Involvement of high mobility group box-1 in imiquimod-induced psoriasis-like mouse model

Tao CHEN,1,2,3,* Li-xin FU,1,* Zai-pei GUO,2 Bin YIN,1 Na CAO,1 Sha QIN2
1Department of Dermatovenereology, Chengdu Second People’s Hospital, 2Department of Dermatovenereology, West China Hospital of Sichuan University, 3Department of Dermatovenereology, Chengdu Qingbaijiang Distinct People’s Hospital, Chengdu, China

ABSTRACT
In the previous work, we have indicated that HMGB1, a pro-inflammatory cytokine, is closely associated with the pathogenesis of psoriasis. To further clarify the role of HMGB1 in the pathogenesis of psoriasis, we investigated the direct function of HMGB1 application and HMGB1 blockade in imiquimod (IMQ)-induced psoriatic mouse model in this study. Mice were treated with imiquimod (IMQ) to induce psoriasis-like inflammation, and consecutively injected with recombinant HMGB1 or phosphate-buffered saline (PBS) i.d. Abundant cytoplasmic expression of HMGB1 was observed in lesional skin from IMQ-treated skin. The injection of HMGB1 into the IMQ-treated skin further aggravated the psoriasis-like disease, enhanced the infiltration of CD3+ T cells, myeloperoxidase+ neutrophils and CD11c+ dendritic cells, increased the number of γδ T cells, and upregulated the mRNA expression of interleukin (IL)-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and IL-17 compared with the PBS injection. Finally, by using anti-HMGB1 monoclonal antibody or HMGB1 inhibitor glycyrrhizin, we indicated that HMGB1 blockade reduced the number of γδ T cells, suppressed the mRNA expression of IL-6, TNF-α, IFN-γ and IL-17, and moderated clinical and histological evolvement in the IMQ-treated skin. Our data suggest that HMGB1 may act as a pro-inflammatory cytokine, and contribute to the development of IMQ-induced psoriasis-like inflammation. HMGB1 blockade may represent a new direction in the suppression of psoriasis.

Key words: glycyrrhizin, high mobility group box-1, imiquimod, inflammation, psoriasis.

INTRODUCTION
Psoriasis is a chronic common inflammatory skin disease characterized by keratinocytes and dermal vascular proliferation, and cellular infiltration of neutrophils, T cells, macrophages and dendritic cells (DC).1–4 It has affected up to 3% of the population worldwide.5 Although it has been recognized that the plentiful productions of T-helper (Th)1/Th17 associated cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and IL-17 are important in the development of this disease.5–8 The potential molecular mechanisms in the development of this disease are not yet fully understood.

High mobility group box-1 (HMGB1), described as a non-histone nuclear protein, is normally located in the nucleus and expressed in almost all mammalian cells.9 Recent evidence reveals that HMGB1 could be translocated to the cytoplasm and secreted to the extracellular space during cellular necrosis or when exposed to inflammatory mediators, for instance, TNF-α, IFN-γ and lipopolysaccharide.10 It has been recognized that extracellular HMGB1 may act as a pro-inflammatory cytokine implicated in the pathogenesis of many autoimmune and inflammatory diseases, for example, ankylosing spondylitis, systemic lupus erythematosus and rheumatoid arthritis.11–13

To investigate whether HMGB1 acts in other roles in the pathogenic mechanism of psoriasis, some preliminary studies have been carried out. Our published work demonstrated that the serum HMGB1 levels were markedly elevated in patients with psoriasis vulgaris (PV) and significantly reduced after glycyrrhizin treatment.14 We also indicated that HMGB1 was abundantly expressed in the cytoplasm of the epidermis of PV patients.14 However, the roles of HMGB1 in the pathogenesis of psoriasis remain unclearly defined.

To further understand the roles of HMGB1 in the pathogenic mechanism of psoriasis and in order to find a possible new therapeutic strategy in the treatment of psoriasis, the direct effects of HMGB1 injection in mice skin and the therapeutic effects of HMGB1 blockade in imiquimod (IMQ)-induced psoriasis-like mouse model were researched in this study.

