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ORIGINAL RESEARCH

Malassezia Globosa Aggravates Atopic Dermatitis by Influencing the Th1/Th2 Related Cytokines in Mouse Models

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Purpose: To establish atopic dermatitis (AD) mouse models infected with *Malassezia globosa* and study its effects and potential mechanisms.

Methods: Twenty - four male BALB/c mice were randomly allocated into four groups: control, AD, M (normal mice treated with olive oil fungus suspension), and AD + M (AD mice treated with the same suspension). DNFB was used to induce the AD model. The M and AD + M groups were treated with *Malassezia* suspension. Body weight, scratching behavior, and skin lesion scores of mice were recorded. Skin tissues underwent HE and PAS staining, viable fungal flora counting, and Th1/Th2 cytokine detection via flow cytometry.

Results: The AD mouse models infected with *Malassezia globosa* were successfully set up. The AD + M group scratched more often. On days 8, 12, and 16, the AD group's skin lesion scores were (9.00±0.89), (10.17±0.87), (9.17±0.75), while those of the AD + M group were (11.00±0.82), (10.83±0.75), (10.83±0.75) ($P<0.05$). The AD + M group had more *Malassezia* colonization ($P<0.001$). The M group displayed a Th1 response. The AD + M group enhanced Th1 response and increased Th2 cytokines like IL - 4 and IL - 10 ($P<0.05$). The control group had normal skin with minimal scratching and low fungal counts.

Conclusion: *Malassezia* causes inflammation in normal and AD - like skin, with worse inflammation when the skin barrier is damaged. Targeting *Malassezia* might alleviate AD inflammation, offering new AD treatment directions.

Keywords: atopic dermatitis, mouse model, immune response, inflammatory cytokines, fungal colonization

Introduction

Atopic dermatitis (AD) is one of the most common chronic relapsing skin inflammatory diseases, characterized by severe itching. Globally, 15% to 25% of children and 2.0% to 17.6% of adults are affected by this disease. This not only seriously impacts the quality of life of patients but also imposes a heavy economic burden.[1,2] The pathogenesis of AD has not been fully elucidated, and the colonization of microorganisms in the skin may trigger immune abnormalities. In recent years, studies have shown that the skin microbiota of AD patients is diverse.

Malassezia is a lipophilic fungus and the most commonly isolated fungal group from AD patients.[3] Malassezia globosa and Malassezia restricta are the dominant fungal flora in AD patients.[4] If there are abnormal changes in the host's skin microenvironment or a weakening of the defense function, Malassezia will multiply in large numbers, thereby inducing various skin diseases. Its pathogenic mechanism may be the lipolytic action triggered by Malassezia. This action induces apoptosis by changing the cell morphology and induces immune damage by affecting the differentiation of helper T cell (Th) subsets in the body, thus exacerbating skin diseases.[5,6]

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Although existing studies have revealed the association between Malassezia and AD, there are still many unknowns regarding the specific role of Malassezia globosa in the pathogenesis of AD. For example, current studies have failed to deeply explain the detailed processes and molecular mechanisms by which Malassezia globosa affects the appearance of skin lesions, histopathological characteristics, and the levels of Th1/Th2-related cytokines. In previous studies, most of them only preliminarily explored the presence and overall impact of the genus Malassezia in AD, and there is a lack of systematic and in-depth research on the unique role of Malassezia globosa, a specific species. This study aims to establish a mouse model of atopic dermatitis infected with Malassezia globosa and deeply investigate the effects of Malassezia globosa on the appearance of skin lesions, histopathological characteristics, and the levels of Th1/Th2-related cytokines in tissues, so as to fill the gap in the research on the role of Malassezia globosa in the pathogenesis of AD and provide a more targeted theoretical basis for the prevention and treatment of AD.

Materials and Methods

Preparation of Malassezia Suspension

In accordance with the routine sample - collection procedures of our outpatient department, the Malassezia globosa was isolated from a patient diagnosed with pityriasis versicolor in our outpatient department. The isolation was followed by identification through fungal morphology and molecular biology techniques. Regarding the use of patient samples, this study was approved by the Research Ethics Committee of Wuhan No.1 hospital (approval number: XS-2024027) and conformed to the Declaration of Helsinki. This patient provided written informed consent prior to his inclusion in the study.

