

ORIGINAL ARTICLE

Subcutaneous injection of recombinant heat shock protein 70 ameliorates atopic dermatitis skin lesions in a mouse model

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Funding information

Chang-hua Christian Hospital, Grant/Award Number: 104-CCH-IRP-046

Abstract

Atopic dermatitis (AD) is a chronic inflammatory skin disease and sometimes is a tough challenge for physicians. We previously reported that in Th2 environment, the production and secretion of thymic stromal lymphopoietin (TSLP) from human keratinocytes was inhibited by recombinant heat shock protein 70 (rHSP70). The present study assessed the therapeutic effectiveness of rHSP70 in a mouse model of AD. An experimental model of AD was reproduced by systemic sensitization and local epicutaneous challenge with ovalbumin (OVA). Treatment of rHSP70 was performed by subcutaneous administration. The levels of OVA-specific IgE, as well as cytokines, were detected by ELISA. Skin samples from patch areas were also taken for histologic examination. Injection of rHSP70 improved the histologic picture by reducing the thickness of epidermis and allergic inflammation. Skin sonography revealed rHSP70 ameliorated skin remodeling. rHSP70 also significantly decreased the protein expression of TSLP of skin from patch areas. Furthermore, in ex vivo studies also showed group of rHSP70 treatment decreased IL-13, RANTES, MIP-1 β and increased IFN- γ secreted from splenocytes stimulated with OVA. The rHSP70 intervention in the mouse model of AD reduced the skin expression of TSLP and attenuated the clinical appearance of OVA-induced AD mice. The effect was achieved by suppressed Th2 immune response in injected skin tissue and enhanced systemic Th1 immune response. These results suggest that rHSP70 have potential as a promising protein for the treatment of AD.

KEYWORDS

atopic dermatitis, heat shock protein, skin barrier, skin inflammation, thymic stromal lymphopoietin

1 | INTRODUCTION

Atopic dermatitis (AD) is the most common chronic, pruritic inflammatory skin disease, which causes a substantial economic and social burden.

Early models of AD were recognized as a disease with skin barrier defect and cutaneous inflammation at the lesion sites. The epidermis performs protective and defensive functions. The defective skin barrier leads to penetration of allergens and impaired tissue immune responses. In AD,

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keratinocytes of damaged epidermis are activated to release cytokines, chemokines, and antimicrobial peptides. Among them, thymic stromal lymphopoietin (TSLP), an IL-7-like chemokine, is involved in the initiation, development, and progression of AD. TSLP binds to its functional heterodimeric receptor complex (TSLPR), which then induces the local network of dendritic cells to secrete eotaxin and IL-8 that then recruit and prime naïve T cells to Th2 cells.¹ Eventually, persistent skin inflammation results in epidermal hyperplasia and skin remodeling such as markedly increased collagen deposition and fibrosis.²

In recent studies, immune activation of AD has been found to beyond the skin.³ In patients with moderate to severe AD, the central and effector memory T cells in blood are polarized and continuously activated. Upregulated T helper 2 (Th2) cells and Th2-related factors including IL-4, IL-5, and IL-13 lead to an increased concentration of immunoglobulin E (IgE) in the plasma. Several activated immune mediators in the circulation have also been reported to correlate with clinical severity and IgE levels.^{4,5} The occurrence of noncutaneous comorbidities in patients with AD also supports the systemic inflammation point of view. In addition to the comorbidities related to allergic conditions, food allergy, asthma, allergic rhinitis (allergic march), cardiovascular, neuropsychiatric, and malignant diseases have been increasingly reported to be associated with AD.⁶ Increasing studies have shown that AD is not only limited to skin lesions but also related to systemic immune activation.

Currently, the main treatments of AD consist of a reconstruction of an intact skin barrier and decreased skin inflammation. Daily use of skin moisturizer is recommended to maintain an intact skin barrier. Therapeutic approaches to skin inflammation include topical corticosteroid creams/calcineurin inhibitors and phototherapy. These topical anti-inflammatory drugs have limited systemic effects. Dupixent (dupilumab) is the first biologic medication approved by the U.S. Food and Drug Administration (FDA) for adults with moderate to severe atopic dermatitis for whom topical treatments have not worked or are not advised. Topical corticosteroids may further cause the skin to be more vulnerable to damage due to the thinning of the skin. In contrast to topical drugs, systemic drugs such as oral corticosteroids and immunosuppressant drugs are given to recalcitrant cases, but these drugs have more apparent side effects and few benefit for skin barrier reconstruction.^{7,8} Since therapeutic options are limited, there is a great need to develop a new treatment that can simultaneously suppress local and systemic inflammation while rebuilding skin barrier function with minimal side effects.

The 70-kDa heat shock protein (HSP70) family is an evolutionarily highly conserved protein family that can be induced by exposure to a variety of stressful conditions. HSP70s can bind to misfolded proteins and help them to adopt a native conformation; therefore, enhanced production of HSPs can promote cell survival in the presence of damaging stimuli.^{9,10} Increasing evidence suggests that HSPs play important roles in both innate and adaptive immunity.^{11,12} HSP70 has also been shown to have anti-inflammatory properties.¹³ The presence of HSP70 proteins in a culture medium can inactivate dendritic cells.¹³ Furthermore, some reports have revealed that HSP70 can promote the production of regulatory T cells (Tregs), which have functions in

tolerance development and immunoregulation.¹⁴ Because of the protective properties of HSP70, it has already been used as a systemic intervention *in vivo* in several disease models.¹⁵

Recently, we have demonstrated that in a Th2 environment, the production and secretion of TSLP from human keratinocyte cell line (HaCaT) are inhibited by preheating or by recombinant heat shock protein 70 (rHSP70).¹⁶ These encouraging results highlight the potential application of HSP70 in decelerating the pathological change of AD. Herein, we revealed that subcutaneous injection of rHSP70 can ameliorate AD in a mouse model induced by systemic sensitization with epicutaneous application of ovalbumin (OVA), and discussed the possible mechanism involved.

