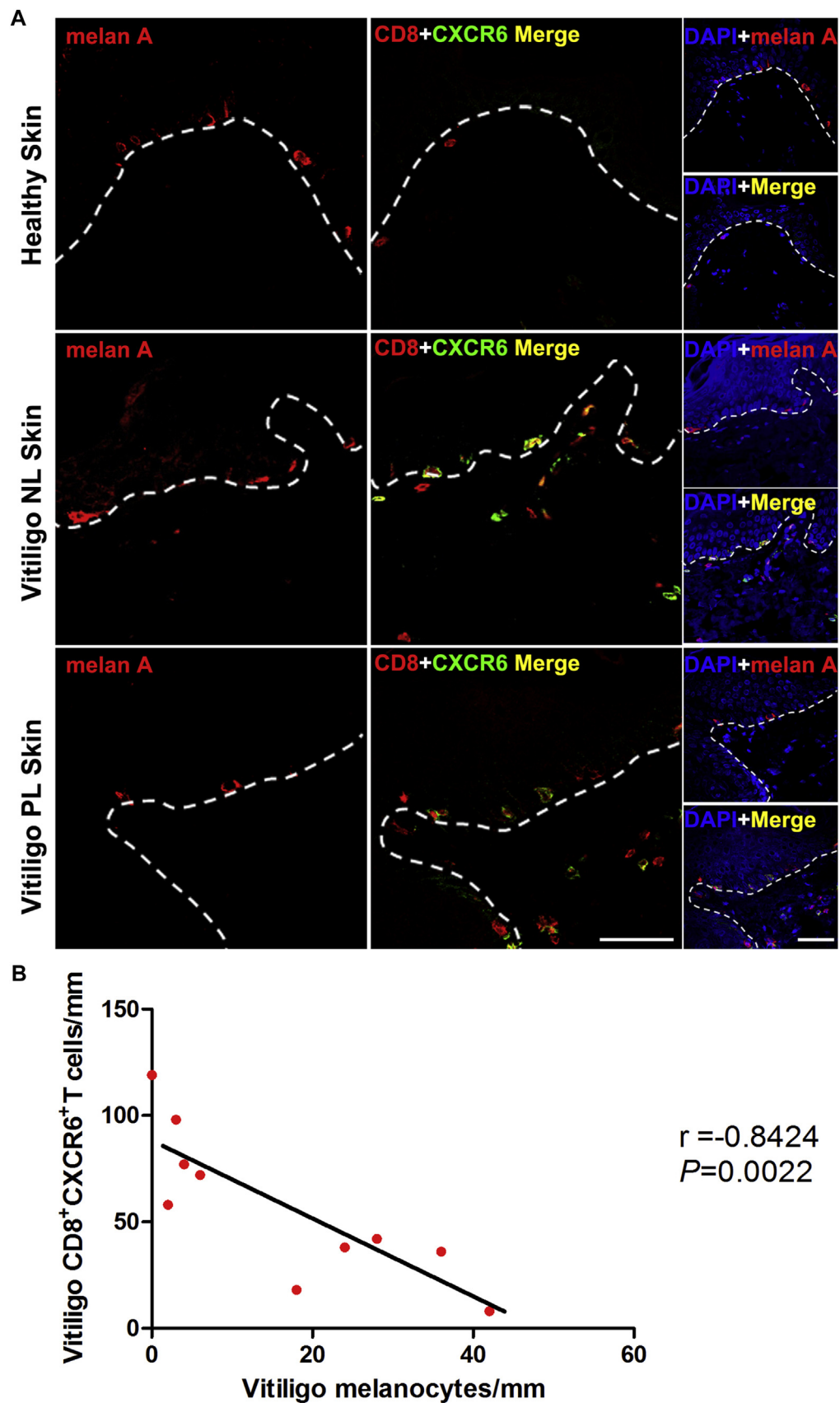


FIG 6. CXCR6⁺CD8⁺ T-cell skin infiltration is accompanied by melanocyte loss in lesions of patients with vitiligo. **A**, CXCR6⁺CD8⁺T cells and MelanA-positive cells in skin of patients with vitiligo. Scale bars = 50 μ m. NL, Nonlesional; PL, perilesional. **B**, Correlations between skin-infiltrated CXCR6⁺CD8⁺ T cells and melanocytes in patients with vitiligo measured by using Spearman correlation ($n = 10$).

