

An Improved Mouse Model of Atopic Dermatitis and Suppression of Skin Lesions by an Inhibitor of Tec Family Kinases

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ABSTRACT

Background: Atopic dermatitis is a chronic or chronically relapsing, pruritic inflammatory skin disease. The incidence of atopic dermatitis has dramatically increased during the past three decades in industrialized countries. We attempted to develop an improved method to induce an animal model of atopic dermatitis and to use it to evaluate the efficacy of a Tec family kinase inhibitor.

Methods: We treated dermatitis-prone inbred mice, NC/Nga, by repetitive epicutaneous applications of a house dust mite allergen and staphylococcal enterotoxin B to induce atopic dermatitis-like skin lesions.

Results: We established a highly efficient protocol to induce skin lesions in NC/Nga mice, which were histologically and immunologically similar to human atopic dermatitis. Similar to human patients, serum IgE levels were increased in dermatitis-induced mice. Consistent with the proposed roles of infiltrated immune cells in the pathogenesis of human atopic dermatitis, skin lesions were treatable with terreic acid, an inhibitor of Tec family kinases, as well as dexamethasone.

Conclusions: We established a highly efficient, highly reproducible protocol to induce skin lesions in NC/Nga mice and successfully applied it to show the efficacy of terreic acid in treating skin lesions. This mouse model of atopic dermatitis will be useful to study the pathogenetic processes of atopic dermatitis and to evaluate the efficacy of drug candidates.

KEY WORDS

allergen, atopic dermatitis, mite, Tec, Th2

INTRODUCTION

Atopic dermatitis (AD) is a chronic or chronically relapsing, pruritic inflammatory skin disease.^{1,2} The incidence of AD has increased two- to threefold during the past three decades in industrialized countries. Systemic immunological abnormalities characteristic of AD include sensitization with various allergens, (e.g., foods, aeroallergens, microbes, and autoallergens), high serum IgE levels, and skin lesions with apoptotic keratinocytes and infiltration with immune cells such as CD4⁺ T cells, eosinophils, and mast cells. These T cells express IL-4, IL-5, and IL-13,³ and numerous studies suggest an association between

AD development and type 2 helper T (Th2) cell-skewed immune dysfunction. However, there are also data suggesting that AD development is independent of IgE, but correlates with an increase in interferon (IFN)- γ -producing Th1 cells.⁴⁻⁶ AD patients often suffer from skin infections and more than 90% of AD patients are colonized with *Staphylococcus aureus*. Activation of the immune cells⁷ and *S. aureus* infection^{8,9} are thought to be critical in the pathogenesis and/or worsening of skin lesions.

Several mouse models of human AD have been developed over the last decade, and have provided insight into the pathogenesis of human AD.¹⁰⁻¹⁶ For example, an ovalbumin epicutaneous sensitization

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model mimicked skin lesions of human AD with regard to infiltration of CD3⁺ T cells, eosinophils, and neutrophils and local expression of mRNAs for IL-4, IL-5 and IFN- γ .¹¹ Differential roles of IL-4, IL-5, and IFN- γ in skin lesion development and leukocyte infiltration in this model were demonstrated using gene-manipulated mice, whereas IgE was not required for skin lesion development in this model.¹⁷

NC/Nga mice spontaneously develop AD-like skin lesions under conventional (non-specific pathogen-free) conditions.¹⁰ Skin lesions in NC/Nga mice were characterized by infiltration of IL-4- and IL-5-producing CD4⁺ T cells, mast cells and eosinophils, as well as Th2 chemokines and their receptors, and the elevation of serum IgE correlated with the onset of skin lesion development.^{10,18} Under conventional conditions these mice were infested with rodent mites,^{19,20} and the eradication of the mites with ivermectin led to healing of skin lesions and reduced IgE levels.¹⁹ However, the incidence of skin lesions in these mice drastically varies from facility to facility (<5% in our facility). Therefore, several groups used mite extracts to induce dermatitis in NC/Nga mice.²¹⁻²⁵ Application of mite *Dermatophagoides* (a common mite on humans) extracts on the skin clearly induced dermatitis and elevated serum IgE, although some studies showed low clinical scores of the induced lesions. In this study, we attempted to increase the efficiency of disease development and the severity of disease by treating NC/Nga mice with agents that are likely to be involved in the pathogenesis of AD, and established a highly efficient mouse model to induce AD-like skin lesions with high clinical scores. Using this model, we found that terreic acid, an inhibitor of Tec family kinases that are crucial to immune cell activation,²⁶ is as efficacious as dexamethasone in treating skin lesions.