2 | MATERIAL AND METHODS

2.1 | Animals

Female C57BL/6 mice aged 6 to 8 weeks were purchased from BioLASCO (Yi-Lan, Taiwan) and kept in a pathogen-free environment with an OVA-free diet and given water *ad libitum*. All the experimental procedures were carried out in strict compliance with the recommendations in the Animal Protection Act of the Council of Agriculture Executive Yuan (Taiwan). The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Changhua Christian Hospital, Taiwan (approval number: CCH-AE-102-017).

2.2 | AD mouse model

The protocols used for systemic and local epicutaneous challenges were designed as previously described.^{17–19} The AD and rHSP70 treatment groups were systemically sensitized with intraperitoneal injection of OVA (Sigma, St. Louis, Missouri) on days 1, 7, and 14, and received a skin OVA patch exposure challenge. The negative control and sham groups were intraperitoneally sensitized with phosphate buffered saline (PBS). The negative control group then received skin PBS patch exposure; however, in the sham group, we simply removed fur without a further skin patch challenge. All the mice were euthanized by CO₂ asphyxiation.

2.3 | OVA systemic sensitization

The OVA (50 µg; Sigma) used in our model was albumin from chicken egg white. This product has 10 mannose residues per mole of OVA and is over 98% pure by agarose gel electrophoresis. OVA with 1 mg of aluminum hydroxide (Thermo, Rockford, Illinois) was dissolved or resuspended in 200 µL PBS, and was injected intraperitoneally per mouse.

2.4 | Skin OVA patch exposure challenge

Briefly, the dorsal fur of mice was gently removed by hair-removing wax and stripped 6 times with a 3 M tape. Next, 2.5 mg of OVA (Sigma) at 500 µg/100 µL in PBS was placed on a 1.5 × 1.5 cm patch

of sterile gauze, which was then fastened to the skin with a Transparent BIOCLUSIVE Dressing (3 M, Brookings, South Dakota). The patch was applied during three 1-week periods of exposure with 2-week intervals.

2.5 | Treatment with rHSP70

Treatment with human rHSP70 with low endotoxin (Enzo Life Science, Farmingdale, New York) was administered by subcutaneous injection near OVA patch on back of mice at doses of 50 $\mu\text{g}/\text{kg}$ dissolved in PBS 100 μL every day between the first, second, and third OVA patch periods. The negative control group was treated with 100 μL of PBS in parallel, and the sham group did not receive any treatment.

2.6 | Histopathological analysis

The skin samples from the patch areas were removed for histological examination after the last epicutaneous application of OVA. Samples were fixed overnight with 10% paraformaldehyde at 4°C, and then embedded in paraffin. Tissue slices were stained with hematoxylin and eosin. The histological preparations were analyzed under a light microscope (Olympus BX50) at $\times 40$, $\times 200$, and $\times 400$ magnifications.

2.7 | Skin echography and transepidermal water loss (TEWL) measurements

The in vivo skin echography was conducted by means of a DermaLab Combo (20 MHz, B mode, mechanical scan, depth 17 mm, $60 \times 200 \mu\text{m}$, Cortex Technology, Denmark). An impaired skin barrier increases TEWL, which leads to dry skin and easy penetration of allergens, resulting in

allergic sensitization.^{20,21} TEWL was measured using the same device with a responding open chamber diffusion gradient probe.

2.8 | Western blot analysis

A small piece of skin tissue was lysed in PBS using a Qiagen TissueLyser II (Qiagen, HK, China) with shaking at 20 Hz for 5 minutes and incubation on ice for 5 minutes. The aforementioned procedure was repeated twice. Then, the lysates were centrifuged at 13000g for 10 minutes (12 000 rpm, 4°C, 10 minutes). After that, an equal amount of each protein sample was loaded into each well and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was incubated overnight at 4°C with the following primary antibodies: a mouse anti-TSLP (1:1000, Genetex) antibody, and an anti- β -actin antibody (1:10 000, Sigma) served as a loading control. The next day, the membrane was incubated with the corresponding secondary antibody after washing. Finally, specific bands were visualized by means of Fusion Fx (Vilber Lourmat) with standard chemiluminescence (Perkin Elmer, Waltham, Massachusetts).

2.9 | Cytokine release assays of splenocytes stimulated by OVA

The spleens were collected aseptically after the last allergen application. Splenocytes ($10^6/\text{well}$) were seeded in 24-well plates, and then stimulated with OVA (10 $\mu\text{g}/\text{mL}$) and incubated for 72 hours in RPMI 1640 medium (GIBCO) containing 10% of fetal bovine serum (FBS; HyClone), gentamicin (800 $\mu\text{g}/\text{mL}$), and 50 μM β -mercaptoethanol at