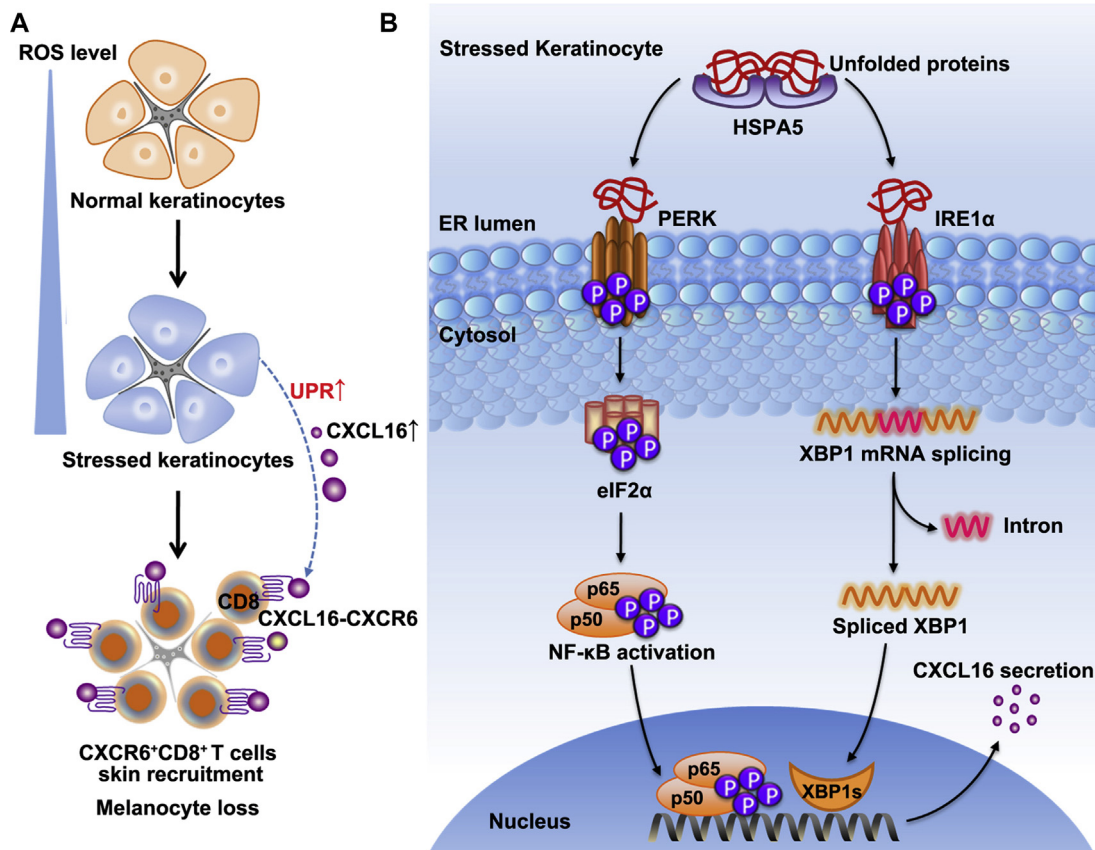


FIG 7. Oxidative stress drives CD8⁺ T-cell skin trafficking in patients with vitiligo through CXCL16 upregulation by activating the UPR in keratinocytes. **A**, CXCL16 secreted by keratinocytes in the ROS-enriched epidermal microenvironment facilitates CXCR6⁺CD8⁺ T-cell traffic to the melanocyte location site. **B**, CXCL16 upregulation in stressed keratinocytes is induced by activation of the PERK-eIF2α and IRE1α-XBP1 branches of the UPR.

data suggested that CXCL16, when compared with CXCL10, has a more important role to play in CD8⁺ T-cell trafficking to stressed skin in patients with vitiligo.

CXCR6⁺CD8⁺ T-cell skin infiltration is accompanied by melanocyte loss in lesions of patients with vitiligo

Compared with healthy skin, in perilesional and nonlesional skin of patients with vitiligo, MelanA-positive cell counts were reduced. Meanwhile, as expected, more CXCR6⁺CD8⁺ T cells were detected at the dermal-epidermal junction (Fig 6, A). More importantly, in the 10 skin samples from 5 patients with vitiligo analyzed, CXCR6⁺CD8⁺ T-cell counts and MelanA-positive cell counts showed a significant negative correlation (Fig 6, B). These results suggested that infiltrating CXCR6⁺CD8⁺ T cells are markedly enriched in lesions of patients with vitiligo and are accompanied by melanocyte loss in lesions of patients with vitiligo.

DISCUSSION

Increasing studies have identified oxidative stress as a key factor in autoimmune initiation. Vitiligo is an autoimmune disease in which increased oxidative stress plays a vital

role.^{5,15,43,44} The current study focused on the role of keratinocytes in CD8⁺ T-cell lesional trafficking in patients with vitiligo undergoing oxidative stress. We describe that increased CXCL16 expression positively correlated with ROS levels in epidermis of patients with vitiligo. In human keratinocytes the expression of CXCL16 can be induced by H₂O₂-induced ER stress, which drives the skin trafficking of CD8⁺ T cells through interaction with its receptor, CXCR6. It is noteworthy that our data provide the first evidence pointing to the involvement of 2 UPR signaling pathways, PERK-eIF2α and IRE1α-XBP1, in chemokine production of stressed keratinocytes in patients with vitiligo (Fig 7).

In patients with vitiligo, CD8⁺ T cells participate in melanocyte-specific autoimmune responses, leading to depigmentation of the skin. Recent studies have found that the IFN-γ-induced CXCL10-CXCR3 chemokine pathway plays a key role in CD8⁺ T-cell skin accumulation and melanocyte destruction in several IFN-γ-specific vitiligo mouse models.²⁸⁻³⁰ In addition to CXCL10, some other chemokines, such as CXCL16, are also closely related to the migration of CD8⁺ T cells.^{31,32} The aforementioned studies do not exclude the involvement of these chemokines in vitiligo development.

