Oxidative Stress—Induced HMGB1 Release from Melanocytes: A Paracrine Mechanism Underlying the Cutaneous Inflammation in Vitiligo

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Vitiligo is a cutaneous depigmentation disorder caused by the destruction of epidermal melanocytes. The generation and the skin infiltration of autoreactive CD8⁺ cytotoxic T cells triggered by oxidative stress play a critical role in vitiligo. High-mobility group protein B1 (HMGB1) is a classic damage-associated molecular pattern molecule with strong proinflammatory effects in inflammatory reactions. A previous study reported an enhanced expression of HMGB1 in vitiligo lesions, but the role of HMGB1 in cutaneous inflammation of vitiligo is still unknown. In the present study, we initially found that HMGB1 was released from the nucleus of melanocytes in vitiligo perilesional skin. Furthermore, cultured normal human melanocytes could release HMGB1 under treatment with hydrogen peroxide. Moreover, HMGB1 facilitated the secretion of CXCL16 and IL-8 from keratinocytes by binding to the receptor for advanced glycation end products and activating NF-kB and extracellular signal—regulated kinase signaling pathways. Subsequently, HMGB1 led to the formation of chemotaxis for the migration of CD8⁺ T cells from patients with vitiligo by increasing the release of CXCL16 from keratinocytes. Additionally, HMGB1 promoted the maturation of dendritic cells from patients with vitiligo. Altogether, our study demonstrates that HMGB1 released from melanocytes contributes to the formation of oxidative stress—induced autoimmunity in vitiligo.


INTRODUCTION

Vitiligo is a chronic inflammatory disease characterized by skin depigmentation caused by the loss of epidermal melanocytes. The destruction of melanocytes in vitiligo has been attributed to several etiologic theories, including the occurrence of oxidative stress that can directly cause the apoptosis of melanocytes and the generation of CD8⁺ cytotoxic T cells that specifically destroy melanocytes (Becatti, 2017; Iian et al., 2014; Li et al., 2017). Notably, recent studies have demonstrated that oxidative stress facilitates the exposure of self-antigens derived from melanocytes, which are essential for the activation of autoreactive T cells (Xie et al., 2016). Moreover, oxidative stress promotes the secretion of chemokines from keratinocytes, which mediate the cutaneous infiltration of T cells in vitiligo lesion (Li et al., 2017). Therefore, oxidative stress can assist in the formation of autoimmune reaction toward melanocytes in the pathogenesis of vitiligo.

High-mobility group protein B1 (HMGB1) is a nucleoprotein that contributes to the stabilization of genome. Intriguingly, HMGB1 can be released from the cells under endogenous or exogenous stress and then acts as a damage-associated molecular pattern molecule that triggers inflammatory responses by binding to certain pattern recognition receptors (PRRs), including the receptor for advanced glycation end products (RAGE), toll-like receptor 2 (TLR2), and toll-like receptor 4 (TLR4) (Bangert et al., 2016; Mou et al., 2017). Kim et al. (2017) reported that HMGB1 was overexpressed in both blood samples and lesional specimens from patients with vitiligo, indicating that HMGB1 could be involved in the immune pathogenesis of vitiligo. Nevertheless, the specific role of HMGB1 in the development of vitiligo has not been clarified.

Oxidative stress is a central regulator of the function of HMGB1 (Mou et al., 2018; Petrović et al., 2017; Yu et al., 2015). In brief, oxidative stress affects the redox status of cysteines in HMGB1, which determines its proinflammatory
activity (Andersson et al., 2018). Moreover, oxidative stress can lead to the secretion of HMGB1 from immune or non-immune cells in a variety of diseases like sepsis and ischemia/reperfusion injury (Deng et al., 2018; Xie et al., 2018). Given that melanocytes are sensitive to oxidative stress in vitiligo lesions (He et al., 2017), we hypothesized that oxidative stress could induce the activated melanocytes to release HMGB1, which further activates the PRRs on the cytomembrane of skin cells like keratinocytes and promotes cutaneous inflammation in vitiligo.

To test our hypothesis, we examined the location of HMGB1 in vitiligo lesion. We found that HMGB1 was released from the nucleus of lesional melanocytes because of oxidative stress, as shown by our further in vitro assays. Subsequently, the role of HMGB1 in the formation of skin inflammation in vitiligo was also investigated.