The Malassezia globosa was inoculated into modified Dixon medium (purchased from Qingdao Haibo Biotechnology, item number: HB9217), and then cultured at 28 °C for 7 days. After that, it was harvested by centrifugation at 3000×g for 3 minutes, washed with PBS for 3 times, the concentration was adjusted to 5×106/mL, and finally suspended in olive oil for use. All experimental procedures involving laboratory animals were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee in Tongji Medical college, Huazhong University of Science and Technology, Wuhan, Hubei, China (approval number: 2024 - s1874). This study is reported in accordance with ARRIVE guidelines.

Animal Studies

Male BALB/c mice of the SPF (pathogen-free) grade (N = 24, 6 weeks old, weighing around 24 g) were purchased from the Hubei Branch of Beijing Vital River Laboratory Animal Technology Co., Ltd. (license number 2022–0030). These 24 specific pathogen-free mice were evenly housed in 4 cages, provided with common feed and unpolluted water. They were raised in a clean environment with constant temperature and humidity, under a 12-hour light and dark cycle, and underwent an adaptive feeding period of 14 days. Exclusion criteria: Mice with abnormal health conditions, inconsistent therapeutic responses, failure to successfully induce atopic dermatitis, or atypical symptoms.

Mice were randomly divided into control, AD, and normal groups coated with olive oil fungus suspension (M group) and AD group coated with olive oil fungus suspension (AD+M group). The backs of the mice were shaved to an area of approximately 2×3 cm for AD induction. 2,4-dinitrofluorobenzene (DNFB) was purchased from Shanghai Jizhi Biochemical Technology Co., Ltd. (item number: F51350); olive oil and acetone were purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd.; and DNFB was prepared with acetone and olive oil (3:1, v/v) at concentrations of 0.5% and 0.3%, respectively, while the control group did not receive any treatment after hair removal. 100μL 0.5% DNFB solution was applied to the back skin of mice in the AD and AD + M groups for AD induction on the second day. A 0.3% DNFB solution was then applied for further induction on days 5th, 8th, 11th, and 14th days. 100μL of Malassezia suspension was applied to the backs of mice in the M and AD+M group from the 4th day of the experiment and repeated every other day seven consecutive times. All the mice were euthanized on the 17th day (Figure 1). Body weight and the number of scratching behaviors were recorded on days 0, 4, 8, 12, and 16. Skin lesions on the backs of mice were scored by dermatologists based on four symptoms: erythema, infiltration/papules, epidermal peeling, and exudation/scab. Each symptom was rated as 0 (none), 1 (mild), 2 (moderate), or 3 (severe), and the total

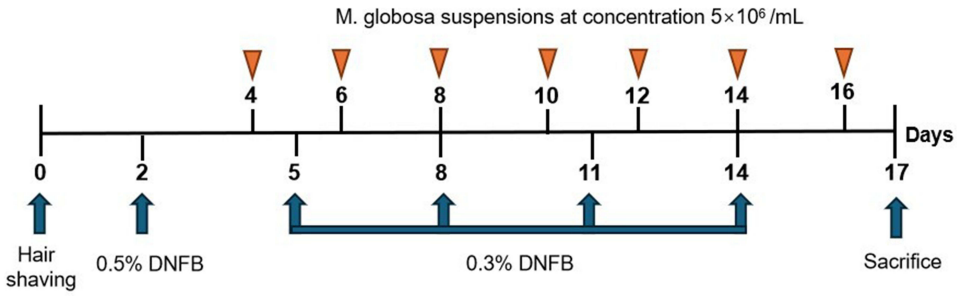

Figure 1 Construction of AD mouse model treated with Malassezia globosa.

score for skin inflammation was 0–12 points.[7] Mice were anesthetized in a chamber containing 2% isoflurane in oxygen and maintained using a face mask with isoflurane. The mice were euthanized by cervical dislocation.

Live Fungal Flora Counts and Histopathological Examination

The back skin lesions were fixed overnight with 4% paraformaldehyde, dehydrated, and embedded in paraffin for sectioning. HE staining was performed and PAS staining was performed simultaneously in the M and AD + M groups. In addition, approximately 0.2 cm^2 of back skin lesions of mice were collected for viable cell counting. The tissue was weighed and homogenized to prepare a tissue suspension, which was diluted in gradient concentrations of 10^{-2}, 10^{-3} and 10^{-4}. 100μL diluted solution was taken from different concentrations and coated onto modified Dixon agar plates. The agar was incubated for 3–5 days at 31°C, and the average fungal flora count was recorded.