Recently, CXCL16 was found to mediate homing of CD8⁺ T cells in human skin. CXCL16 is constitutively expressed in keratinocytes and functions as a chemokine by binding to its receptor, CXCR6, which is expressed on a subset of type 1 polarized

peripheral blood cells, including CD4⁺ and CD8⁺ T cells, $\gamma\delta$ T cells, natural killer T cells, natural killer cells, monocytes, and B cells.^{32,45-48} In the current study we observed an increased CXCL16 level in the vitiligo microenvironment; more importantly, the expression of CXCL16 showed a positive correlation with H₂O₂ levels in both lesions and peripheral blood of patients with vitiligo. *In vitro* experiments with human primary keratinocytes and HaCaT cell lines confirmed the clear inducible CXCL16 expression by H₂O₂.

These findings suggest a causal role of oxidative stress stimuli in CXCL16 expression in patients with vitiligo. To the best of our knowledge, this association has never been reported, supporting a potential unrecognized link between oxidative stress and CXCL16 production in patients with vitiligo.

In addition to melanocytes, ER dilation in keratinocytes has been also observed at the periphery of lesions of patients with vitiligo.⁴⁹⁻⁵¹ Our data identified that H₂O₂ activates the PERK-eIF2 α pathway and IRE1 α -XBP1 arm of the UPR in keratinocytes. In addition, we also confirmed the activation of these 2 pathways in lesions of patients with vitiligo. Moreover, our data demonstrated that NF- κ B contributes to upregulation of CXCL16 induced by H₂O₂ in keratinocytes. To be specific, we identified that (1) the -149 to -388 fragment of the CXCL16 promoter has a consensus NF- κ B p65 direct binding region (-194 to -202 region); (2) NF- κ B knockdown withdraws the CXCL16 upregulation induced by H₂O₂; and (3) phosphorylation of NF- κ B p65, which is known to initiate downstream target gene transcription, was also induced by H₂O₂ in keratinocytes. Initiation of PERK signaling leads to phosphorylation of eIF2 α , which is necessary and sufficient to suppress the expression of I κ B, which in turn promotes phosphorylation of p65.⁵² It is noteworthy that activation of the PERK-eIF2 α pathway is of great importance for NF- κ B-dependent production of CXCL16 induced by H₂O₂ in keratinocytes.

Previous genome-wide association studies have implicated that XBP1 polymorphisms are associated with increased risk of vitiligo development.^{53,54} XBP1 exerts its functions in the immune system by activating MHC class II gene expression, regulating plasma cell differentiation, and mediating inflammatory response to ER stress.⁵³ In melanocytes XBP1 increases production of the immune mediator IL-6 and the IL-8 expression response to ER stress.¹⁰ We found blocking XBP1 activation by either inhibitors or gene knockdown both resulted in reduced production of CXCL16 in keratinocytes exposed to H₂O₂, whereas overexpression of XBP1 enhanced CXCL16 expression. These results suggested that XBP1 activation plays a critical role in CXCL16 production in response to H₂O₂, providing further support for involvement of XBP1 in the pathogenesis of vitiligo.

In this study we confirmed that CXCL16 attracted peripheral blood-derived CD8⁺ T cells in patients with vitiligo and that CXCR6⁺CD8⁺ T cells accumulated in vitiligo skin perilesions of patients with vitiligo. Moreover, our data demonstrated that infiltration of CXCR6⁺CD8⁺ T cells was accompanied by melanocyte loss, suggesting the potential destructive effect toward melanocytes by this subpopulation. Because CXCR3 is expressed on antigen-specific CD8⁺ T cells in skin lesions of patients with vitiligo, as described previously,²⁹ whether CXCR3 coexpresses on CXCR6⁺CD8⁺ T cells and consequently contributes to the migration in patients with vitiligo is unknown. However, our *in vitro* migration data revealed that CD8⁺ T-cell migration induced by ROS relied more on the CXCL16-CXCR6 pair than

the CXCL10-CXCR3 pair, suggesting that the specific roles CXCL16-CXCR6 plays within the stressed skin of patients with vitiligo are important and will require further study to offer direct evidence.

Previous studies have proved that ROS-induced melanocyte stress initiates inflammation that leads to autoimmunity. Kroll et al¹¹ report that stressed melanocytes secrete exosomes that contain melanocyte-specific antigens and damage-associated molecular pattern molecules, which can activate nearby dendritic cells and induce their maturation into efficient antigen-presenting cells. Heat shock protein 70 is another factor that can be induced in melanocytes of patients with vitiligo, which alerts infiltrating dendritic cells and worsens depigmentation in these patients.¹²⁻¹⁴ In addition, melanocytes under oxidative stress can produce IL-6 and IL-8, which can promote autoimmune targeting of melanocytes.¹⁰ Because CXCL16 can be induced by ROS, it can participate in ROS-initiated inflammation and autoimmunity, thus contributing to the onset of vitiligo. Moreover, persistent oxidative stress in patients with vitiligo can sustain CXCL16 production, leading to continuous skin infiltration of CXCR6⁺CD8⁺ T cells, which might contribute to the spread of vitiligo lesions. Therefore we tend to think that CXCL16 is involved in not only the initiation but also the progression of vitiligo.