**RESULTS**

**Oxidative stress induces the release of HMGB1 from melanocytes in vitiligo**

In order to observe the release of HMGB1 from melanocytes in vitiligo, we performed immunofluorescence analysis using skin specimens collected from five patients with vitiligo and five healthy donors. As a result, the translocation of HMGB1 into cytoplasm was seen in the melanocytes of vitiligo perilesional skin but not in that of healthy controls (Figure 1a), indicating that HMGB1 could be released by epidermal melanocytes in vitiligo. We then examined the HMGB1 serum level of patients with vitiligo by ELISA assay. It turned out that HMGB1 serum levels were significantly higher in the patients at active progressive phase than in the patients at slowly progressive phase or in healthy controls (Figure 1b).

We proceeded to investigate the influence of oxidative stress on HMGB1 in epidermal cells of vitiligo lesion. To this end, normal human melanocytes (NHM) and normal human keratinocytes (NHK) were treated with hydrogen peroxide ($H_2O_2$) at a concentration of 0.5 mM to establish oxidative stress models in vitro. Our quantitative real-time reverse transcription-PCR (QRT-PCR) and western blotting assays showed that the mRNA and protein expressions of HMGB1 were elevated in NHM treated with $H_2O_2$ but not in NHK with the same treatment (Figure 1c and d). A similar phenomenon could be observed in corresponding cell lines; treatment with $H_2O_2$ facilitated the mRNA expression of HMGB1 in PIG1 cells, an immortalized human melanocyte cell line, but failed to affect the mRNA level of HMGB1 in HaCaT cells, an immortalized human keratinocyte cell line (see Supplementary Figure S1a online). Furthermore, cytoplasmic HMGB1 level increased after $H_2O_2$ stimulation in NHM, whereas nuclear HMGB1 level decreased dramatically (Figure 1e). Subsequent immunofluorescence assay revealed that HMGB1 was located in the nucleus of NHM or PIG1 cells but was distributed throughout the cytoplasm after $H_2O_2$ treatment (Figure 1f and see Supplementary Figure S1b online). Moreover, $H_2O_2$ treatment led to enhanced accumulation of HMGB1 in the culture medium of NHM or PIG1 cells but not in that of NHK or HaCaT cells, as shown by ELISA assay (Figure 1g and see Supplementary Figure S1c online). Taken together, our findings verified that oxidative stress promotes the release of HMGB1 from melanocytes in vitiligo.

**HMGB1 released by melanocytes under oxidative stress enhances the production of CXCL16 and IL-8 in keratinocytes**

It is known that the chemokines secreted by activated keratinocytes play a key role in the cytotoxic T-cell response in vitiligo (Rashighi et al., 2014). Given that HMGB1 is a proinflammatory factor that can induce chemokines (Amin and Islam, 2014; Zhang et al., 2017), we speculated that HMGB1 released by melanocytes under oxidative stress could activate keratinocytes and promote chemokine production in a paracrine way in vitiligo. To test this, NHK were treated with recombinant human HMGB1 (rhHMGB1) and examined for the expression of chemokine (C-X-C motif) ligand 9 (CXCL9), chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-X-C motif) ligand 11 (CXCL11), chemokine (C-X-C motif) ligand 16 (CXCL16), and interleukin-8 (IL-8), all of which are elevated in vitiligo. It turned out that rhHMGB1 increased the mRNA levels of these chemokines, except CXCL9 in NHK, in a concentration-dependent way (Figure 2a). Subsequent ELISA assay showed that under the stimulation with 100 ng/ml rhHMGB1, the release of CXCL16 and IL-8 from NHK was elevated only in the groups with longer stimulation (Figure 2b). More importantly, the treatment with the culture medium of NHM exposed to $H_2O_2$ for 48 hours caused remarkable secretion of CXCL16 and IL-8 from NHK, which, however, could be inhibited by the addition of HMGB1-neutralizing antibody into the culture system (Figure 2c). Collectively, these results demonstrated that oxidative stress—induced HMGB1 release from melanocytes could enhance the production of CXCL16 and IL-8 in keratinocytes.