Flow Cytometry

The CBA multi-factor detection kit for flow cytometry was purchased from the BD Company (item number 562246). The back skin lesion was homogenized, and the supernatant was collected and mixed with IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ capture microspheres, centrifuged, and labeled according to the protocols. CBA software FCAP Array v3 was used to draw a standard curve, and the cytokine content in the sample was automatically calculated.

Statistical Methods

GraphPad Prism 8.0 software was used for analysis and plotting, and all data were expressed as mean±standard deviation (SD). Repeated measures ANOVA was used to compare the skin lesion scores, scratching times, and weight changes of mice among groups. *t*-test was used to compare the viable Malassezia flora counts and cytokine levels of skin lesions, and $P<0.05$ was considered statistically significant.

Results

Malassezia Globosa Aggravated Skin Lesions and Increased the Scratching Behavior in AD Mouse Model

The back skin of DNFB-induced AD mice showed edema, erythema, and a large number of white scales, with some blood scabs. Compared with the AD group, the AD+M group had aggravated back skin erythema and more obvious exudation, scaling, and scabbing accompanied by capillary dilation. Compared to the control group, the M Group showed slight flaking, scabbing, and scratching without obvious erosion or exudation (Figure 2A). The skin lesion score and scratching behavior frequency of mice in group M were significantly higher, but their body weight was significantly reduced, compared to the control group ($P<0.05$); Compared with the AD group, the AD+M group showed a significant increase in skin lesion scores and scratching behavior frequency ($P<0.05$), and both groups showed a significant decrease in body weight (Figure 2B-D). The situation of SCORED Assessment in mice is shown in the following table (Table 1).

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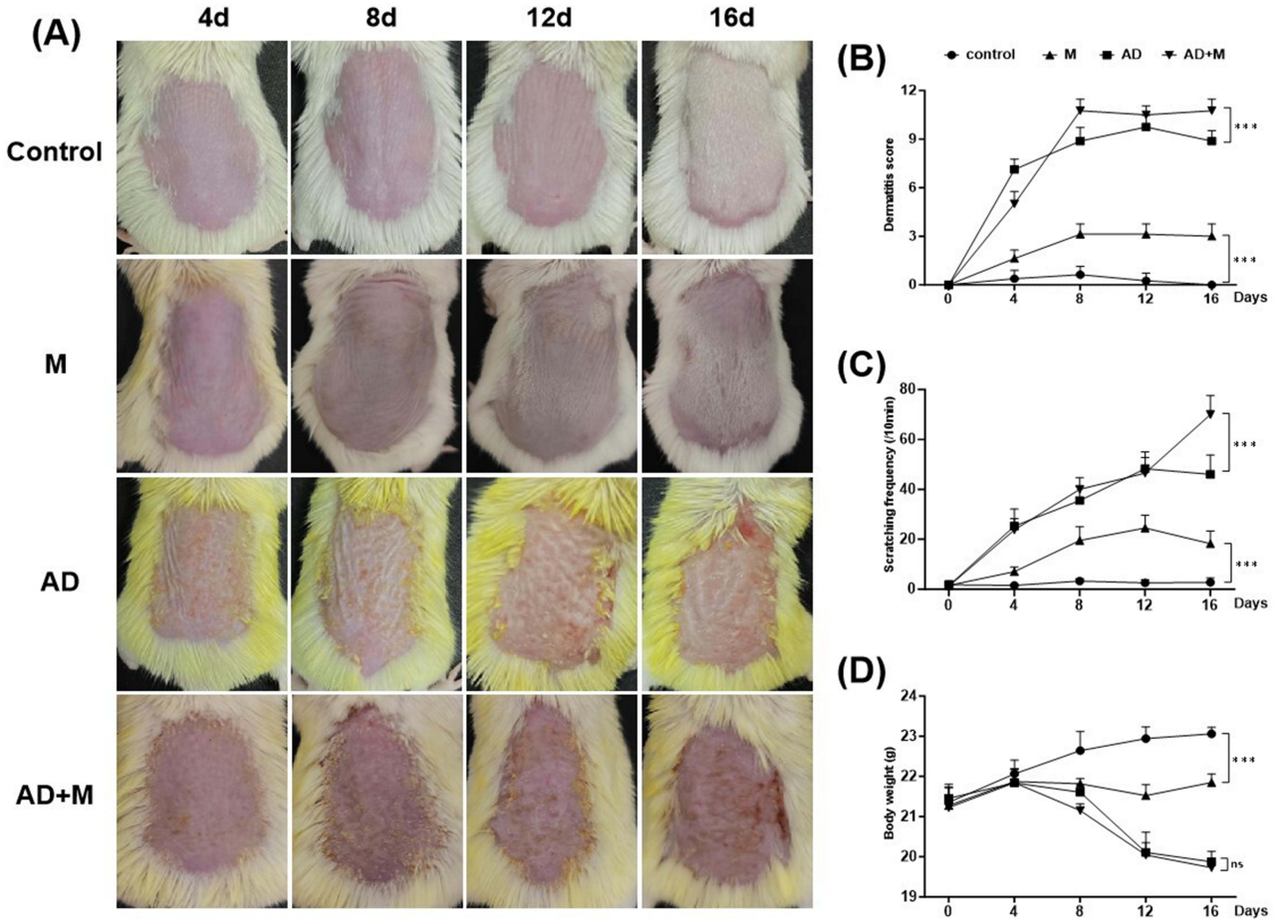