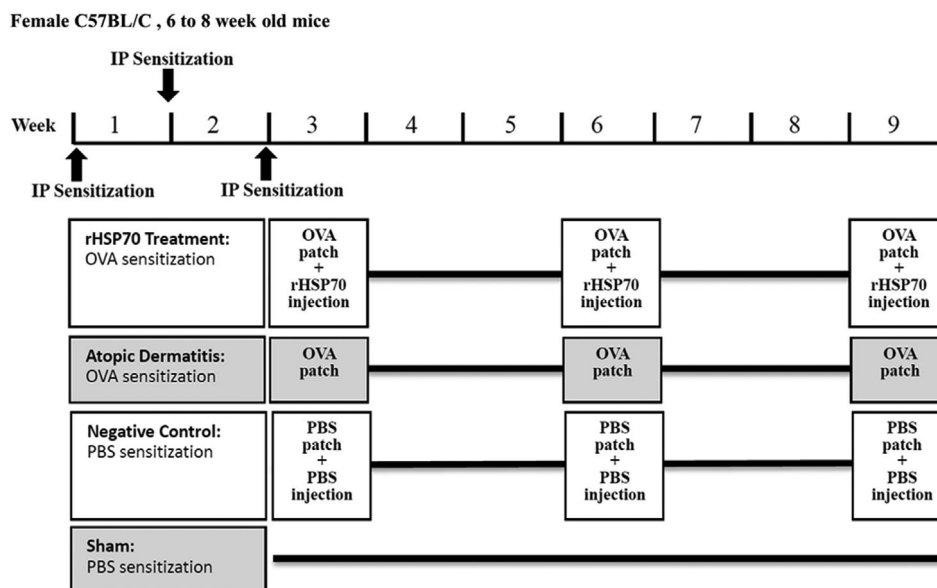


FIGURE 1 Protocol for the mouse model of atopic dermatitis (AD) and rHSP70 treatment. The AD and rHSP70 treatment groups were sensitized with 50 μg of ovalbumin (OVA), while the negative control and sham groups were sensitized with phosphate buffered saline (PBS). The 200 μL suspension for sensitization was intraperitoneally injected. After three sensitization procedures, the AD and rHSP70 treatment groups were challenged with a sterile patch containing 2.5 mg OVA (500 μg per 100 μL of PBS), while the negative control group was treated with 100 μL of PBS via a sterile patch. The mice had three 1-week treatments with the patch separated by 2-week intervals. The interventions—subcutaneous injection of rHSP70 (500 $\mu\text{g}/\text{kg}$) or PBS (as a negative control)—were performed during the periods of the OVA patch challenge

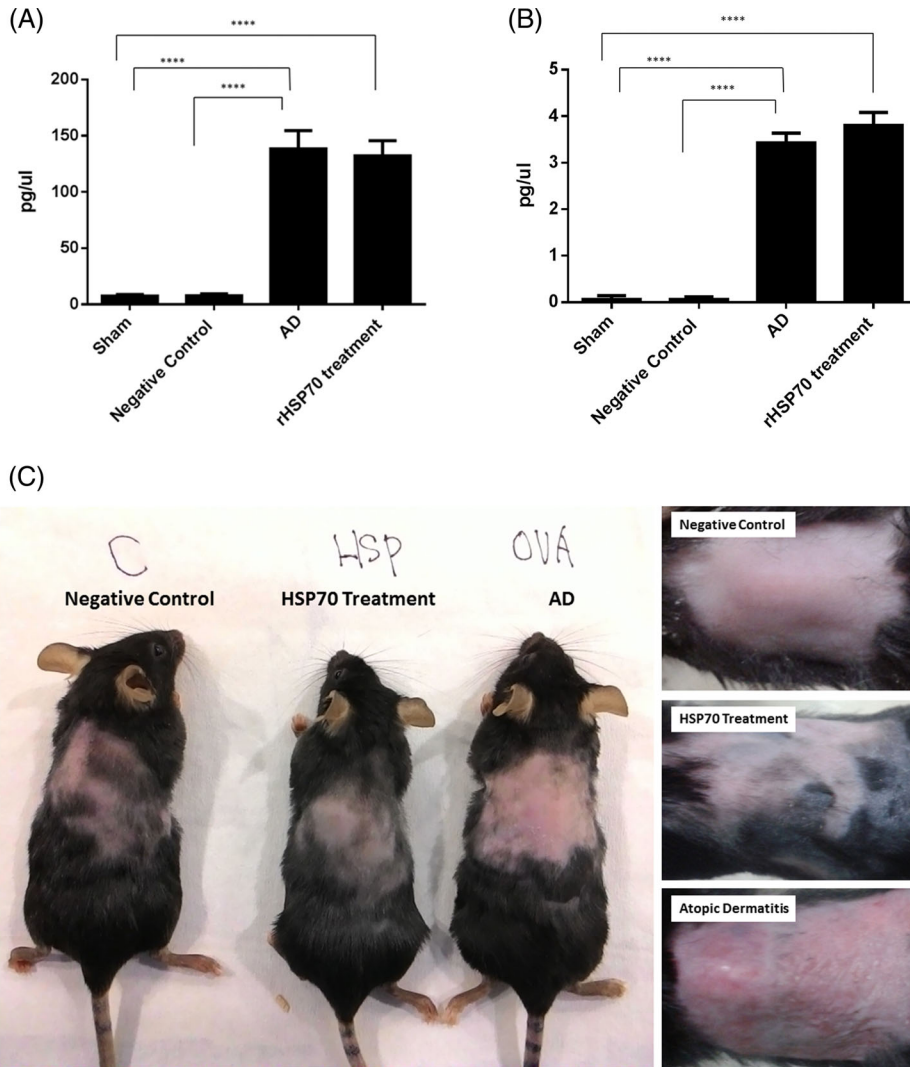


FIGURE 2 Successful induction of atopic dermatitis (AD) in C57BL/6 mice and rHSP70 treatment improved macroscopic lesions. After three sensitization procedures, the levels of, A, total IgE and, B, ovalbumin (OVA)-specific IgE in serum were quantified by an enzyme-linked immunosorbent assay. C, Images of skin manifestations in C57BL/6 mice of different groups. The results are presented as mean $\pm$ SE ($n = 8$ in each group). * $P < .05$; ** $P < .01$; *** $P < .001$

37°C and 5% CO₂ in a humidified incubator. The concentrations of cytokines in supernatants were determined by an ELISA (Bio-Plex Multiplex immunoassay system; Bio-rad, California).