**RAGE is the key receptor that mediates the secretion of CXCL16 and IL-8 from keratinocytes induced by HMGB1**

To clarify the receptor that mediates the proinflammatory effects of HMGB1 in keratinocytes in vitiligo, we initially examined the expressions of the three HMGB1-related PRRs in vitiligo lesion by immunofluorescence. We found that the expression of RAGE was prominently increased at the epidermis layer of vitiligo lesion compared with healthy tissue (Figure 3a), whereas TLR2 and TLR4 showed weak expression in the skin specimens from either source (see Supplementary Figure S2a and b online). Since HMGB1 could promote the expression of its own receptors (Li et al., 2017), we performed immunofluorescence analysis in vitro and discovered that the treatment with rhHMGB1 elevated the expression of RAGE rather than TLR2 or TLR4 in NHK (Figure 3b and see Supplementary Figure S2c and d online), which was consistent with the results of the western blotting assay (Figure 3c). To explore whether RAGE mediated the production of chemokines in keratinocytes induced by HMGB1, NHK were firstly transfected with RAGE small interfering RNA (interference efficiency seen in Supplementary Figure S2e online) and then stimulated by rhHMGB1 or the culture medium of $H_2O_2$-treated NHM. Subsequent ELISA assay showed that with the knockdown of RAGE, the release of CXCL16 and IL-8, from NHK induced by either rhHMGB1 or the culture
were both suppressed (Figure 3d and e). Therefore, it can be concluded that HMGB1, released from melanocytes under oxidative stress, enhances the secretion of CXCL16 and IL-8 from keratinocytes by binding to RAGE.

HMGB1 induces the release of CXCL16 and IL-8 from keratinocytes via NF-κB p65 and extracellular signal-regulated kinase pathways

The production of chemokines in keratinocytes requires the activation of several signaling pathways, especially...
Consequently, HMGB1 released from keratinocytes via NF-κB pathways including p38 and JNK were not activated by rhHMGB1 (Figure 4a), but two other mitogen-activated protein kinase pathways (Santos et al., 2018; Xie et al., 2018), the activity of which strongly repressed the phosphorylation levels of p65 and ERK binding of HMGB1 on RAGE since the knockdown of RAGE confirmed that the activation of p65 and ERK required the phosphorylation levels of p65 and extracellular signal regulated kinase (ERK) were upregulated by rhHMGB1 in NHK (Figure 4b), but two other mitogen-activated protein kinase pathways including p38 and JNK were not activated by rhHMGB1 (see Supplementary Figure S3 online). We further confirmed that the activation of p65 and ERK required the binding of HMGB1 on RAGE since the knockdown of RAGE strongly repressed the phosphorylation levels of p65 and ERK in NHK treated with rhHMGB1 (Figure 4b).

In order to verify the role of p65 and ERK in the proinflammatory effects of HMGB1 on keratinocytes, we took advantage of p65 siRNA or U1026 to block HMGB1-induced activation of p65 or ERK, respectively, in NHK (Figure 4c and d). Our ELISA assay revealed that either p65 siRNA or U1026 successfully reduced the secretion of both CXCL16 and IL-8 from NHK stimulated by the culture medium of H2O2-treated NHM (Figure 4e and f). Consequently, HMGB1 released from melanocytes under oxidative stress could promote the secretion of CXCL16 and IL-8 from keratinocytes via NF-κB p65 and ERK pathways.

**Figure 2.** HMGB1 released by melanocytes under oxidative stress facilitates the formation of CXCL16 and IL-8 in keratinocytes. (a) The mRNA levels of CXCL9, CXCL10, CXCL11, CXCL16, and IL-8 in NHK treated with rhHMGB1 for 24 hours. (b) Secretional levels of CXCL9, CXCL10, CXCL11, CXCL16, and IL-8 from NHK treated with rhHMGB1. (c) Secretional levels of CXCL16 and IL-8 from NHK stimulated by rhHMGB1 or the culture medium of NHM with different treatments as indicated. For graphical representation, data are presented as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. CM, culture medium of melanocytes; CXCL16, chemokine (C-X-C motif) ligand 16; H1M-Ab, HMGB1-neutralizing antibody; HMGB1, high-mobility group protein B1; H2O2-CM, CM of melanocytes treated with H2O2; NHM, normal human melanocytes; NHK, normal human keratinocytes; rhHM1, rhHMGB1; rhHMGB1, recombinant human HMGB1.