Figure 2 Effects of Malassezia on DNFB-induced AD-like Mice. (**A**) General appearance of dorsal skin lesions in mice of different treatment groups on days 4, 8, 12, and 16. (**B**) Dermatitis scores of mice in different treatment groups. (**C**) Scratching frequencies of mice in different treatment groups. (**D**) Changes in body weights of mice in different treatment groups. ns $P > 0.05$, *** $P < 0.001$.

Malassezia Globosa Aggravated Pathological Changes and Enhanced the Fungal Flora Colonization Unit in AD Mouse Model

Pathological examination of the back skin lesions showed that the control group had a thin epidermis, regular collagen in the dermis, and normal appendages without inflammatory cell infiltration. The M Group exhibited excessive keratinization of the epidermis, thickening of the stratum spinosum, and infiltration of inflammatory cells into the dermis. In

Table 1 SCORED Assessment Scale for BALB/c Mouse Model

	Control						M					
Mouse	1#	2#	3#	4#	5#	6#	1#	2#	3#	4#	5#	6#
Day0	0	0	0	0	0	0	0	0	0	0	0	0
Day4	1	1	0	0	0	0	2	1	3	2	1	1
Day8	1	1	1	1	0	0	4	3	3	3	3	3
Day12	0	1	0	1	0	0	3	3	4	3	2	3
Day16	0	0	0	0	0	0	3	3	4	2	2	3

(*Continued*)

Table 1 (Continued).

	AD						AD+M					
Mouse	1#	2#	3#	4#	5#	6#	1#	2#	3#	4#	5#	6#
Day0	0	0	0	0	0	0	0	0	0	0	0	0
Day4	7	7	8	6	7	8	4	6	5	6	5	5
Day8	9	9	10	8	9	8	10	11	11	12	10	11
Day12	10	11	11	9	10	8	11	10	11	11	9	11
Day16	9	10	9	8	9	8	11	11	11	10	10	11

the AD group, hyperkeratosis and parakeratosis of the epidermis, thickening of the granular and spinous layers, intercellular edema, and extensive infiltration of mononuclear cells into the upper layer of the dermis were observed. The AD + M group showed more significant thickening of the granular and spinous layers than the AD group, with denser infiltration of inflammatory cells in the dermis layer, mainly neutrophils and lymphocytes (Figure 3A). PAS staining showed that a large number of Malassezia spores colonized and clustered in the epidermis and expanded hair follicles in the M and AD + M groups. Spore aggregation in the AD+M group was significantly higher than that in the M group (Figure 3B). The live count results showed the number of Malassezia colonies forming unit per gram skin tissue in AD+M group mice (800315±70016) was significantly higher than that in M group (461256±44,388), $P<0.001$ (Figure 3C). No Malassezia colonization was detected in the skin of the control or AD groups.