Glycyrrhizin, a main active ingredient in licorice root, has been widely used in the therapy of various inflammatory skin diseases including psoriasis in China. However, the pharmaceutical functions of glycyrrhizin in the therapy of psoriasis were still ill-defined. Recently, it has been recognized that...
glycyrrhizin is a HMGB1 inhibitor, which can bind directly to HMGB1 protein and inhibit its cytokine activities. Therefore, we also observed the effects of glycyrrhizin on HMGB1 expression and psoriatic inflammation in IMQ-induced skin in this study.

METHODS

Animals

Eight-week-old BALB/c mice were chosen in this study and fed under specific pathogen-free conditions. The experimental use of animals has been approved by the Institutional Animal Care and Use Committee of Chengdu Second People’s Hospital.

IMQ-induced psoriasis-like mouse model and drug treatment

The IMQ-induced psoriatic mouse model was induced as previously described. Briefly, a daily topical dose of 62.5 mg commercially available IMQ cream (5%, Mingxing, Chengdu, China) or Vaseline cream (as control) was applied to the shaved back skin of BALB/c mice for 6 days.

For HMGB1 priming, mice were injected with either 500 ng recombinant mouse HMGB1 (Sino Biological, Beijing, China) or equal volume of phosphate-buffered saline (PBS) i.d. daily for 4 days. In another experiments, mice were injected with recombinant mouse HMGB1 500 ng or equal volume of PBS i.d. daily for 4 days during IMQ treatment (days 3–6).

For glycyrrhizin treatment, mice were injected i.p. with glycyrrhizin (20 mg/kg; Nippon Kayaku, Tokyo, Japan) or equal volume of PBS once every 2 days three times during IMQ treatment (days 2, 4 and 6).

For anti-HMGB1 monoclonal antibody (mAb) treatment, mice were injected i.p. with anti-HMGB1 mAb (2 mg/kg; Shinohara, Tokyo, Japan) or matching immune complex (IC) mAb (2 mg/kg; Sigma-Aldrich, St Louis, MO, USA) once every 2 days three times during IMQ treatment (days 2, 4 and 6).

After treatment, the psoriasis symptoms and disease severity of the back skins were observed and evaluated with a clinical score system. The system was built on the Psoriasis Area and Severity Index and scored from 4 to 0 (i.e. 4, very marked; 3, marked; 2, moderate; 1, slight; and 0, none), and independently on a scale of thickening, erythema and scaling. The cumulative score served as a measure of the severity of inflammation (scale 0–12). Then, mice were killed. Skin samples in different group were obtained.

Histological examination and immunohistochemical staining

Tissue sections (~5 μm) were stained by hematoxylin–eosin (HE) following standard protocol for histological observation and immunohistochemistry using antibody against HMGB1 (Epitomics, Burlingame, CA, USA), CD3 (Abcam, Cambridge, UK), CD11c (Beijing Biosynthesis, Beijing, China), and myeloperoxidase (MPO; Beijing Biosynthesis, Beijing, China). The severity of psoriasis was assessed according to a Baker score system, which were quantitative assessments of pathological changes, observed in five random fields (×100) from each HE-stained section. The epidermal thickness was also measured to indicate epidermal proliferation.

Real-time quantitative polymerase chain reaction

The mRNA expression of IL-6, TNF-α, IFN-γ, IL-17, IL-4, IL-10, IL-27, IL-23p19 and IL-12p40 in skin of all groups was detected by real-time quantitative polymerase chain reaction (qPCR). Total RNA of all samples were isolated and purified by Trizol reagent according to the instructions. The experiment was performed using SYBR Green Master Mix, qPCR primers and our previously established systems. The following primers were used: IL-6 (MQP036632; GeneCopoeia, Rockville, MD, USA); TNF-α (MQP031019; GeneCopoeia); IFN-γ (MQP027401; GeneCopoeia); IL-17 (MQP029457; GeneCopoeia); IL-4 (MQP032451; GeneCopoeia); and IL10 (Catalog: MQP029453 GeneCopoeia, USA). The expression levels and fold change relative to the control of these cytokines were analyzed in triplicate and calculated relative to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase using the 2^(-ΔΔCt) method.