3 | STATISTICAL ANALYSIS

The data are shown as mean $\pm$ SE. Statistical analysis was carried out in the Prism 6.0 software (Turku Center for Biotechnology University of Turku, Finland). The significance of the results was determined by ANOVA. Differences were considered significant at $P < .05$.

4 | RESULTS

4.1 | Treatment with rHSP70 improved skin manifestations of mice with AD

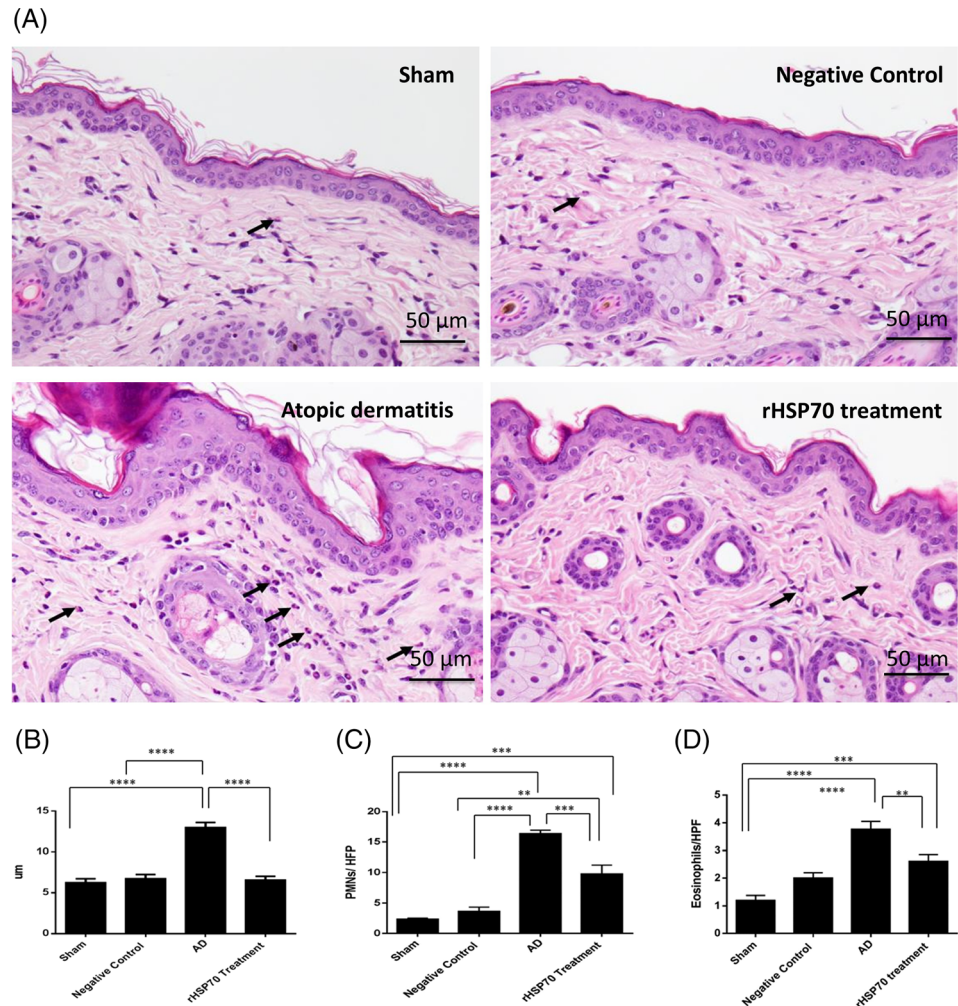
Female C57BL/6 mice were subdivided into four groups: "sham," "negative control," "AD," and "AD with rHSP70 treatment" (abbreviated as "rHSP70 treatment"; Figure 1). The mice were successfully intraperitoneally sensitized with an OVA/Al(OH)₃ suspension (50 μ g/mg per milliliter

(Figure 2A,B) and were then subjected to repeated application of a 2.5-mg OVA patch to the shaved back to induce AD-like lesions. Grossly, the epicutaneous OVA patch challenges led to local inflammation of the skin, which manifested itself as erythema and/or hemorrhage, mild edema, some erosion, and dryness. Rubbing behavior developed in AD group, especially after second time patch exposure. Obviously, all these symptoms including scratching behavior were alleviated (ie, suppressed) in the rHSP70 treatment group (Figure 2C).

4.2 | Treatment with rHSP70 attenuated cellular skin inflammation in skin lesion of mice with AD

Skin samples were taken after the last OVA or PBS exposure and the histological analysis was performed. Compared to groups sham and negative control, the AD group showed hyperkeratosis with necrosis and moderately thickened dermis with diffuse infiltration of mixed leukocyte including eosinophils. In group rHSP70 treatment, the hyperplasia of epidermis and dermis was significantly reduced (Figure 3A,B). In addition, the OVA-induced increased polymorphonuclear leukocytes and eosinophils were also moderately attenuated by rHSP70 treatment (Figure 3C,D).