Malassezia Globosa Promoted the Th1/Th2 Related Cytokines Expression in AD Mouse Model

Compared to the control group, the secretion of TNF-α and IFN-γ in the M group increased significantly, whereas IL-2, IL-12, IL-4, IL-6, and IL-10 showed no significant changes. Compared with the AD group, the secretion of IFN-γ in the AD + M group increased significantly, whereas there were no significant changes in TNF-α, IL-2, and IL-12. The levels of the Th2 cytokines IL-4 and IL-10 were significantly increased. In addition, IL-1β levels were significantly elevated after Malassezia infection in both the M and AD + M groups (Figure 4).

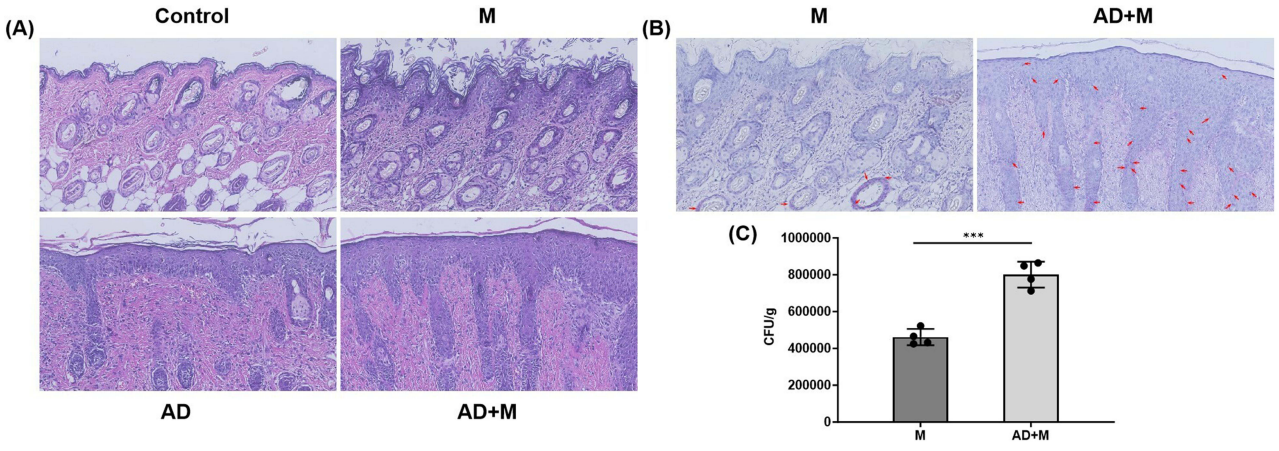

Figure 3 Results of Histopathological Manifestations and live Malassezia population counts. (**A**) Histopathological manifestations of dorsal skin lesions of mice in different treatment groups (HE × 200). (**B**) Colonization of Malassezia globosa in dorsal skin lesions of mice in M Group and AD+M Group. Malassezia spores were indicated by red arrows (PAS × 200). (**C**) Results of live Malassezia population counts in skin lesion tissues of mice in M Group and AD+M Group. Each data point represents one mouse, with n = 6 mice in each group. The results were expressed as mean ± standard deviation. The *t*-test was used to determine statistical significance. *** $P < 0.001$.