HMGB1 released from melanocytes under oxidative stress facilitates the formation of T-cell chemotaxis created by keratinocytes in vitiligo

We subsequently investigated whether HMGB1 released from melanocytes could facilitate the T-cell chemotaxis created by chemokines from keratinocytes in vitiligo. Using flow cytometry, CD8+ T cells were sorted from the patients with vitiligo. Subsequent transwell assay showed that the supernatant of NHK treated by rhHMGB1 or the culture medium of H2O2-treated NHM led to more migrated T cells, which, however, were abolished by the pretreatment with HMGB1-neutralizing antibody in the stimulating system for NHK or by the addition of CXCL16-neutralizing antibody into the transwell system (Figure 5a). IL-8-neutralizing antibody failed to affect the T-cell chemotaxis, which was consistent with the fact that IL-8 is a chemokine that mainly mediates the migration of neutrophils rather than T cells (Lee et al., 2018; Singhto and Thongboonkerd, 2018). We further observed that the serum levels of both CXCL16 and IL-8 were significantly higher in patients with vitiligo than in healthy controls (Figure 5b and c). Moreover, HMGB1 serum level showed positive correlations with CXCL16 and IL-8 serum levels in patients with vitiligo (Figure 5d and e). Taken together, our findings indicate that HMGB1 released from melanocytes under oxidative stress could facilitate the T-cell migration of neutrophils rather than T cells (Lee et al., 2018; Singhto and Thongboonkerd, 2018). We further observed that the serum levels of both CXCL16 and IL-8 were significantly higher in patients with vitiligo than in healthy controls (Figure 5b and c). Moreover, HMGB1 serum level showed positive correlations with CXCL16 and IL-8 serum levels in patients with vitiligo (Figure 5d and e). Taken together, our findings indicate that HMGB1 released from melanocytes under oxidative stress could facilitate the T-cell migration of neutrophils rather than T cells (Lee et al., 2018; Singhto and Thongboonkerd, 2018). We further observed that the serum levels of both CXCL16 and IL-8 were significantly higher in patients with vitiligo than in healthy controls (Figure 5b and c). Moreover, HMGB1 serum level showed positive correlations with CXCL16 and IL-8 serum levels in patients with vitiligo (Figure 5d and e). Taken together, our findings indicate that HMGB1 released from melanocytes under oxidative stress could facilitate the T-cell
chemotaxis created by keratinocytes by promoting the production of CXCL16 in vitiligo.

**HMGB1 released from melanocytes under oxidative stress could promote the maturation of dendritic cells in vitiligo**

It is reported that dendritic cells (DCs) play a critical role in the pathogenesis of vitiligo for their function of self-antigen presentation (Mosenson et al., 2013). Because HMGB1 could accelerate the activation of DCs (Kim et al., 2014; Saidi et al., 2008), we next explored the effects of HMGB1 on DCs in vitiligo. Initially, our immunofluorescence assay showed that HMGB1 receptors, including RAGE, TLR2, and TLR4, were colocalized with CD11c, which is the marker of DCs in vitiligo lesions (Figure 6a). We then treated the blood cells from patients with vitiligo with rhHMGB1 or the culture medium of H2O2-treated NHM. Subsequent flow cytometry
analysis revealed that the maturation markers of DCs, including CD80, CD86, and HLA-DR, were all upregulated in the CD11c<sup>+</sup> DCs treated with either rhHMGB1 or the culture medium of H<sub>2</sub>O<sub>2</sub>-treated NHM, but failed to react to the treatment containing HMGB1-neutralizing antibody (Figure 6b–e). Altogether, these results demonstrated that HMGB1 released by melanocytes under oxidative stress could facilitate the maturation of DCs in vitiligo.

**DISCUSSION**

Damage-associated molecular pattern molecules have been proven to take a part in the pathogenesis of vitiligo (Mosenson et al., 2013; Mosenson et al., 2014; Zhang et al., 2014). It has been reported that heat shock protein 70 secreted by melanocytes induced an inflammatory DC phenotype and was necessary for depigmentation in vitiligo mouse models (Mosenson et al., 2013; Mosenson et al., 2014).
melanocytes in vitiligo perilesional skin. Further in vitro abnormally localized in the cytoplasm of the remaining 2018). In the current study, we found that HMGB1 was inflammation by integrating with PRRs (Tisserand et al., 2017), atopic dermatitis (Karuppagounder et al., 2017). As a classic damage-associated molecular pattern molecule, HMGB1 is involved in the development of many autoimmune skin diseases, including psoriasis (Zhang et al., 2017), atopic dermatitis (Karuppagounder et al., 2015; Wang et al., 2018), and lichen planus (de Carvalho et al., 2018). Recently, some studies have reported that HMGB1 may directly induce the apoptosis of melanocytes in vitiligo (Becatti, 2017; Mou et al., 2018). We found that HMGB1 secreted by melanocytes under oxidative stress was able to promote the secretion of chemokines from keratinocytes via the activation of NF-κB signaling pathway (Tisserand et al., 2017). Moreover, the present study demonstrates that oxidative stress could also indirectly result in the secretion of CXCL16 from keratinocytes via a paracrine way.