METHODS

INDUCTION AND TREATMENT OF AD-LIKE SKIN LESIONS

Mice were anesthetized and their backs were shaved. Solutions of 500 ng of staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, MO, USA) and 10 μ g of *Dermatophagoides farinae* extract (Der f) (100 μ g/ml, Greer Laboratories, Lenoir, NC, USA) were pipetted on a 1 cm $\times$ 1 cm square gauze pad placed on the shaved area. This portion of the back skin was occluded with a TegadermTM Transparent Dressing (3M HealthCare, St. Paul, MN, USA) using bandages. Three days later, the dressings were replaced with a new one. Another 4 days later, the dressings were removed and the mice were kept without treatment for the next week. This 2-week cycle was repeated one or two more times. The healthy skin mice used as a control throughout this project were treated with vehicle alone. Clinical severity was scored by an investigator who did not know the identities of mice 2 days after

removing the dressings in the last cycle. Clinical scores were based on the severity (0, no symptoms; 1, mild; 2, intermediate; 3, severe) of four possible symptoms (redness, bleeding, eruption, and scaling). The mice with a clinical score of 9 or higher were used for treatment with terreic acid (Kirin Brewery Co., Tokyo, Japan), dexamethasone (Sigma-Aldrich), or ampicillin (Sigma-Aldrich). Skin lesions were covered with a piece of gauze spotted with 100 μ l of these reagents or vehicle and occluded with TegadermTM. This treatment with fresh agents was repeated every other day (four times in total). Clinical scoring and tissue sampling were done 2 days after the last treatment. Animal experiments were approved by the Institutional Review Board and followed the guidelines described in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press.

HISTOLOGICAL ANALYSIS

Dorsal skin samples were fixed in 10% formaldehyde, paraffin embedded and cut into 6 μ m sections. Deparaffinized sections were stained with hematoxylin and eosin (H&E), toluidine blue (pH 4.0) or Congo red. The remaining part of the skin was embedded in an OCT compound, snap frozen in liquid nitrogen and stored at -80°C until use. Frozen sections cut at 6 μ m were incubated with rat antibodies to CD4 (1 : 100, Santa Cruz Biotechnology), CD8 (1 : 15, BD Biosciences Pharmingen), or Mac-1 (1 : 12.5, BD Biosciences Pharmingen). Secondary antibodies were horseradish peroxidase-conjugated goat antibody to rat IgG (1 : 50, BD Biosciences Pharmingen). Bound antibodies were detected using the ImmunoCruzTM Staining System (Santa Cruz Biotechnology) followed by hematoxylin counterstaining. Cells were counted under a microscope at a magnification of $\times$ 400 and expressed as the total number of the cells in five high-power fields per section.

SERUM IMMUNOGLOBULINS

Serum IgE and IgG levels were measured using enzyme-linked immunoassay kits purchased from BD Biosciences Pharmingen (San Diego, CA, USA) and Roche Applied Science (Indianapolis, IN, USA), respectively.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS OF CYTOKINE GENE EXPRESSION

Skin lesions were sampled using a paper puncher. RNA was extracted from axillary lymph nodes and skin samples using a Trizol One Step RNA Reagent (BioPioneer Inc., San Diego, CA, USA). cDNA synthesis was prepared with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using the