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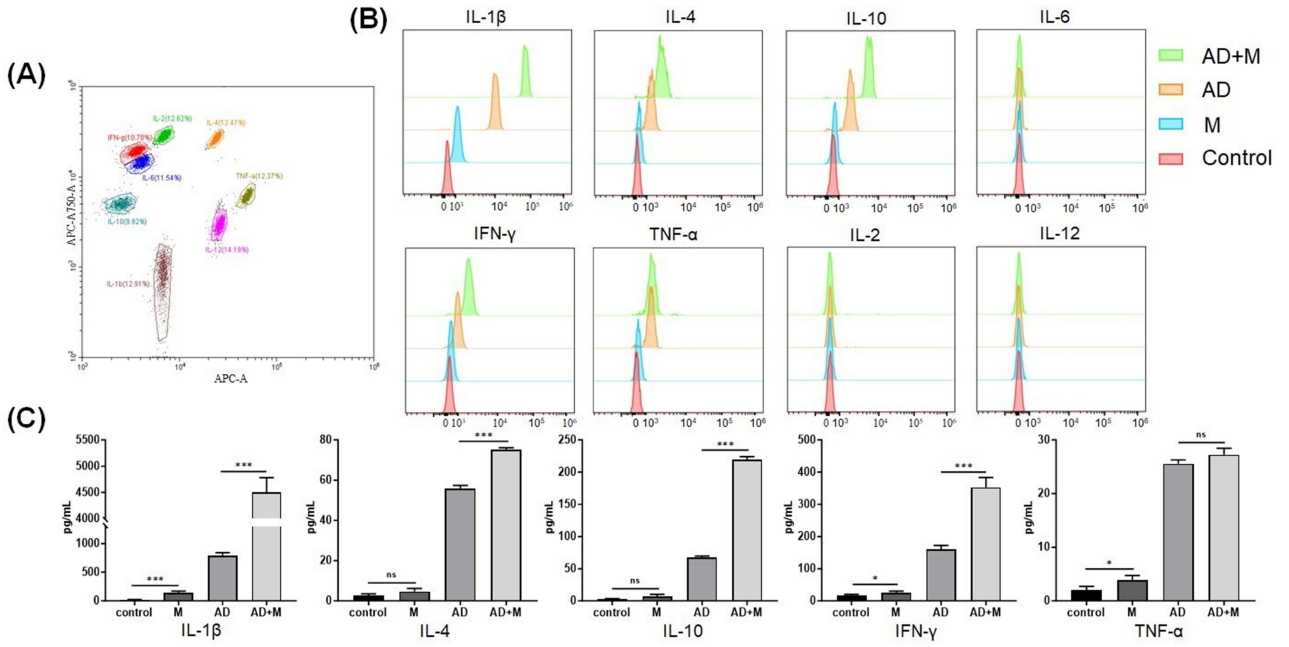

Figure 4 Malassezia Colonization Aggravated Th1/Th2-Related Inflammatory Responses in Normal and AD-like Mice. (**A**) Scatter plot of cytokine distribution. (**B**) Secretion of cytokines (IL-1β, IL-4, IL-6, IL-10, IFN-γ, TNF-α, IL-2, IL-12) in dorsal skin lesions of mice in different treatment groups. (**C**) Comparison of cytokines (IL-1β, IL-4, IL-10, IFN-γ, TNF-α) in dorsal skin lesions of mice in different treatment groups. The values were shown as the mean ± standard deviation of 6 mice. Unpaired t-tests were used among four groups. ns P > 0.05, *P < 0.05, *** P < 0.001.

Discussion

AD results from multiple factors working together and Malassezia may be involved. There is limited research on animal models of AD with Malassezia infection. Recent studies have shown that the changes in the epidermal barrier function of patients with atopic dermatitis (AD) are the cause of the excessive growth of Malassezia.[8–10] The structural and functional disorders of the skin barrier create an environment conducive to the colonization of Malassezia, which in turn leads to epidermal hyperplasia and changes in the body's immune state.[11] Currently, there are multiple ways to construct a mouse model of atopic dermatitis infected with Malassezia. Sparber et al damaged the epidermal barrier of mouse ear skin mildly by repeatedly using Scotch tape adhesion, and then applied olive oil with Malassezia solution suspension to create a model.[12] They found that excessive proliferation of Malassezia restricta and Malassezia sympodialis could exacerbate the inflammatory response in mouse skin with damaged epidermal barrier and exhibit AD-like dermatitis. However, widespread damage to the skin barrier caused by physical means, such as scotch tape, cannot truly simulate the skin barrier damage caused by genetic factors or immune response abnormalities in AD. The DNFB-induced mouse model is currently recognized as an AD-like animal models.[13,14] In addition to the main skin changes, DNFB can simulate the systemic inflammatory state of AD. In this study, we used DNFB to induce AD-like skin lesions on the backs of mice to avoid physical damage to the skin barrier, and the back was easier to observe than the ears.

In this study, DNFB was used as a sensitizer to repeatedly stimulate the backs of mice and create an AD model. The AD mice group showed typical skin lesions such as erythema, edema, exudation, and scab formation, successfully establishing an AD mouse model, and compared with the control group, the M group showed a small amount of mild inflammatory symptoms such as erythema and scales. Compared with the AD group, the AD + M group of mice showed an increased inflammatory response and scaling of skin lesions, indicating that Malassezia infection can exacerbate the skin inflammatory response as well as the clinical manifestations in AD mice. Itching is the main symptom of AD. The cytokines secreted by T cells in patients are associated with an IgE-mediated immune response.[15] Antigen - which triggers the release of histamine, leading to itching. Skin surface damage caused by scratching further increased the probability of Malassezia infection; in this study, compared with the control group, the scratching behavior in the M group increased significantly, accompanied by weight loss. The scratching behavior in the AD + M group also

increased significantly compared to that in the AD group. This may be due to the binding of antigens derived from Malassezia to IgE, leading to an exacerbation of itching.[16] The histopathological results showed that after the colonization of Malassezia globosa, the epidermal thickness of the skin lesions increased, the infiltration of inflammatory cells increased, and the PAS staining and viable fungal flora counts in the AD+M group showed the spore population significantly higher than in the M group, indicating excessive proliferation of Malassezia on the skin surface with barrier disruption.