Generally, HMGB1 stays in the nucleus and is a vital regulator of gene transcription by interacting with chromatin structures of DNA (Andersson et al., 2018). Nonetheless, when cells are under endogenic or environmental stress, HMGB1 can be either actively secreted or passively released upon cell death into extracellular milieu, and then promote inflammation by integrating with PRRs (Tisserand et al., 2018). In the current study, we found that HMGB1 was abnormally localized in the cytoplasm of the remaining melanocytes in vitiligo perilesional skin. Further in vitro experiments showed that under the treatment with 0.5 mM H$_2$O$_2$, which is known as the concentration of H$_2$O$_2$ in vitiligo lesion (Li et al., 2017), HMGB1 was translocated from nucleus to cytoplasm in melanocytes and subsequently released into extracellular space, which was consistent with the findings reported by Mou et al. (2018). Keratinocytes also failed to release HMGB1 under the same treatment, implying that melanocytes are the main source of proinflammatory HMGB1 in vitiligo. Additionally, HMGB1 serum level was increased in the patients in the active progressive phase but showed no change in the slowly progressive phase, which indicates that HMGB1 is a crucial inflammatory factor involved in the progression of vitiligo. Therefore, serum HMGB1 level is a promising biomarker for monitoring disease activity in patients with vitiligo, and inhibiting the melanocytic release of HMGB1 could be a potential strategy for preventing the rapid progression of vitiligo.

Our previous research on psoriasis showed that HMGB1 facilitated the production of a variety of cytokines in keratinocytes, especially IL-18, which promoted CD4$^+$ Th17 cell response in the development of psoriasis (Zhang et al., 2017). The current study focused on the effect of HMGB1 on the production of chemokines in keratinocytes for their significant role in vitiligo. Previously, we verified that oxidative stress could directly induce the production of CXCL16 in keratinocytes via the activation of NF-κB signaling pathway and unfolded protein response (Li et al., 2017). Moreover, the present study demonstrates that oxidative stress could also indirectly result in the secretion of CXCL16 from keratinocytes via HMGB1 from melanocytes in a paracrine way.
Other than CXCL16, IL-8 was another increased chemokine from HMGB1-treated keratinocytes; this result is consistent with the findings reported by Dejean et al. (2012). IL-8 is known to induce the chemotaxis for neutrophils; however, the migration of neutrophils depends not only on the chemotactic function of IL-8 but also on the polarity of neutrophils, which is mediated by Rho GTPases (Pantarelli and Welch, 2018). Recently, some studies have found that 8-hydroxydeoxyguanosine, an oxidative stress-induced DNA product that is elevated in vitiligo (Vaseghi et al., 2017; Wei et al., 2013), could inhibit the function of Rho GTPases (Park et al., 2017). Therefore, oxidative stress may inhibit the migratory ability of neutrophils and thus suppress the cutaneous infiltration of neutrophils in vitiligo, even though the level of IL-8 is elevated in the disease.

It is known that HMGB1 must bind to certain PRRs to exert its proinflammatory functions. Although all of the three HMGB1-binding PRRs, including RAGE, TLR2, and TLR4, are constitutively expressed in the membrane of keratinocytes (Zhang et al., 2017), our study found that only RAGE was increased in vitiligo lesion as well as in cultured keratinocytes stimulated by rhHMGB1. Additionally, the knockdown of RAGE significantly reduced the production of CXCL16 and IL-8 in keratinocytes treated with rhHMGB1. These findings suggest that RAGE may be the predominant PRR that mediates the proinflammatory effects of HMGB1 in keratinocytes.

Figure 6. HMGB1 released from melanocytes under oxidative stress could promote the maturation of DCs in vitiligo. (a) Representative images of the expressions of HMGB1 receptors in CD11c+ DCs in vitiligo skin. (b) Representative flow charts of the expressions of CD80, CD86, and HLA-DR in CD11c+ DCs from patients with vitiligo (n = 5) with various treatments as indicated. (c–e) Statistical analysis of the expressions of CD80, CD86, and HLA-DR in CD11c+ DCs from patients with vitiligo (n = 5) with various treatments as indicated. For graphical representation, data are presented as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001. CM, culture medium of melanocytes; DCs, dendritic cells; HM1-Ab, HMGB1-neutralizing antibody; HMGB1, high-mobility group protein B1; H2O2-CM, CM of melanocytes treated with H2O2; rhHM1, rhHMGB1; rhHMGB1, recombinant human HMGB1. Scale bar = 50 μm.
in vitiligo, though the role of TLR2 and TLR4 still needs to be investigated in future studies.