Flow cytometry

Skin cells were isolated individually from each group and washed twice with PBS. Single-cell suspensions (200 μL) containing 1 × 10⁶ cells were thoroughly mixed with 2 μL of anti-CD3e–PE and anti-γ–δ fluorescein isothiocyanate monoclonal antibodies and incubation at 4 °C for 30 min in the dark. Then, the cells were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA), and data were processed using CellQuest Pro software (Becton Dickinson).

Statistical analysis

Values in the figures are expressed as mean ± standard deviation. One-way ANOVA or Student’s t-test were used to compare data between groups with Graphpad Prism 5 software (GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered statistically significant. All experiments were carried out three times.

RESULTS

Altered distribution of HMGB1 in IMQ-induced psoriasis-like lesional skin

The distribution of HMGB1 in lesional skin of IMQ-induced psoriasis-like mouse with or without glycyrrhizin treatment was detected by immunohistochemistry. As presented in Figure 1, the expression of HMGB1 was almost completely located in the nucleus of the epidermis of the Vaseline control group. Moreover, HMGB1 was abundantly expressed in the cytoplasm of the epidermis from psoriasis-like lesional skin induced by IMQ. In contrast, glycyrrhizin markedly blocked the cytoplasmic translocation of HMGB1 in the epidermis from IMQ-induced mice.

HMGB1 exacerbated the psoriasis-like phenotype on IMQ-treated skin

To assess whether HMGB1 could induce psoriatic lesions on the mouse skin, we first injected HMGB1 or PBS i.d. into normal mice daily for 4 days. As showed in Figure 2(a), HMGB1-treated
skin showed no obvious changes in phenotype and epidermal thickness when compared with the PBS-treated group. As HMGB1 expression in the IMQ-induced psoriatic lesion is highly cytoplasmic, it should be involved in the evolution of psoriasis. Therefore, HMGB1 or PBS was continuously injected into IMQ-treated skin i.d. Mice treated with PBS + IMQ gradually developed the clinical outcome of psoriasis with thickness, erythema and scales (Fig. 2b). Mice treated with HMGB1 + IMQ exhibited worse psoriatic phenotype with more severe thickness and scales of skin as compared with the control group (Fig. 2b). Moreover, by using a clinical score system to assess disease severity, we found that HMGB1 obviously increased the clinical score at 7 days of IMQ treatment (Fig. 2c). Consistent with psoriatic phenotype and clinical scores, histological findings in lesional skin from day 7 of mice treated with HMGB1 + IMQ showed more intense inflammatory cell infiltrates, severer epidermal hyperplasia and more elongational rete ridges relative to the control group (Fig. 2b).

Furthermore, it was showed that in comparison with the control group, HMGB1 markedly increased the average epidermal thickness (Fig. 2d). In addition, we also found that the Baker scores, confirmed by the pathological score, were obviously increased in HMGB1-injected mice as compared with the PBS-injected group (Fig. 2e).

**HMGB1 injection enhanced the number of CD3+ T cells, MPO+ neutrophils and CD11c+ DC infiltrating IMQ-treated skin**

T cells, neutrophils and DC infiltrating skin are believed to be important in the development of psoriasis. As presented in Figure 3(a), immunohistochemistry showed the infiltration of CD3+ T cells, MPO+ neutrophils and CD11c+ DC in the skin dermis of PBS-injected mice. However, HMGB1-injected mice revealed the more plentiful infiltration of CD3+ T cells, MPO+ neutrophils and CD11c+ DC in the skin dermis compared with PBS controls.