Under normal circumstances, the expression levels and functions of Th1 and Th2 cells interact in a dynamic balance to maintain normal cellular immune functions in the body. Th1 cells mainly secrete IL-2, TNF-α, and IFN-γ, which promote the proliferation, differentiation, and function of cytotoxic T lymphocytes, while Th2 cells mainly secrete IL-4, IL-6, and IL-10, enhancing the proliferation, differentiation, and antibody production of B cells.[17] In AD, Th2 immune cells are considered the main driving factor, but Th1 cells also play an important role in chronic inflammation.[18,19] This study showed that compared with the control group, there was no significant change in Th2 related cytokines, such as IL-4, IL-6, and IL-10, in group M, while Th1 related cytokines such as TNF-α and IFN-γ, as well as pro-inflammatory cytokine IL-1β, were significantly increased, which was consistent with the results of in vitro cell experiments.[20] This suggests that Malassezia colonization infection in normal skin can lead to a Th1 immune response in the body to resist microbial invasion. Compared with the AD group, the AD + M group showed a significant increase in the pro-inflammatory cytokines IL-1β and Th2 related cytokines like IL-4 and IL-10. The level of the Th1 cytokine, IFN-γ, was also significantly increased. This suggests that for skin with impaired barrier function, the colonization of Malassezia globosa not only promotes the Th2 immune response of AD itself, but also upregulates Th1 cytokines to exacerbate the inflammatory response.

Currently, there are limited studies on the animal models of AD with Malassezia infection. In this study, a mouse model of AD was established by inducing with DNFB. It was found that Malassezia infection could exacerbate the skin inflammation and clinical symptoms of AD mice, and also affect the expression of cytokines related to Th1/Th2. This indicates that the interaction between Malassezia and the skin immune system may be one of the causes of AD inflammation, and treatment targeting Malassezia can effectively alleviate AD inflammation. Jain C et al found that the isolation rate of Malassezia was higher in patients with AD through studies on patients and healthy controls, and there were significant differences in the gene polymorphisms of IL10 - 819/592C/T and IFNγ + 874A/T. This suggests that differences at the genetic level may affect the host's susceptibility to Malassezia, and in turn, influence the occurrence and development of AD.[21] This complements the findings in this study that Malassezia infection exacerbates AD inflammation, collectively indicating the important role of host genetic factors in Malassezia-mediated AD inflammation. Szczepańska M et al mentioned that the skin barrier function of patients with AD is impaired, leading to dysbiosis of the mycobiota, especially an increase in Malassezia. Malassezia activates the innate and adaptive immune responses through its metabolites and allergens Mala s11 and s13, promotes Th2 and Th17-type inflammatory responses, and may exacerbate AD symptoms through IgE-mediated allergic reactions.[22] This study, from the perspective of the balance of Th1/Th2 cytokines, further clarifies the specific role of the imbalance of the immune response caused by Malassezia infection in the exacerbation of AD inflammation, providing an experimental basis for understanding the pathogenic mechanism of Malassezia in AD.

Although this study has achieved certain results in the research on the relationship between Malassezia and AD, there are still potential biases and limitations in aspects such as the sample size and the experimental cycle. Therefore, in future research, the sample size can be increased, the observation period can be extended, and relevant indicators can be comprehensively detected, so as to explore the pathogenesis of Malassezia in AD more deeply and provide a more reliable theoretical basis for the clinical treatment of AD.

Conclusion

In summary, Malassezia can cause inflammatory reactions in both normal skin and AD-like skin and can even worsen skin barrier function. The underlying mechanism may be overexpression of Th1/Th2 cytokines caused by Malassezia infection. Targeting Malassezia may be a potential method to alleviate AD inflammation.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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