The presentation of self-antigens derived from melanocytes via mature DCs is essential for the activation of cytotoxic T cells that target melanocytes in vitiligo (Moseenson et al., 2013). In fact, blocking the activation of DCs can facilitate the regpimentation of vitiligo lesion (Taleb and Seneschal, 2013). Previous studies have reported that HMGB1 is able to accelerate the maturation of DCs (Kim et al., 2014). Consistently, our study showed that endogenous HMGB1 from melanocytes upregulated the expression of several DCs maturation markers from patients with vitiligo. Therefore, oxidative stress–induced HMGB1 released from melanocytes may play a dual role in the autoimmune pathogenesis of vitiligo by promoting both the cutaneous infiltration and the activation of cytotoxic T cells.

A recent study by Zhang et al. (2019) reported that HMGB1 could be released from melanocytes exposed to UVB and promote the activation of ERK pathway in melanocytes in an autocrine way, which is consistent with the paracrine effects of HMGB1 on keratinocytes observed in our study. Notably, UVB phototherapy is a commonly used strategy for treating vitiligo by activating melanocyte stem cells and suppressing the function of autoreactive T cells (Bulat et al., 2011; Goldstein et al., 2018). However, the therapeutic effect of UVB phototherapy is limited, given that a considerable portion of patients with vitiligo is not responsive (Silpa-Archa et al., 2018), which may be due to the proinflammatory effects of UVB-induced HMGB1 on keratinocytes, according to our findings.

In summary, we demonstrated the function of HMGB1 in facilitating the secretion of chemokines from keratinocytes and the maturation of DCs in vitiligo. Our findings suggest that HMGB1 is a pivotal damage-associated molecular pattern molecule that links oxidative stress to the formation of autoimmunity in the pathogenesis of vitiligo. Moreover, HMGB1 could mediate the proinflammatory cross-talk between melanocytes and other cells, including keratinocytes and DCs, in the cutaneous microenvironment under oxidative stress. Therefore, HMGB1 may be a promising therapeutic target for the treatment of vitiligo.

MATERIALS AND METHODS

Patients and samples
Peripheral blood samples and skin specimens were collected from patients diagnosed with vitiligo, according to clinical and histological manifestation, and healthy donors at the Department of Dermatology of the Xijing hospital of the Fourth Military Medical University. Perilesional skin and control skin samples were collected from five patients with vitiligo and five healthy donors, respectively, that underwent cosmetic surgery at the Xijing Hospital. Peripheral blood samples were collected from 21 healthy donors and 21 patients with vitiligo, of which 15 patients were in the slowly progressive stage and 6 patients were in the active progressive stage, based on their Vitiligo European Task Force assessment scores of +1 to +3 and +4 to +5, respectively (Taleb et al., 2007). All of the patients and the control subjects consented by written and informed agreement for inclusion in this study. The patients and controls were pair-matched in age and gender. 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 the Fourth Military Medical University.

Cell culture and treatments
Details for cell culture and treatments are described in Supplementary Materials and Methods (online).

RNA interference
RNA interference was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, USA), as described in Supplementary Materials and Methods.

RNA isolation and QRT-PCR analysis
Details for RNA isolation, reverse transcription, and QRT-PCR analysis are described in Supplementary Materials and Methods.

Western blotting analysis
Details for protein isolation and western blotting analysis are described in Supplementary Materials and Methods.

ELISA assay
Details for ELISA analysis are described in Supplementary Materials and Methods.

Immunofluorescence analysis
Details for immunofluorescence assay are described in Supplementary Materials and Methods.

Transwell assay
Details for transwell assay are described in Supplementary Materials and Methods.

Flow cytometry analysis
Details for flow cytometry are described in Supplementary Materials and Methods.

Statistical analyses
Data analysis was performed using GraphPad Prism software, version 6.0 (GraphPad Software, San Diego, CA). Dual comparisons were made with unpaired two-tailed Student t test. Groups of three or more were analyzed by one-way analysis of variance with Dunnett posttests. Correlation analysis was performed by Spearman’s rank correlation test. A P-value of less than 0.05 was considered significant. Data are presented as mean ± standard deviation for at least three independent experiments.