**HMGB1 injection upregulated the expression of IL-6, TNF-α, IFN-γ and IL-17 in IMQ-treated skin lesions**

T-helper 1/Th17-associated cytokines like IL-6, TNF-α, IFN-γ and IL-17 are important in the pathogenesis of psoriasis. The mRNA expression of cytokines (including IL-6, TNF-α, IFN-γ, IL-17, IL-4, IL-10, IL-27, IL-23p19 and IL-12p40) in the mice lesional skins were assayed by real-time qPCR. As listed in Figure 3(b), HMGB1 caused an effective increase in the expressions of IL-6, TNF-α, IL-17 and IFN-γ in lesions when compared with PBS-injected controls. However, expression of IL-4, IL-10, IL-27, IL-23p19 and IL-12p40 were unchanged in lesional skin of IMQ-induced mice after HMGB1 injection. Thus, we suggest that HMGB1 injection enhanced Th1/Th17-related cytokine expression in the IMQ-treated skin.

**HMGB1 injection increased the number of γδ T cells in IMQ-treated skin lesions**

The number of γδ T cells in skin was detected by flow cytometry. As shown in Figure 3(c), HMGB1-injected mice revealed a greater number of γδ T cells in skin compared with PBS controls.

**HMGB1 blockade improved clinical and histological psoriatic changes in IMQ-treated skin**

To investigate whether HMGB1 blockade could improve the development of psoriasis, we injected anti-HMGB1 mAb, IC
mAb, glycyrrhizin or PBS during the IMQ treatment by i.p. HMGB1 antibody-injected and glycyrrhizin-injected mice presented improvement in the clinical scores compared with IC mAb-injected mice (Fig. 4a,b) or PBS-injected mice (data not shown). Consistent with psoriatic phenotype and clinical scores, histological analyses of Baker scores and epidermal thickness in HMGB1 antibody-injected and glycyrrhizin-injected mice exhibited significant improvement (Fig. 4c,d).

Figure 2. Exacerbation of imiquimod (IMQ)-induced psoriasis-like skin inflammation by high mobility group box-1 (HMGB1) injection. (a) The macroscopic presentation and histological analyses of mouse back skin are shown. In each group, mice were injected with phosphate-buffered saline (PBS) or HMGB1 onto back skin. (b) Phenotypical presentation of mouse back skin which was injected with PBS or HMGB1 and IMQ was applied and hematoxylin-eosin (HE) staining of tissue sections treated as described. (c) A composite clinical score (CS) was based on the clinical Psoriasis Area and Severity Index to assess disease severity. (d) Average epidermal thickness of all groups (mean ± SD, n = 6 per group). (e) Quantitative assessments of pathological score (mean ± SD, n = 10 per group), based on a Baker score system, were performed in 10 random fields (original magnification ×100) of HE-stained sections to evaluate the severity of psoriasis. Statistical analysis was conducted using Student’s t-test. Values are expressed as mean ± standard deviation, n = 6 per group. P < 0.05 was statistically significant. *P < 0.05. Scale bars, 200 µm.

HMGB1 blockade reduced the number of CD3+ T cells, MPO+ neutrophils and CD11c+ DC infiltrating IMQ-treated skin

CD3+ T cells, MPO+ neutrophils and CD11c+ DC infiltrating skin was observed by immunohistochemistry. Anti-HMGB1 mAb and glycyrrhizin obviously decreased the number of CD3+ T cells, MPO+ neutrophils and CD11c+ DC infiltrating dermis relative to IC mAb-injected mice (Fig. 5a) or PBS-injected mice (data not shown).

Figure 3. (a) Immunohistochemistry using antibody against CD3, CD11c and myeloperoxidase (MPO). Mice were treated as described above. These results were representative of multiple experiments and microscopic fields. (b) The levels of interleukin (IL)-17, interferon (IFN)-γ, IL-6 and tumor necrosis factor (TNF)-α mRNA from back skins in mice (n = 6 per group). Glyceraldehyde 3-phosphate dehydrogenase was used as internal reference gene. Statistical analysis was conducted using Student’s t-test. (c) Fluorescence-activated cell sorting plots show the γ6+ CD3+ staining profile after resetting of large cell size forward scatter (FSC) and high side scatter (SSC). Quadrant gates were set using isotype controls. Values are expressed as mean ± standard deviation, n = 6 per group. P < 0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars, 200 µm.
Involvement of HMGB1 in psoriasis mice

(a) Staining images of CD3, MPO, and CD11c in IMQ+PBS and IMQ+HMGB1 groups.