Interestingly, our data imply that keratinocytes make great contributions to the aberrant CXCL16 level in the epidermal microenvironment in response to excessive oxidative stress in patients with vitiligo, which, accordingly, attracts CXCR6⁺CD8⁺ T-cell trafficking to the lesional sites. It is noteworthy that keratinocytes are important but very likely not the only source of CXCL16 in the vitiligo microenvironment. Previous studies revealed that CXCL16 secretion could also be induced by Langerhans cells in human epidermis.³⁴ In addition, CXCL16 can be expressed on the surface by monocytes/macrophages,^{55,56} which are frequently upregulated in patients with vitiligo. Further studies are needed to determine the role of these cell types in the production of CXCL16 in patients with vitiligo.

In line with previous reports, we demonstrate increased ROS levels in the epidermal microenvironment of patients with vitiligo (ie, both keratinocytes and melanocytes are affected by oxidative stress). The cell origin of ROS in the epidermis of patients with vitiligo, especially for that in keratinocytes, is still not clarified. However, given the occupation of keratinocytes in the epidermis, it is quite unlikely that ROS diffuses to the mass of keratinocytes from the few melanocytes. Intriguingly, although ROS exists in the whole epidermis, only melanocyte loss is obvious in lesions of patients with vitiligo. This could be because melanocytes are more susceptible to ROS than other epidermal cells or because ROS-induced autoimmunity specifically targets melanocytes, both of which warrant further investigation. It is worth mentioning that some studies have observed that keratinocytes also underwent bystander apoptosis in lesions of patients with vitiligo, which can be caused by lesional cytokines, including IFN- γ and TNF- α , that can sensitize keratinocytes to apoptosis.²⁵

According to our findings in the present study, we propose that the increased expression of CXCL16 by impaired keratinocytes in response to oxidative stress plays a critical role in CD8⁺ T-cell skin migration in patients with vitiligo. Thus targeting the UPR-CXCL16-CXCR6 axis might shed new light on vitiligo treatment. Further studies will be required to test the therapeutic implications of our observations.

We thank Professor Boquan Jin (Department of Immunology, Fourth Military Medical University) for his valuable advice and suggestions. We also thank the patients with vitiligo and healthy donors for their participation in this study.

Key messages

- The CXCL16-CXCR6 chemokine pair mediates recruitment of CD8⁺ T cells in response to oxidative stress in lesions of patients with vitiligo.
- The CXCL16 expression in keratinocytes induced by ROS occurs, at least in part, through 2 UPR pathways: PERK-eIF2 α and IRE1 α -XBP1.
- Blocking CXCL16-CXCR6 interaction alleviates CD8⁺ T-cell migration, suggesting that targeting the ROS-CXCL16-CXCR6 axis might be a promising therapy for vitiligo.