FIGURE 3 The rHSP70 treatment improved histological presentation of atopic dermatitis (AD) by reducing the allergic inflammation. A, Representative photomicrographs of H&E-stained slices showed the epidermal thickness in various groups. The rHSP70 treatment significantly attenuated the epidermal thickening. The black arrows indicate eosinophils. B, Representative photomicrographs of slices stained with hematoxylin and eosin (scale bars, 50 μ m; $\times 400$). The black arrows indicate eosinophils. C, The number of polymorphonuclear cells in the skin lesions. In each slide (tissue slice), polymorphonuclear cells were counted in five random high-power fields of view ($\times 400$). D, The number of eosinophils in the skin lesions. In each slide (tissue slice), eosinophils were counted in five random high-power fields of view ($\times 400$). Data are presented as mean $\pm$ SE (n = 8 in each group). * $P < .05$; ** $P < .01$; *** $P < .001$



4.3 | Treatment with rHSP70 decreased TSLP expression in skin lesion of mice with AD

We next confirmed the efficacy of rHSP70 at suppression of TSLP production by immunohistochemical staining and western blots. The immunohistochemical staining of TSLP revealed that few epidermal keratinocytes being stained in basal and para-basal layers in the negative control and sham group. In contrast, the AD group expressed abundant TSLP in all layers of the epidermis including the stratum spinosum and granulosum. Furthermore, the rHSP70 treatment significantly attenuated TSLP expression (Figure 4A,B). As shown in Figure 4C, the production of TSLP was significantly inhibited by rHSP70 as compared to the AD group by Western blot analysis of the tissue lysates.

4.4 | Treatment with rHSP70 ameliorated skin remodeling and enhanced repair of skin barrier function in skin lesions of mice with AD

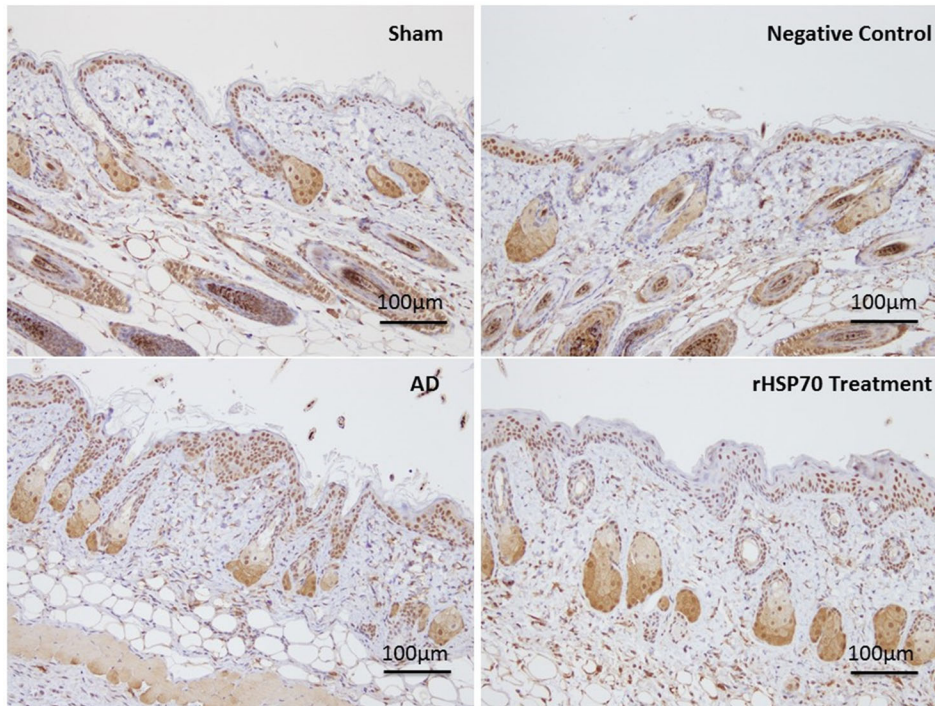
High-frequency ultrasound is a noninvasive tool that allows for clear identification of the skin layers in vivo. The increased dermal collagen deposition of skin remodeling in the AD group results in a green-dark color in dermal image compared to a normal bright yellow color. As

shown in Figure 5A, rHSP70 treatment successfully rescued the skin remodeling process in mice with AD. TEWL increased markedly in mice with AD as compared to sham and negative control groups. From the second period of the OVA patch exposure, rHSP70 started to attenuate TEWL with a trend toward significance ($P = .08$) (Figure 5B).

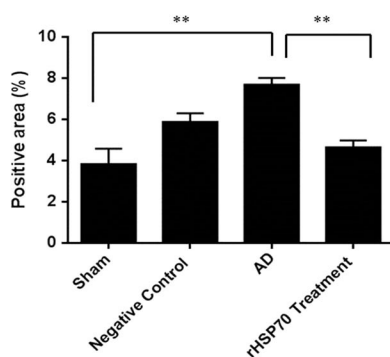
4.5 | The influence of rHSP70 on the systemic immune response in mice with AD

To elucidate the influence of rHSP70 on Th2 immune responses in mice with AD, serum OVA-specific IgE concentrations were measured when mice were euthanized. The rHSP70 treatment significantly attenuated the upregulation of OVA-specific IgE as shown in Figure 6A. Results of ex vivo studies further supported the mechanisms. The secretion of Th2-related cytokines into the supernatants from OVA-stimulated murine splenocytes of different groups was also evaluated. IL-4, IL-13, and two CC-chemokines, RANTES and MIP-1 β , were secreted in significantly greater amounts from splenocytes in the AD group compared to the sham and negative control groups. The rHSP70 treatment attenuated the upregulation of IL-13, RANTES, and MIP-1 β but not IL-4 (Figure 6B-E). We also assessed the levels of IFN- γ , which is a key marker of the Th1 immune response. The lowest

(A)



(B)



(C)