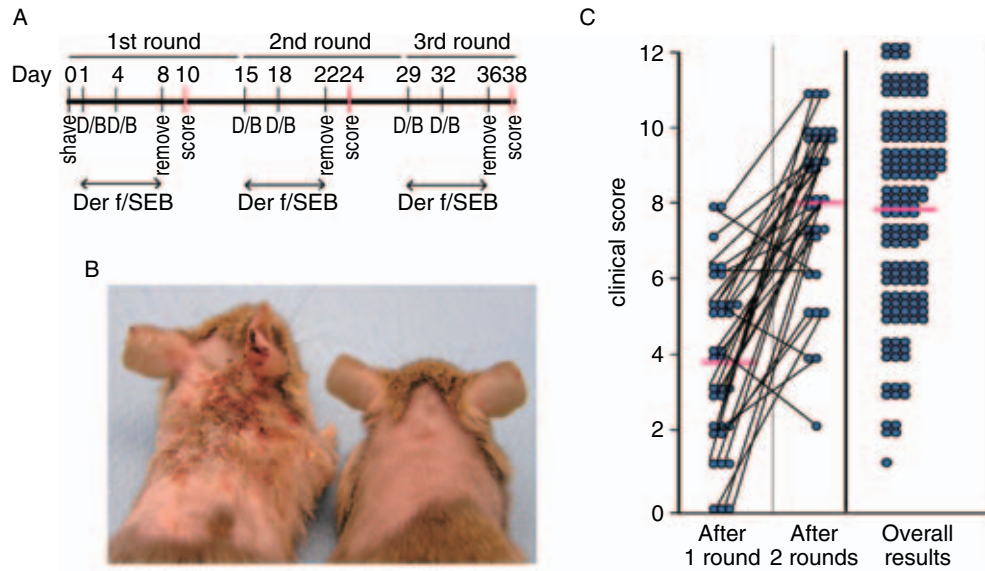


Fig. 1 Der f/SEB-induced Skin Lesions. **(A)** The schedule for dermatitis induction is depicted. D/B, treatment with Der f and SEB. The periods when Der f/SEB-treated back skin is occluded with Tegaderm™ are also shown at the bottom. **(B)** A representative mouse (Left) with skin lesions of a clinical score of 12 is shown along with a mouse (Right) with healthy skin. **(C)** Clinical scores after one and two rounds of Der f/SEB induction (Left). Each mouse is connected between their score after one round and that after two rounds of treatment to indicate that the scores are increased after two rounds in a majority of mice. Cumulative clinical data on skin lesions induced by two or three rounds of Der f/SEB treatment are also shown (Right, $n = 153$). Note that 76 of 153 mice showed clinical scores of 9 or higher.

isolated RNAs as templates and oligo (dT) as a primer. PCR was performed on the cDNAs using appropriate primers for amplification of IL-4, IL-13 and IFN- γ . The PCR products were analyzed by agarose gel electrophoresis and quantified using a ChemDoc XRS system densitometer and Quantity One software (Bio-Rad, Hercules, CA, USA).

RESULTS

After several trials and errors, we found that epicutaneous application with Der f (house dust mite allergen) and SEB is particularly efficient at inducing skin lesions (Fig. 1). Clinical scores increased from 3.7 ± 2.2 after one round of one-week treatment with Der f and SEB and one-week resting to 7.97 ± 2.2 after two rounds of the same treatment (Fig. 1C). It is noteworthy that approximately 50% of the mice that underwent two or three rounds of Der f/SEB treatment exhibited clinical scores of 9 or higher. This allowed for easy evaluation of the effect of a drug candidate in treating dermatitis. Cumulative results ($n = 153$) demonstrated that this is a very efficient method to induce skin lesions (Fig. 1C).

Microscopic analysis indicated that skin lesions exhibit mild spongiosis, epidermal hyperplasia, and parakeratosis, as well as infiltration with leukocytes (Fig. 2A). Immunohistochemical staining demon-

strated that the numbers of CD4⁺ T cells and Mac-1⁺ monocytes/macrophages, but not CD8⁺ T cells, are increased in skin lesions (Figs. 2A, B). Cell type-specific stainings and H&E staining showed that the lesions contained increased numbers of mast cells, eosinophils, and neutrophils as well (Figs. 2A, B). This pattern of leukocyte infiltration is quite similar to human AD lesions and spontaneously developed skin lesions in NC/Nga mice. Also similar to human AD, disease-borne mice had increased serum IgE levels (average values $\pm$ sd: 55.1 ± 4.1 μ g/ml for Der f/SEB-treated mice on day 21 *vs.* <0.5 μ g/ml for skin lesion-free mice). Consistent with the role for Th2 cells in the development of human AD and mouse AD models,^{12,17} draining lymph nodes had increased levels of IL-4 mRNA (Fig. 3). However, IL-13 mRNA levels in skin and lymph nodes were comparable between skin lesion-borne and -unborne mice. Like human AD and other AD mouse models including spontaneously developed skin lesions in NC/Nga mice,^{13,14,17,27} IFN- γ mRNA levels also showed a tendency toward an increase (not statistically significant) in Der f/SEB-induced skin lesions (Fig. 3).

Application of the same Der f/SEB-resting cycle to other strains of mice, C57BL/6, BALB/c and 129/SvJ, showed that NC/Nga mice are the most susceptible to this form of dermatitis induction and that 129/