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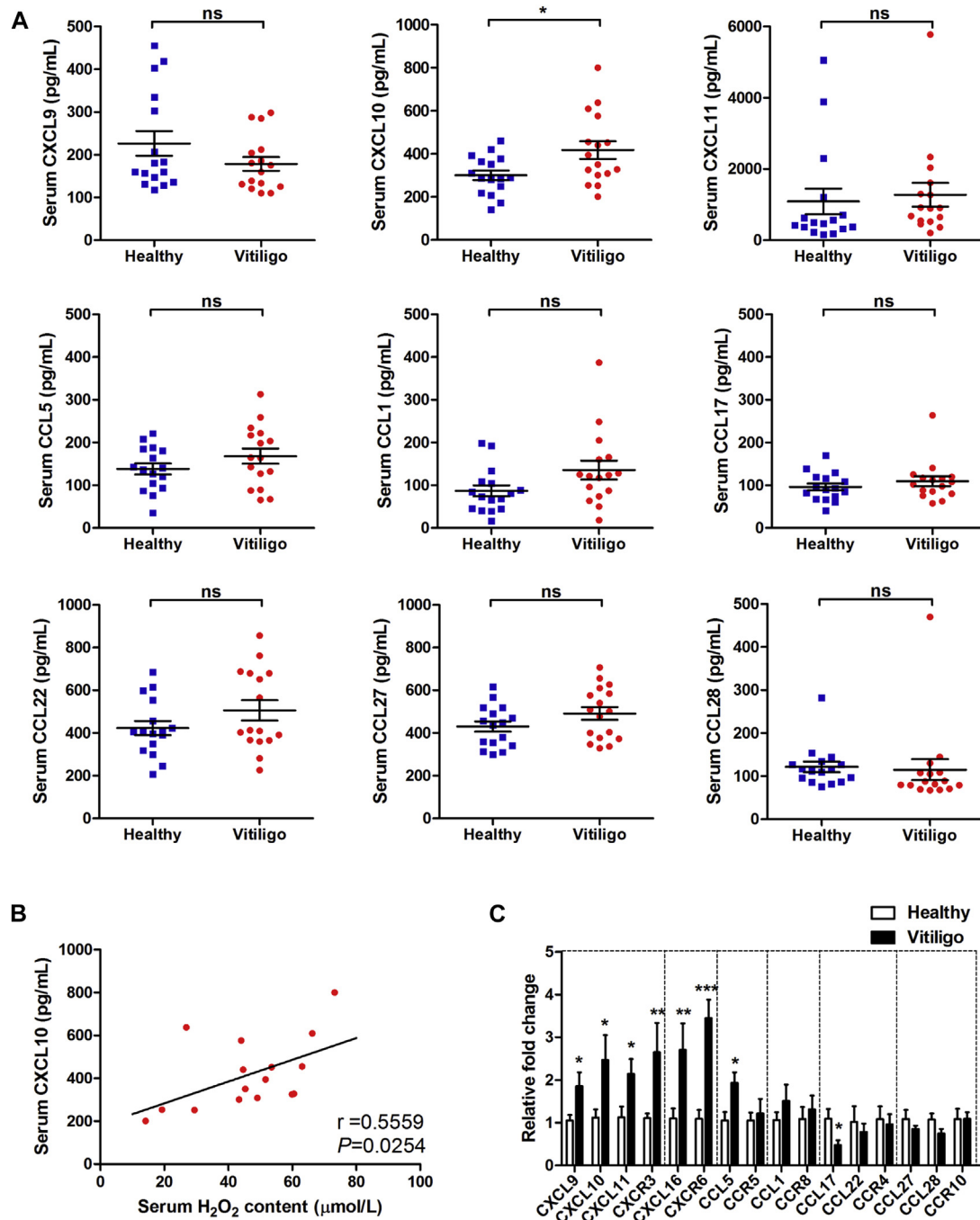


FIG E1. Expression of putative T-cell skin-homing chemokines in patients with vitiligo. **A**, Serum concentration of putative T-cell skin-homing chemokines in healthy control subjects and patients with vitiligo ($n = 16$). **B**, Correlations between H₂O₂ concentrations and serum CXCL10 levels. **C**, Skin mRNA levels of chemokines in skin of patients with vitiligo ($n = 8$). ns, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

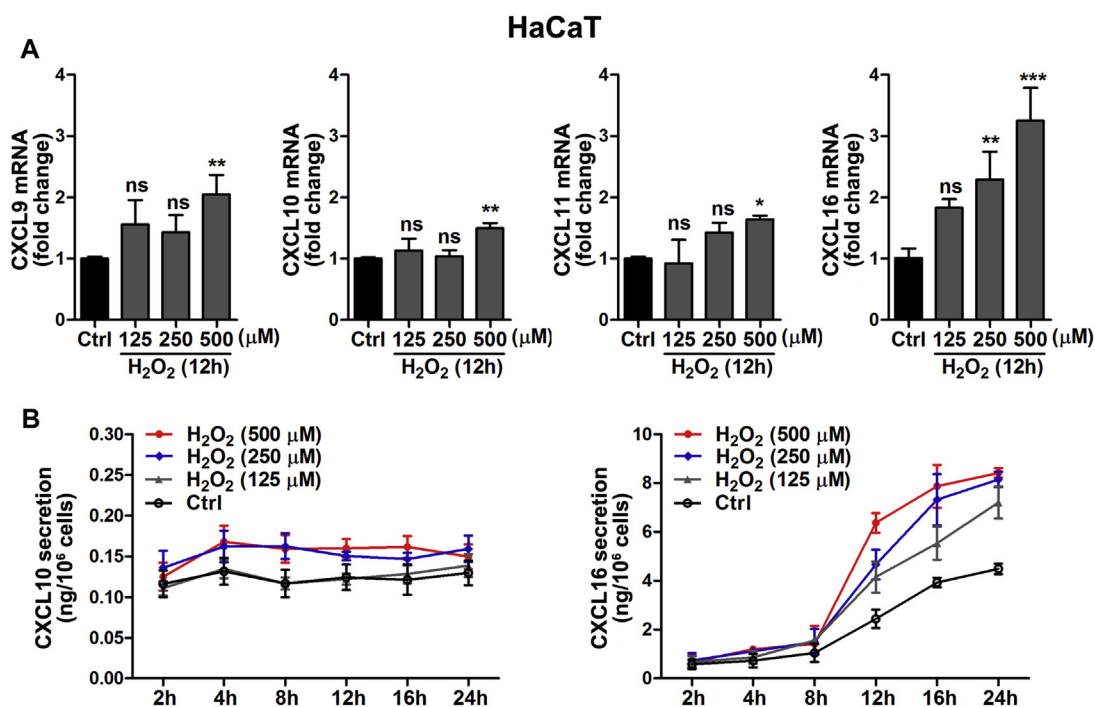


FIG E2. H₂O₂ induces chemokine CXCL16 expression in the keratinocyte cell line HaCaT. **A**, Expression of the chemokines CXCL9, CXCL10, CXCL11, and CXCL16, as determined by using qRT-PCR in HaCaT cells treated with indicated concentrations of H₂O₂ for 12 hours (n = 3). **B**, Secretion of CXCL10 and CXCL16 determined by using ELISA in HaCaT cell exposure to H₂O₂ at the indicated times (n = 3). ns, Not significant. *P < .05, **P < .01, and ***P < .001.