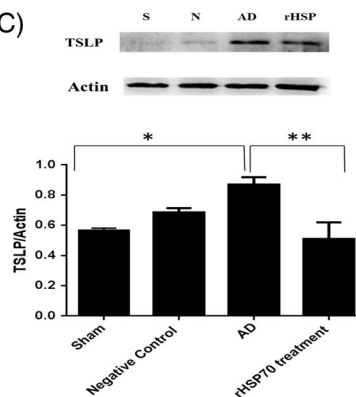


FIGURE 4 The rHSP70 treatment reduces thymic stromal lymphopoietin (TSLP) expression in skin lesions of mice with atopic dermatitis (AD). A, Representative images show immunohistochemical (IHC) staining of TSLP in lesioned skin in experimental groups. Original magnification $\times 200$. B, The area of TSLP staining was quantified by the Image-pro plus software (Media Cybernetics). The results indicated that TSLP expression decreased following rHSP70 treatment. All images were acquired and analyzed using identical hardware and software settings. C, TSLP protein expression in skin lesions via western blotting. The upper panel shows the representative western blots of TSLP expression in skin lesions (30 μ g) from different groups. Actin expression served as a loading control. S: sham, N: negative control. The lower panel shows a summary histogram of a relative TSLP band density in each group normalized to total actin expression. The results are presented as mean $\pm$ SE ($n = 8$ in each group). * $P < .05$; ** $P < .01$; *** $P < .001$

concentration of IFN- γ was detected in the AD group; however, rHSP70 treatment significantly enhanced the release of IFN- γ (Figure 6F). Furthermore, to assess the possibility that Tregs established global tolerance, we analyzed the population of CD4⁺CD25⁺Foxp3⁺ T cells (Tregs) from splenocytes as well as the levels of IL-10 in the supernatants of OVA-stimulated splenocytes from the four groups. Neither the Treg proportions nor IL-10 levels showed significant difference among the groups (Figure 6G,H), indicating Tregs play little role in establishing global tolerance.

5 | DISCUSSION

The pathophysiology of AD is complex, including skin barrier dysfunction, alterations in immune responses, environmental factors, and gene. AD is a dominant Th 2 immune response with increased levels of IL-4, IL-13, IL-31, and chemokine ligand 18 cytokines.²² The IL-4 and IL-13 play a key role in pathogenesis of AD which contribute to a

defective Skin barrier and promote the overproduction of IgE.^{22–24} Moreover, IL-4 and IL-13 also inhibit production of antimicrobial peptides and down regulate the Th1 and Th17 dependent skin defense mechanism.^{22,25} In chronic AD, Th2 and Th22 responses are increased with parallel activation of the Th1 axis. IL-22 induces epidermal hyperplasia, whereas the IL-31 cytokine is associated with disease severity.²² In addition to adaptive immune, there are evidences that supports the role of IL-1 in AD. IL-1 ligands (IL-18) is related with the clinical and histopathologic features of chronic AD lesions which may due to dysfunctional inflammasome processing.^{26,27} Recently, IL-33, a newly described member of the IL-1 family, has been found to play an important role in inducing Th2 immune responses and provide novel insights for addressing AD.²⁸

Several mouse models of human AD have been developed that have provided insights into the pathogenesis of human AD.^{17,18} Epicutaneous sensitization is a method which reveals characteristic clinical and histological features of AD. Our model combined systemic

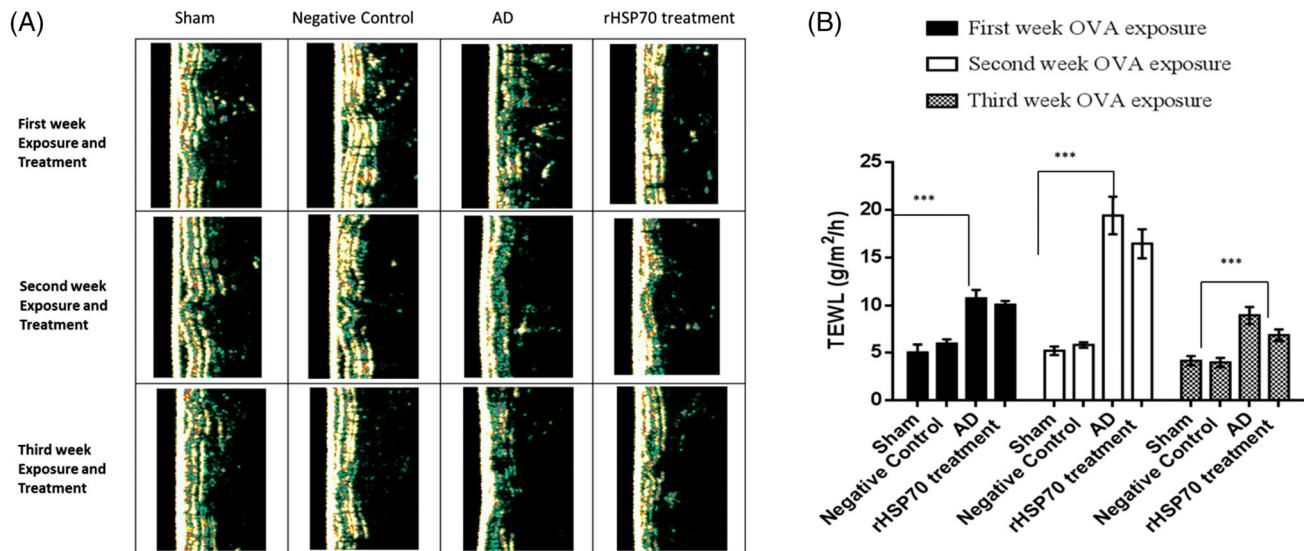


FIGURE 5 The rHSP70 treatment ameliorates skin remodeling and skin barrier dysfunction of mice with atopic dermatitis (AD). A, Ultrasonography of the skin clearly shows the dermal fibrotic remodeling changes in the AD group and the treatment efficacy of rHSP70. B, The rHSP70 treatment was almost significant for decreasing thymic stromal lymphopoietin (TEWL) ($P = .08$). TEWL values in different groups were measured by means of a TEWL meter. The results are presented as mean $\pm$ SE ($n = 8$ in each group). * $P < .05$; ** $P < .01$; *** $P < .001$

sensitization and a local skin challenge, which is compatible with the clinical pictures of AD. More encouragingly, we demonstrated for the first time that subcutaneous administration of rHSP70 attenuated the process of skin remodeling as well as the endpoint in our AD model.