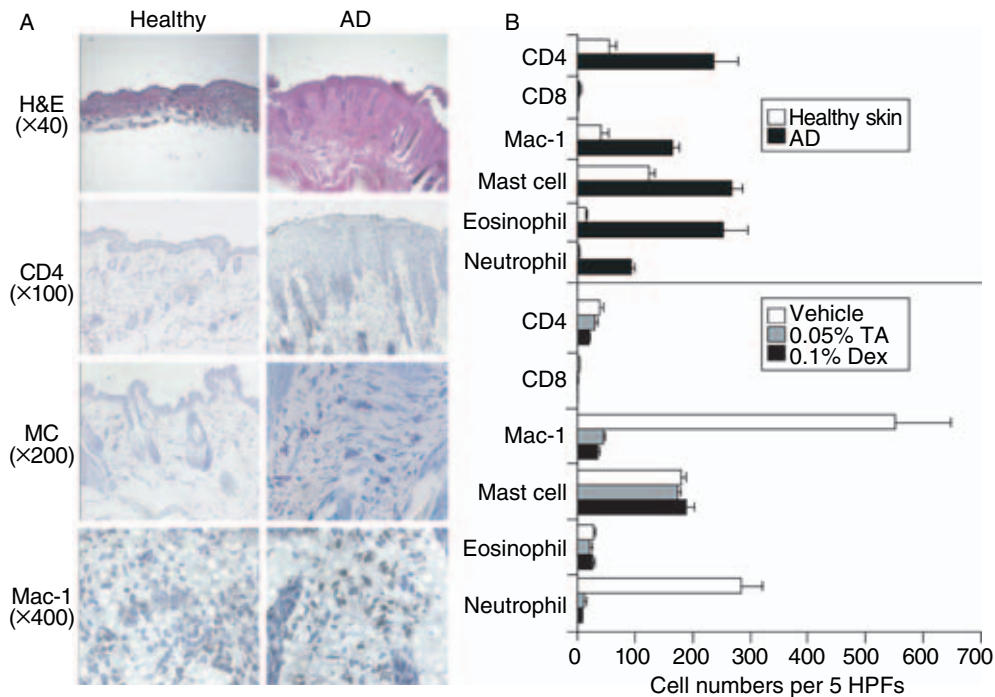


Fig. 2 Histological Analysis of Skin Lesions in Der f/SEB-treated Mice. **(A)** Untreated healthy skin and Der f/SEB-induced skin lesions were stained with H&E, anti-CD4, anti-Mac-1, and toluidine blue (to stain mast cells, MC). **(B)** Cellular data of skin lesions before and after treatment with Der f/SEB are shown (Top), combined with cell counts in the skin after therapeutic treatments with vehicle, terreic acid (TA) or dexamethasone (Dex)(Bottom). Samples were taken from the therapeutic experiments shown in Figure 4C. Error bars represent standard deviation.

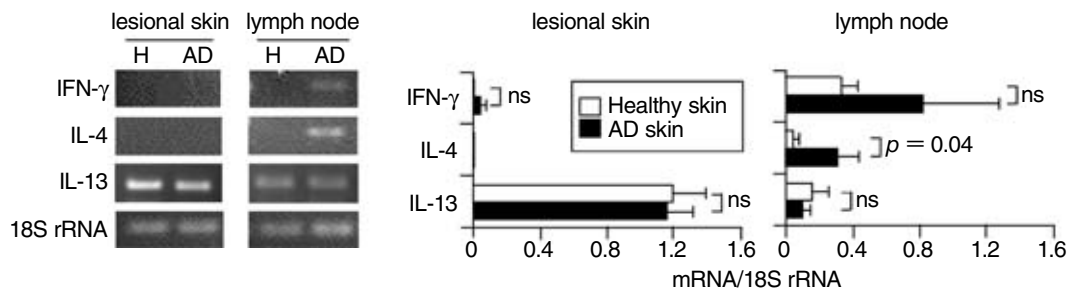


Fig. 3 RT-PCR Analysis of Cytokine Gene Expression in Der f/SEB-induced Skin Lesions and Draining Lymph Nodes. Untreated healthy skin (H) and Der f/SEB-induced AD-like skin lesions (AD) were sampled and RNA isolated. RT-PCR analysis was performed to measure mRNAs for IL-4, IL-13 and IFN-γ. 18S RNA was used for normalization. Representative agarose gels (Left) and cumulative data from 6 mice (Right) are shown. Error bars represent standard deviation. P values obtained by Student's t test are shown. NS denotes statistically not significant.

SvJ and BALB/c are the least susceptible (average scores $\pm$ sd: 2.625 ± 2.5 for C57BL/6 [$n = 8$]; 0.125 ± 0.35 for BALB/c [$n = 8$]; 0.00 for 129/SvJ [$n = 8$]).