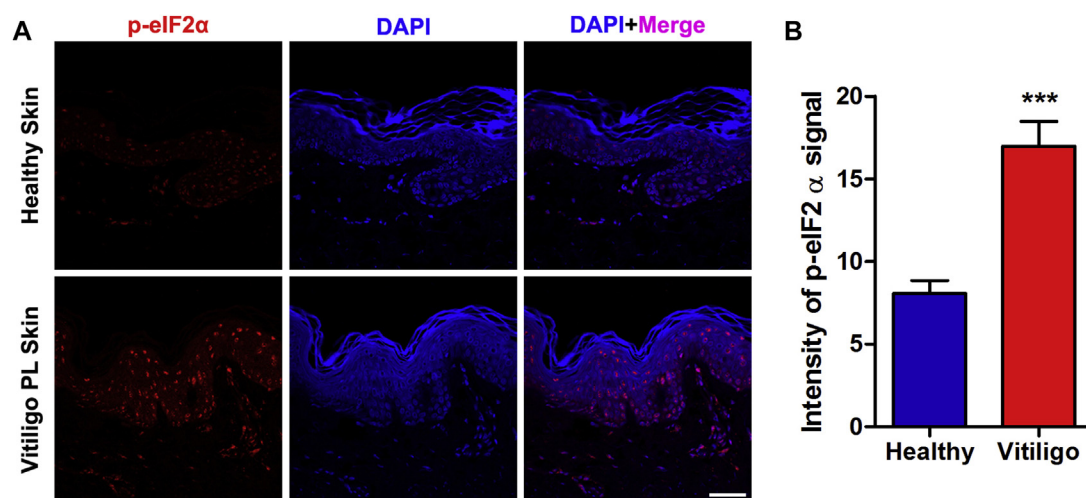


FIG E3. Phosphorylation of eIF2 α in skin of patients with vitiligo. **A**, Levels of phosphorylated eIF2 α in perilesions of patients with vitiligo (*PL Skin*) and healthy control subjects. *Scale bars* = 50 μ m. **B**, Statistical results of the intensity of phosphorylated eIF2 α signal ($n = 5$). *** $P < .001$.

NF-κB p65 (-1930 to -1939)

agctctccagctctctataatgcagttggggggagatggcatcaggctggaggaggtgcttgggaatccaggggcccgagtcctcc -1916

NF-κB p65 (-1868 to -1877) NF-κB p65 (-1836 to -1854)

ccagcctcagataccagatttctataccgatgcagccgggcatctcccacagtaagctgtcgggcttccggggaatccccagagc -1831

NF-κB p65 (-1816 to -1826)

ccatgggacaggccaagtgtgacagaggagagcatagggcccaaggagccaggctccttggtccctgagccccagcttgcggttcc -1746

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aataccctacggggctagggcaagtaacctcactgagtaaaagggtctgggttagagagggcacgctccttctaaaggggacagctgc -1491

tacagaggaccagaatgcgctgcatgcagaaaagttagggccactgtcagagcttgagattttcttgggaatgtgtaaaatccag -1406

acttctggatacttttttcttgcaatgttttaaacattaaattaaaaaaacccccactgtgggtcacaataaaacaccttttc -1321

aggccagattggcctgaaggccagcactcagaactagtggcaggggagggcttccgaaggaggatggctgggcaagggttaggg -1236

caggggcagccctgggttcttaccactcctctgtcccttctcttctgggtcttgttctgttctccacctgtctctcggcag -1151

NF-κB p65 (-1074 to -1084)

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taaggctagagaggagagggctaaatcctatcggaacgggttgggtggggagggaagcatttttgatgcgtctgtggggtatgg -216

NF-κB p65 (-194 to -202)

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tccggtacctgcagccgcccacttcttgcgaaggtaaatacctcgcggtggagggtgcgcgggtgcggttgtagcgtcagggtg -46

5'UTR (+1)

ccgagccctcgccggtcagcgggcgccacgcccccggtcGGTGCGTCCGCGGGTGGCTGCCCGCAGGTGCGCGCGGCC +40