HSP70s are a group of proteins in a large family of HSPs and are among the more extensively studied HSPs. HSP70 has been shown to be constitutively expressed in human skin and can be upregulated as a result of exposure to stressors.²⁹ In addition to the attenuation of pathologically systemic inflammatory status of AD in mice, we demonstrated for the first time that administration of rHSP70 also substantially decreased skin lesion inflammation. The rHSP70 treatment decreased polymorphonuclear leukocytes and eosinophils in skin lesions, which could be due to the suppression of TSLP expression in the lesional area. TSLP, an epithelial-derived cytokine, participates in the communication between epithelial and dendritic cells during the development of an allergic inflammation, and has been shown to be highly upregulated in the skin lesions of patients with AD.¹ TSLP-activated dendritic cells create a Th2-permissive microenvironment and thus polarize naïve CD4⁺ T cells into Th2 cells that produce IL-4, IL-5, IL-13, and TNF- α .³⁰ TSLP and/or TSLPR moreover have been found to mediate skin fibrosis.³¹

Although HSP70 has been described as a proinflammatory protein, increasing evidence indicates that HSP70 has a potent anti-inflammatory function.³² Numerous experimental studies have revealed the systemic effects of exogenous HSP70 in inflammatory diseases.^{33,34} In AD, systemic immune activation may trigger barrier dysfunction and/or aggravate inherited barrier defects through a positive feedback loop. The suppressed influence of rHSP70 on systemic Th2 immune responses in our AD model was demonstrated by decreased serum OVA-specific IgE concentrations and attenuated secretion of IL-13 and RANTES from splenocytes stimulated by OVA.

Our results showing enhanced IFN- γ secretion from splenocytes also suggest that rHSP70 simultaneously induced a systemic Th1 immune response, which should have contributed to the clinical improvement in the mice with AD.

The defective epidermal permeability of AD is a well-recognized feature and a driver of disease activity. Unlike topical corticosteroids, which suppress inflammation of the skin but may delay the recovery of the skin barrier function, rHSP70 attenuated the process of skin remodeling and skin barrier repair in mice with AD. In the rHSP70 treatment group, the hyperplasia of epidermis and dermis was significantly reduced compared to the AD group. Although the results were not significantly different in our study, rHSP70 started to attenuate TEWL from the second period of OVA patch exposure. Our results showed rHSP70 improved the defective epidermal permeability and skin barrier function. The reason why rHSP70 cannot induce complete recovery of the skin barrier may be explained by its inability to suppress all of the inflammatory molecules in AD such as IL-4, which is associated with innate barrier dysfunction.²⁴

High-frequency ultrasound is a noninvasive tool that allows for clear identification of the skin layers and thus could be used for in vivo tissue assessment. After processing, the cross-sectional echo intensity of the skin could be visualized in colors according to strength. Normally, epidermis is expressed as a white color belt. The dermis was expressed as a yellow or red color, and subcutaneous fatty tissue as a green or black color. From the sonographic images, it is clear that after rHSP70 treatment, the dermis of AD lesions re-acquired a variety of intensities similar to the control groups. High frequency ultrasound allows for the real-time imaging of skin structures in a noninvasive manner such that in daily medical practices, it could be a potentially valuable method for treatment assessment and lesion monitoring of patients with AD.