(b) Relative mRNA expression levels of IL-17, IL-6, IFN-γ, TNF-α, IL-27, IL-12p40, and IL-23p19 in IMQ+PBS and IMQ+HMGB1 groups.

(c) Flow cytometry analysis showing γδ T cells and CD3 levels in IMQ+PBS and IMQ+HMGB1 groups.
HMGB1 blockade obviously decreased the expression of IL-6, TNF-α, IFN-γ and IL-17 in IMQ-treated skin lesions

Finally, we also indicated the effects of HMGB1 blockade on mRNA expression of IL-6, TNF-α, IFN-γ, IL-17, IL-4, IL-10, IL-27, IL-23p19 and IL-12p40 in IMQ-treated mice by real-time qPCR. As displayed in Figure 5(b), the mRNA levels of IL-6, TNF-α, IFN-γ and IL-17 showed a markedly increase in skin lesions with IMQ treatment. However, expression of IL-4, IL-10, IL-27, IL-23p19 and IL-12p40 showed no changes in lesional skin of IMQ-induced mice (data not shown). Treatment with anti-HMGB1 mAb and glycyrrhizin significantly attenuated these changes.

HMGB1 blockade decreased the number of γδ T cells in IMQ-treated skin lesions

Compared with control animals, the ratio of skin γδ T cells in the model group was higher. Moreover, the ratio of skin γδ T cells was decreased in anti-HMGB1 mAb- and glycyrrhizin-treated animals when compared with IMQ + IC mAb-treated animals (Fig. 5c).

DISCUSSION

Our previous work first investigated the involvement of HMGB1 with PV, and demonstrated the altered HMGB1 distribution in the epidermis and enhanced HMGB1 serum levels of PV patients.14 In this study, abundant expression of HMGB1 was also detected in cytoplasm of IMQ-induced psoriatic skin lesions. All the data suggested that HMGB1 may be involved in the pathogenesis of psoriasis.

As showed above, extracellular HMGB1, acts as a pro-inflammatory cytokine, and is associated with many autoimmune and inflammatory diseases. To further explore the role of HMGB1 in the pathogenetic mechanism of psoriasis, an experiment was performed in BALB/c mice and IMQ-induced
Figure 5. (a) Immunohistochemistry using antibody against CD3, CD11c and myeloperoxidase (MPO). Mice were treated as described above (six mice per group). Skin sections were stained with antibodies against CD3, CD11c and MPO. These results were representative of multiple experiments and microscopic fields. Scale bars, 100 μm. (b) The levels of interleukin (IL)-17, interferon (IFN)-γ, IL-6 and tumor necrosis factor (TNF)-α mRNA from back skin in mice (n = 6 per group). Glyceraldehyde 3-phosphate dehydrogenase was used as internal reference gene. One-way analysis was performed and found that the levels of IL-17, IFN-γ, IL-6 and TNF-α mRNA from back skin in mice treated with high mobility group box-1 (HMGB1) inhibitor were significantly lower than the imiquimod (IMQ) group. (c) Fluorescence-activated cell sorting plots show the γδ⁺ CD3⁺ staining profile after pregating of large cell size (FSC) and high side scatter (SSC). Quadrant gates were set using isotype controls. \( P < 0.05 \) was considered statistically significant. \( \cdot P < 0.05 \), \( \cdot\cdot P < 0.01 \), \( \cdot\cdot\cdot P < 0.001 \), compared with IMQ group; \( * P < 0.05 \), \( ** P < 0.01 \), \( *** P < 0.001 \), compared with Vaseline control group.
psoriatic mouse model in vivo. It was indicated that HMGB1-injected mice showed no obvious phenotypical and epidermal thickness changes. In line with this, our unpublished work also indicated that HMGB1 did not induce cell proliferation in cultured human keratinocytes by CCK8 assay (data not shown). On the other hand, in the current study, we indicated that HMGB1 injection skin, together with IMQ treatment, further exacerbated the phenotype of psoriasis with more abundant CD3+ T cells, MPO+ neutrophils, CD11c+ DC and γδ T cells infiltrating the dermis and increased the mRNA expression of Th1/Th17-associated cytokines (including IL-6, TNF-α, IFN-γ and IL-17). Furthermore, a previous study indicated that HMGB1 can lead to the activation of Th17 cells in rheumatoid arthritis patients.19 induce the production of pro-inflammatory cytokines in human monocytes and neutrophils,20,21 and increase the production of chemokine and adhesion molecules in human dermal microvascular endothelial cells.22 Our unpublished work also showed that HMGB1 can induce the release of pro-inflammatory cytokines and chemokines in keratinocytes. Therefore, we suggest that the mechanism by which HMGB1 contributes to the development of IMQ-induced psoriatic skin lesions may be associated with the pro-inflammatory effects of HMGB1 in these cell types.