GGGGCTGGCGGCGACTCTCTCCACCGGCCCGCCGGGAGGCTCATGCAGCGCGGTGGGTCCGCGGCGCCCGGATCGGGGAAGT +125

GAAAGTGCTCGGAGGAGGAGGGCGGTCCGGCAGTGCAGCCGCTCACAGGTGCGCGGACGGGCCAGGCGGGCGGCTCCTGAA +210

CCGAACCGAATCGGCTCCTCGGGCCGTCGTCCTCCCGCCCTCCTCGCCGCGCGCGGAGTTTCTTTTGGTTTCTTCAAGATT +295

CCTGGCTTCCCTCGACGGAGCCGGGCCAGTGCGGGGCGCAGGGCGCGGGAGCTCCACCTCCTCGGCTTCCCTGCGTCCAGA +380

GGCTGGCATGGCGCGGGCCGAGTACTGAGCGCACGGTCGGGGCACAGCGGGCGGGGGTGCAGCTGGCTCGCGCCTCCTCTCC +465

CDS

GGCCGCGCTCTCTCCGGTCCCGCGGAAAGCCATTGAGACACCAGCTGACGTCACGCGCGGAGCATGTCTGGGAGTCAGAGC +550

FIG E4. Potential binding sites of the transcription factor NF-κB p65 in the -2000 to +1 region of the *CXCL16* promoter predicted by using the JASPAR database shown in red and overlined.

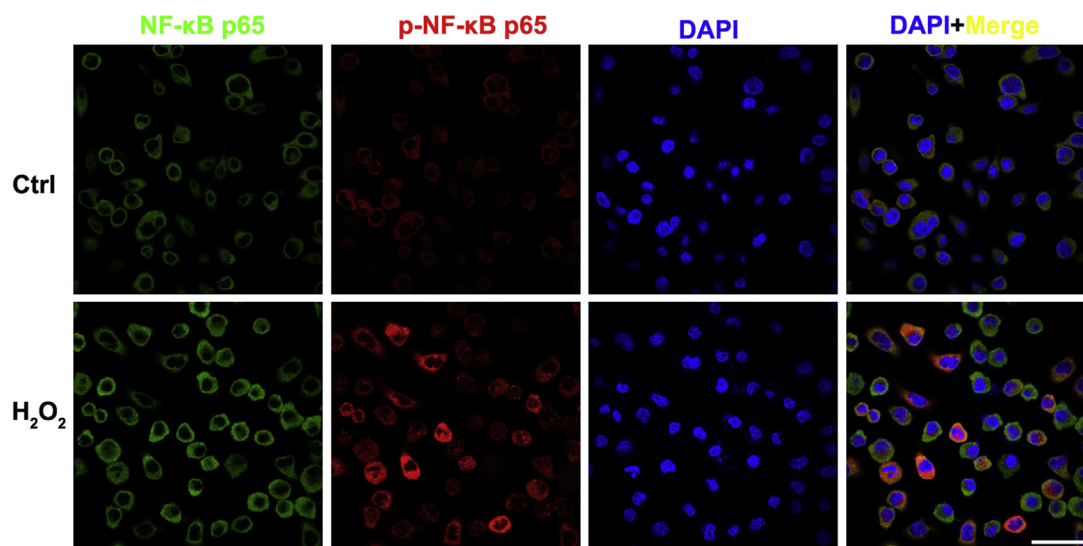


FIG E5. Treatment with H₂O₂ induces phosphorylation of NF-κB p65 in human keratinocytes. Phosphorylation of NF-κB p65 was evaluated in HaCaT cells exposed to H₂O₂ (500 μmol/L) for 3 hours. *Scale bars* = 50 μm.

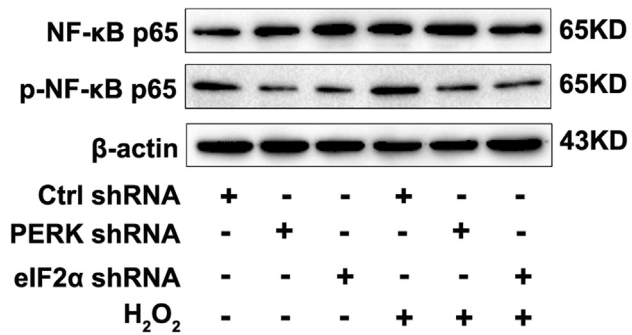


FIG E6. Activation of NF-κB p65 through the PERK-eIF2α pathway. Phosphorylation of NF-κB p65 was detected by using Western blot analysis in HaCaT cells transduced with PERK or eIF2α shRNA treated with 500 μmol/L H₂O₂ for 12 hours. β-actin was used as a control.