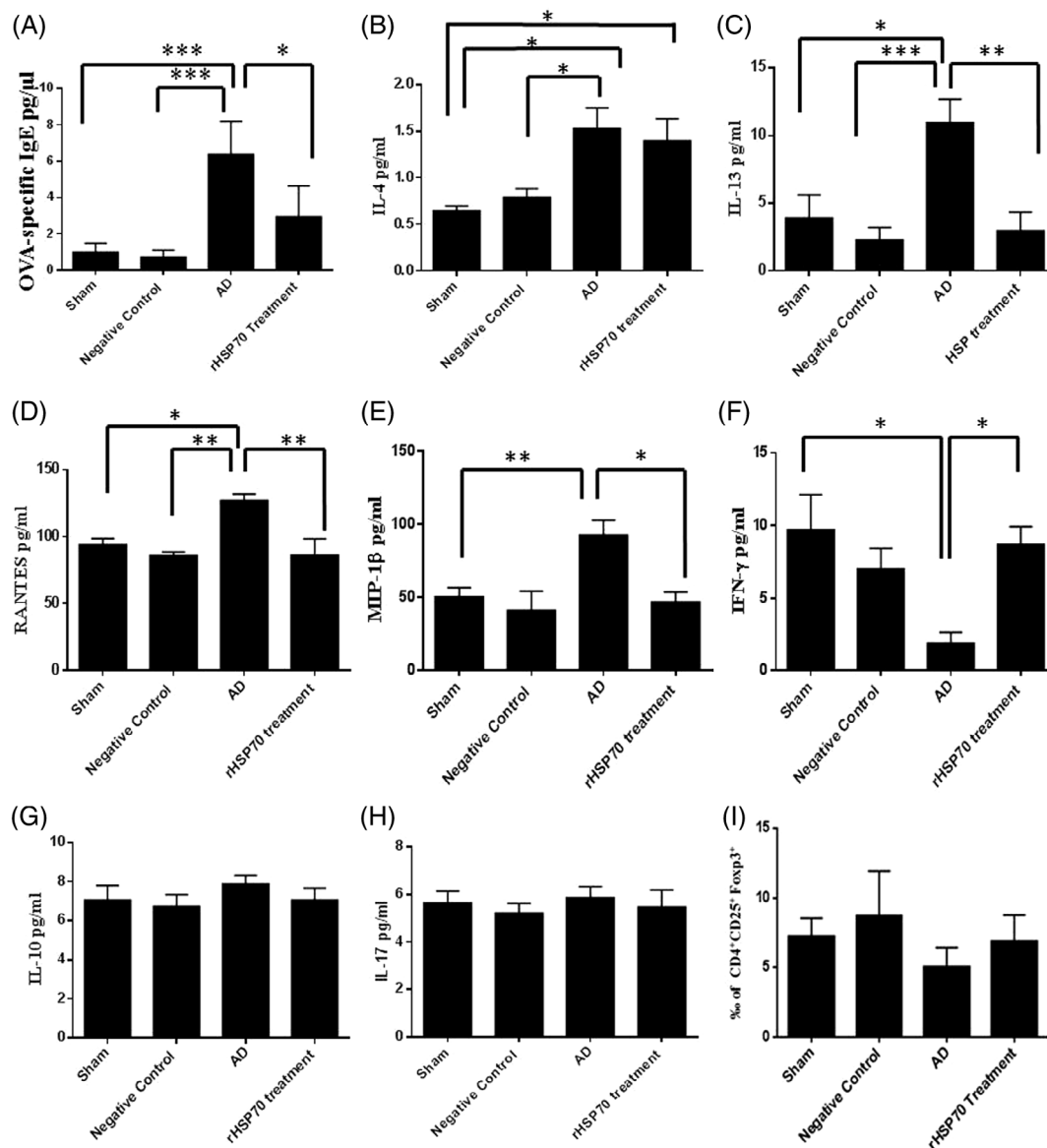


FIGURE 6 The influence of rHSP70 treatment on immune response. A, The rHSP70 treatment decreased serum ovalbumin (OVA)-specific IgE concentration. The levels of OVA-specific IgE in serum were quantified by an enzyme-linked immunosorbent assay (ELISA). B–H, The levels of cytokines in supernatants of OVA-stimulated murine splenocytes from different groups (incubated with OVA for 72 hours) were measured by ELISAs. B, IL-4; C, IL-13; D, RANTES; E, MIP-1 β ; F, INF- γ ; and G, IL-10. H, The proportion of CD4⁺CD25⁺Foxp3⁺ T cells among murine splenocytes of different groups was quantified by flow cytometry and presented as mean per million cells. I, IL-17. Data are presented as mean $\pm$ SE ($n = 8$ in each group). * $P < .05$; ** $P < .01$; *** $P < .001$

Although subcutaneous injection of microbial HSP70 has been shown to increase the population of Tregs, which is a subgroup of T cells that play a role in the maintenance of immunological tolerance and suppression of excessive immune responses, they were not upregulated due to the rHSP70 treatment in excised draining lymph nodes.³⁵ Moreover, epicutaneous immunization of mice with OVA has been shown to trigger the activation of IL-17-producing T cells in the draining lymph nodes and spleen, and the concentrations of IL-17 did not significantly differ among our groups of mice (Figure 6I).³⁶ It is clear that subcutaneous rHSP70 intervention in our mouse model of AD enhanced the systemic Th1 immune reaction and suppressed the

systemic Th2 immune reaction, which was not due to the Tregs or a Th17 immune response.

Current systemic treatments for AD offer limited efficacy and are often restricted by safety concerns. With an increased understanding of the cytokine pathways of AD, biologic medicines for specific targets have been developed.^{37,38} However, because of the significantly greater costs, biologics are to be used after failure of conventional systemic agents.³⁹ A systematic review and meta-analysis evaluated different biologics for AD patients and the results revealed that Dupilumab (IL-4 and IL-13 receptor antagonist) is currently the only biologic with strong evidence of efficacy in AD.⁴⁰ Nemolizumab (IL-31

receptor antagonist), lebrikizumab (IL-13 monoclonal antibody), and tralokinumab (IL-13 monoclonal antibody) show promise but need further studies. Disappointingly, the clinical trials of Omalizumab (IgE monoclonal antibody) and Mepolizumab (IL-5 monoclonal antibody) did not provide convincing evidence of efficacy.

In conclusion, we demonstrated that administration of rHSP70 reduced the skin expression of TSLP and attenuated the skin inflammatory appearance of mice with OVA-induced AD. Treatment with rHSP70 also suppressed the systemic Th2 immune response and induced a Th1 type of immune reaction, based on the results showing a decline in anti-OVA-specific IgE, IL-13, and RANTES as well as the increase in IFN- γ after rHSP70 treatment. Furthermore, rHSP70 treatment enhanced the repair of skin barrier dysfunction in mice with AD. These results highlight the potential clinical application of rHSP70 in the treatment of AD.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How to cite this article: Kao J-K, Hsu T-F, Lee M-S, et al. Subcutaneous injection of recombinant heat shock protein 70 ameliorates atopic dermatitis skin lesions in a mouse model. *Kaohsiung J Med Sci.* 2020;1–10. <https://doi.org/10.1002/kjm2.12163>