Previously, Mollica et al. had reported that the mitogenic and chemotactic activities of HMGB1 can be inhibited by glycyrrhizin. Also, glycyrrhizin can bind directly to HMGB1 protein and confirm its binding site.23,24 Additionally, glycyrrhizin has been recognized as a HMGB1 inhibitor and potentially block the extracellular release of HMGB1 from the cell nucleus.15,25 In East Asia, glycyrrhizin has been recognized as a natural anti-inflammatory agent and now extensively used for the treatment of various inflammatory skin diseases including psoriasis. In our study, it was also demonstrated that glycyrrhizin obviously blocked the cytoplasmic translocation of HMGB1 in IMQ-induced psoriatic skin lesions.

In addition, our study demonstrated that anti-HMGB1 mAb and glycyrrhizin effectively induced clinical and histological improvements, decreased the number of infiltrated CD3+ T cells, MPO+ neutrophils, CD11c+ DC and γδ T cells, and inhibited the mRNA levels of IL-6, TNF-α, IFN-γ and IL-17 during the IMQ treatment. Our study heighted the possibility that the pharmaceutical effects of glycyrrhizin for its clinical use may be at least partly associated with the direct inhibitory effects on HMGB1 activity in psoriasis. Our data also indicates the involvement of HMGB1 in the pathogenic mechanism of psoriasis in vivo; to our knowledge, this has been previously unreported.

Interleukin-17 plays an important role in psoriasis. Anti-HMGB1 mAb and glycyrrhizin inhibited the expression of IL-17 in the mouse skin. Because the cells secreting IL-17 in the skin during psoriasis were mainly Th17 cells and γδ T cells, we measured the number of skin γδ T cells in these mice. HMGB1-injected mice revealed a greater number of γδ T cells in skin compared with PBS controls. Compared with control animals, the ratio of skin γδ T cells in the model group was higher. Moreover, the ratio of skin γδ T cells was decreased in anti-HMGB1 mAb- and glycyrrhizin-treated animals when compared with IMQ + IC mAb-treated animals. This implies that the γδ lineage may be one of the cell types targeted by HMGB1 mAb or glycyrrhizin.

Taken together, our results provide novel insights into the pathogenetic mechanism of psoriasis. Our work indicates the accelerating effects of HMGB1 in psoriasis. In addition, it also indicates that HMGB1 blockade (anti-HMGB1 monoclonal antibody and glycyrrhizin) can reduce inflammatory response and clinical severity in IMQ-induced mouse models of psoriasis. Hence, we indentified for the first time that HMGB1 is involved in the pathogenetic mechanism of IMQ-induced psoriatic inflammation. It will open novel therapeutic ways of psoriasis in targeting HMGB1 by anti-HMGB1 mAb or glycyrrhizin.

ACKNOWLEDGMENTS: The study was supported, in part, by the Natural Science Foundation of China (no. 81470143), and a research grant from the Health and Family Planning Commission of Sichuan Province (no. 150005).

CONFLICT OF INTEREST: None declared.

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