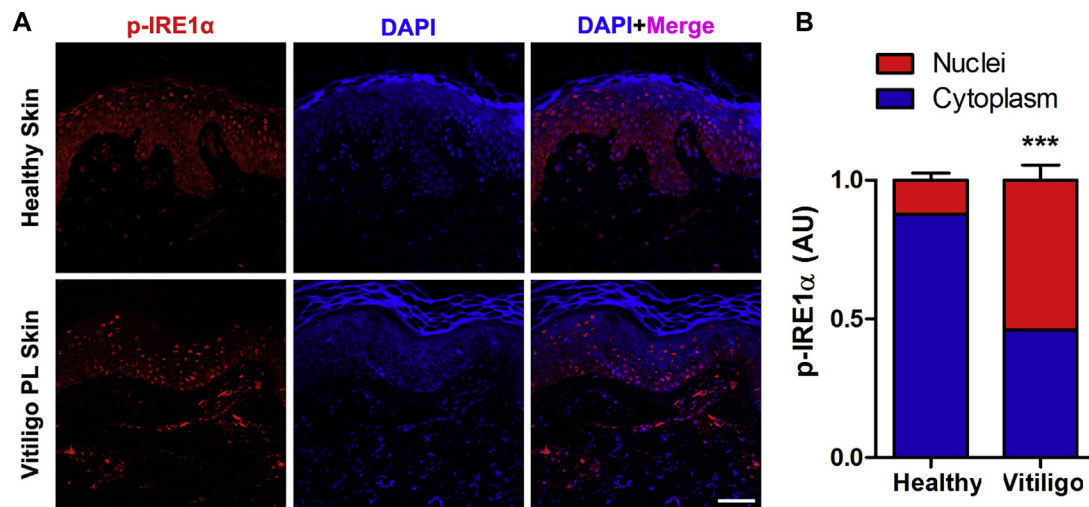


FIG E7. Phosphorylation of IRE1 α in skin of patients with vitiligo. **A**, Levels of phosphorylated IRE1 α in perilesions of patients with vitiligo (*PL Skin*) and healthy skin. Scale bars = 50 μ m. **B**, Statistical results of the nuclear to cytoplasm ratio of phosphorylated IRE1 α signal (n = 5). *** P < .001.

agctctccagctctctaaatgcagttggggggagatggcatcaggctggaggaggtgcttgggaatccagggcccgagtcctcc -1916
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 ccatgggacaggccatgctgtgacagaggagagcataggcccaaggagccaggtccttggtccctgagccccagcttgcggttcc -1746
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 XBP1 (-615 to -628)
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 XBP1 (-50 to -63)
 tccggtacctgcagccgccaccacttttcgaaggtaaatacctcgcggtggagggtgcgcgggtgcggttggtgacgtcagggtc -46
 5'UTR (+1)
 ccgagccctgcgcggctcagcgggcgccacgcccccgggctcGGTGCCTCCGCGGGTGGCTGCCCGCAGGTGCGCGCGGCC +40
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 GGCTGGCATGGCGCGGGCCGAGTACTGAGCGCACGGTCCGGGACACAGAGGGCCGGGGGTGCAGTGGCTCGCGCTCTCTCTCC +465
 CDS
 GGCCCGCGTCTCTCCGGTCCCGGCCGAAAGCCATTGAGACACCACTGGACGTACGCGCCGGAGCATGTCTGGAGTCAGAGC +550

FIG E8. Potential binding sites of XBP1 in the -2000 to +1 region of the *CXCL16* promoter predicted by using the JASPAR database and shown in red and overlined.

TABLE E1. Primers used for real-time PCR assays

Gene	Direction	Primer sequence (5' to 3')
CXCL9	Forward	TCTAAACCCAGATTTCAGCA
	Reverse	CTCCTTTGGAATGATAGCG
CXCL10	Forward	CCTCCAGTCTCAGCACCAT
	Reverse	AAATTGGCTTGCAGGAATA
CXCL11	Forward	AGCAGTGAAAGTGGCAGAT
	Reverse	GATTTAGGCATCGTTGTCC
CXCL16	Forward	GCCATCGGTTCAAGTTCA
	Reverse	CAATCCCCGAGTAAGCAT
CCL5	Forward	CGCGCCCACATCCAAGAGTATTTCTA
	Reverse	TCTTCTCTGGATTGGCACACACCT
CCL1	Forward	AGGCCTCTTTGCCTCTCTTC
	Reverse	ATGCAGATCATCACCACAGC
CCL17	Forward	GAGCCATTCCCCTTAGAAAG
	Reverse	AGGCTTCAAGACCTCTCAAG
CCL22	Forward	ATTACGTCCGTTACCGTCTG
	Reverse	TAGGCTCTTCATTGGCTCAG
CCL27	Forward	CTACAGCAGCATTCTACTGC
	Reverse	ATGGAGCTTTCTCTCTTGGTG
CCL28	Forward	AGAAGCCATACTTCCCATTGC
	Reverse	AGCTTGCACCTTCATCCACTG
CXCR3	Forward	GATTCATCCTGGTCTGAGACC
	Reverse	TACGCCATGCCTTGTACTCC
CXCR6	Forward	ATGGCAATGTCTTTAATCTCGACAA
	Reverse	TGAAAGCTGGTCATGGCATAGTATT
CCR5	Forward	AATGAGAAGAAGAGGCACAGG
	Reverse	CTTGAGTCCGTGT CACAAGC
CCR8	Forward	AGTATGCACATCTTGGATGG
	Reverse	TGTAGTCTACGCTGGAGGA
CCR4	Forward	GAAGAAGAACAAGGCGGTGAAGAT
	Reverse	ATGGTGGACTGCGTGTAAGATGAG
CCR10	Forward	GTTTCCTGGGGCCATTAC
	Reverse	ACAGCGTCGTTGGCCTTC