Data availability statement
The authors confirm that the data supporting the findings of this study are available in the article and its supplementary materials. Any additional data that support the findings of this study and/or more information about the study design are available from the corresponding author, PS, upon reasonable request.
CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: PS, CL; Data Curation: SL, YY, JC; Formal Analysis: XY, PK; Funding Acquisition: PS; Investigation: TC, XY; Methodology: LL, ZJ; Project Administration: KL; Supervision: GW, TG; Validation: XC; Visualization: YC; Writing - Original Draft Preparation: TC; Writing - Review and Editing: WZ, PS

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at 10.1016/j.jid.2019.03.1148.

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and treatments
The NHM, NHK, PIG1 cells, and HaCaT cells were obtained and cultured as described in our previous studies (Chang et al., 2017). Based on our previous report (Li et al., 2017), H2O2 (Sigma-Aldrich, St. Louis, MO) was used to treat cells at the concentration of 0.5 mM and in our CCK8 assays (see Supplementary Figure S4 online). Recombinant human Dsulfide-HMGB1 (HMGBiotech, Milano, Italy) was used to stimulate NHK or HaCaT cells at different concentrations as indicated previously. Peripheral blood samples and peripheral blood mononuclear cells were obtained from patients with vitiligo, with informed consent, and cultured in Modified Medium RPMI 1640 (HyClone, Logan, UT) with 10% fetal bovine serum (Sijiqing, Hangzhou, China) before subsequent flow cytometry analysis. The supernatants of NHM were used by mixing them with the culture medium for NHK at a ratio of 1:1. HMGB1-neutralizing antibody (Chicken polyclonal to HMGB1, Shino-test, Japan) was used at a concentration of 100 ng/ml. For inhibiting ERK signals, U1026 was used at a concentration of 20 μM.

RNA interference
Cells were seeded at 2 × 10^5 cells per well the day before transfection. Cells were transfected in 2 ml of culture medium with 3 μl of RAGE siRNA or p65 siRNA or control siRNA (Songon, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA), following the manufacturer’s protocol.

RNA isolation and QRT-PCR analysis
Total RNA from cultured cells was isolated using Trizol reagent (Invitrogen), and then reversely transcribed to cDNA using the PrimeScript RT reagent Kit (Takara, Ohtsu, Japan). QRT-PCR analysis was performed using SYBR Premix Ex Taq II (Takara) with the iQ5 PCR Detection System (Bio-Rad, Hercules, CA). The relative mRNA expression was normalized to the β-actin gene. The primers used in this study were as follows: CXCL9, forward: TCTAAACCCAGATTCAGCA, reverse: CTCCATTGGGAATGATAGCC; CXCL10, forward: CCTCCAGTCTCAGGACC; CXCL11, forward: AAATTTGCCTGGCAGGAATA; CXCL11, reverse: AGCAGTGAAGTGGCAGAT; reverse: GATTTAGGCATCGTTGTCC; CXCL16, forward: GCCATCGGTTGAGTCCA, reverse: CAATCCCCAGTAAAGCAT; and IL-8, forward: CACTGCACCAACACAGAAAT, reverse: GCCATTGAAGTTCTGCGATC.

Western blotting analysis
Cells were lysed with cell lysis solution (DSL, Webster, IA) or put through nuclear-cytoplasmatic fractionation using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA). Each protein sample was quantified using the DC protein assay Kit (Pierce, Rockford, IL). Equal amounts of protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Millipore, Bedford, MA). After blocking in a solution of 5% non-fat dry milk diluted in tris-buffered saline for 1 hour, the membranes were washed and then incubated with primary antibodies overnight at 4 °C. After extensive rinsing, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (Zhongshan Biotechnology, Beijing, China) for 2 hours at room temperature. The immunoreactive bands were detected using the ECL western blotting detection system (Millipore, Billerica, MA). The primary antibody against HMGB1 (ab18256) was purchased from Abcam (Burlingame, CA). Other primary antibodies against p65 (8242), phospho-p65 (3033), ERK (4695), phospho-ERK (4370), p38 (9212), phospho-p38 (9216), JNK (9255), and phospho-JNK (4671) were purchased from Cell Signaling Technology (Danvers, MA). The bands were quantified using Image J software.

Immunofluorescence analysis
For skin specimens, deparaffinized tissue sections of 5 μm were first put through heat-mediated antigen retrieval with Tris-EDTA buffer (pH 9.0). For cultured cells in coverslips, they were fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.1% TritonX-100 for 5 minutes. Subsequently, the skin sections or the cells were blocked at room temperature with 5% goat serum in phosphate buffered saline for 30 minutes, and incubated with primary antibodies (Rabbit monoclonal to HMGB1, Abcam; Mouse monoclonal to Melan-A, Abcam; Rabbit monoclonal to RAGE, Abcam; Rabbit monoclonal to TLR2, Abcam; Rabbit monoclonal to TLR3, Abcam; Mouse monoclonal to CD11c, CST) at 4 °C overnight, followed by incubation with corresponding secondary antibodies (Goat anti-mouse IgG, Red Cy3 Conjugated; Goat anti-mouse IgG, Green Fluorescein Isothiocyanate Conjugated; Goat anti-Rabbit IgG, Red Cy3 Conjugated; Goat anti-mouse IgG, Green Fluorescein Isothiocyanate Conjugated), all purchased from Abcam, at a dilution of 1:200 for 45 minutes at room temperature in the dark. DAPI (Dako, Denmark) was used to mark cell nucleus. All the specimens were analyzed by FV-1000/ES confocal microscope (Olympus America, Melville, NY). ImageJ 64 software was utilized to analyze the fluorescence intensity.

Transwell assay
NHK were treated or transfected as indicated. After 48 hours, culture supernatants were collected for the chemotaxis assay. Peripheral blood mononuclear cells—derived vitiligo CD8+ T cells (1 × 10^5 cells, cultured in 100 μl medium) were sorted for the chemotaxis assay, separated from the cell supernatants using a 5.0 μm polycarbonate membrane (Corning Life Sciences, Corning, NY). In addition, CXCL16 neutralization antibody (AF976; R&D Systems, Minneapolis, MN) and IL-8 neutralization antibody (mab266, R&D Systems) were used to block CXCL16 or IL-8. Plates were left at 37 °C for 3 hours. Migrated cells in the lower chamber were counted by flow cytometry (Beckman Coulter, Brea, CA).
Flow cytometry analysis
The detection of the surface markers of DCs was performed according to previous reports (Sousa et al., 2018; Yu et al., 2018). Generally, fresh peripheral blood from patients with vitiligo was treated as indicated, for 24 hours at 4 °C, and then the cells were collected by centrifugation at 1200 r.p.m. for 10 minutes and incubated with fluorochrome-conjugated anti-human CD11c-FITC (eBioscience, Thermo Fisher Scientific) with each antibody against the mature markers of DCs including anti-human CD80-PE (eBioscience), anti-human HLA-DR-PERCP (eBioscience), and anti-human CD86-APC (eBioscience), respectively, at 4 °C for 30 minutes in the dark. Subsequently, the red blood cells were removed with red blood cell lysis buffer, and the remaining cells were washed with phosphate buffered saline and then resuspended in 200 μl of phosphate buffered saline. Flow cytometry assays were performed using FACS Canto II (BD Biosciences, Heidelberg, Germany) and the results were analyzed with BD FACSDiva Software, version 6.1.2.

SUPPLEMENTARY REFERENCES
Supplementary Figure S2. The expressions of TLR2 and TLR4 are not significantly changed in vitiligo. (a, b) Representative images of TLR2 and TLR4 expression in vitiligo skin versus healthy skin. (c, d) Representative images of TLR2 and TLR4 expression in rhHMGB1-treated NHK. (e) Representative immunoblots for interference efficiency of RAGE siRNA in NHK. NHK, normal human keratinocytes; RAGE, receptor for advanced glycation end products; rhHMGB1, recombinant human HMGB1; siRNA, small interfering RNA; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4. Scale bar = 50 μm.

Supplementary Figure S3. Representative immunoblots of the activation levels of p38 and JNK pathways in NHK treated with rhHMGB1. NHK, normal human keratinocytes; rhHMGB1, recombinant human HMGB1.
Supplementary Figure S4. H$_2$O$_2$ at the concentration of 0.5 mM was safe to the viability of NHM, NHK, PIG1 cells, and HaCaT cells. The viability of (a) NHM, (b) NHK, (c) PIG1 cells, and (d) HaCaT cells with H$_2$O$_2$ treatment was detected by CCK8 assay. For graphical representation, data are presented as mean ± standard deviation. *P < 0.05, ***P < 0.001. NHK, normal human keratinocytes; NHM, normal human melanocytes; ns, not significant.