Current therapeutics for AD includes treatment with emollients, topical corticosteroids, tacrolimus, or pimecrolimus.^{1,28} In light of side effects of the current

therapies and some recalcitrant cases, we were interested in developing an alternative therapeutic approach. Activation of infiltrating immune cells, such as T cells, eosinophils, mast cells, and monocytes/macrophages, are assumed to play critical roles in the pathogenesis. Protein-tyrosine kinases of the Tec family such as Itk, Btk, and Tec are important in the

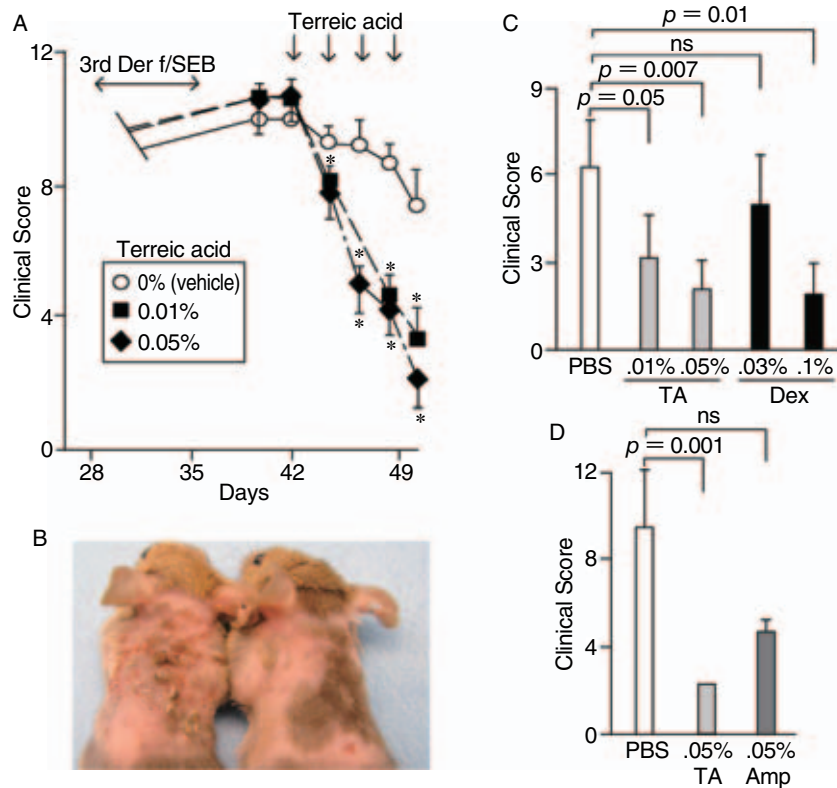


Fig. 4 Therapeutic Effects of Terreic Acid on Der f/SEB-induced Skin Lesions. (A) NC/Nga mice were induced to develop skin lesions by three rounds of Der f/SEB treatments (see Fig. 1A for the treatment schedule). Mice with clinical scores of ≥ 9 were treated with 0% (vehicle), 0.01%, or 0.05% terreic acid on days 42, 44, 46, and 48. Skin lesions were scored on days 40, 42, 44, 46, 48, and 50. Error bars represent standard deviation and asterisks indicate statistically significant ($p < 0.05$) differences between terreic acid-treated and vehicle-treated mice. $N = 6$ for each group. Representative data out of 5 independent experiments are shown. (B) A representative mouse with terreic acid-treated skin lesions (Right) is shown along with a mouse treated with a vehicle (Left). (C) Experiments were performed similar to those shown in A ($n = 6$ each group). The clinical scores after treatment with the indicated concentrations of terreic acid and dexamethasone are shown. Error bars represent standard deviation. P values obtained by Student's t test are shown. NS denotes statistically not significant. (D) Effects of ampicillin are shown. Experiments were performed similar to those shown in A ($n = 6$ each group).

activation of the immune cells.²⁹ We previously identified terreic acid, a quinone epoxide that was originally isolated as an antibiotic, as an inhibitor of this class of kinases.²⁶ To evaluate the effect of terreic acid on skin lesions, we first checked the change in skin lesions for 15 days after the last Der f/SEB treatment. Clinical scores of the mice that had scores of 9 or higher at day 40 stayed at high levels (> 8.5) for the next 8 days and started to decline at day 50 (14th day after the last Der f/SEB treatment) (vehicle-treated mice in Fig. 4A). When terreic acid was repeatedly (four times before scoring) applied to skin lesions, clinical scores were significantly improved

over vehicle-treated controls (Figs. 4A, B). The extent of clinical improvement with 0.05% terreic acid was approximately the same as that with 0.1% dexamethasone-treated mice (Fig. 4C). Unlike dexamethasone treatment that left the skin reddish, however, terreic acid treatment did not have such an adverse effect. Microscopic analysis of vehicle-treated lesions demonstrated that, compared to the skin lesions before the treatment, numbers of CD4⁺ T cells and eosinophils returned to basal levels 14 days after the last Der f/SEB treatment and mast cell numbers also decreased, whereas numbers of monocytes/macrophages and neutrophils dramatically increased

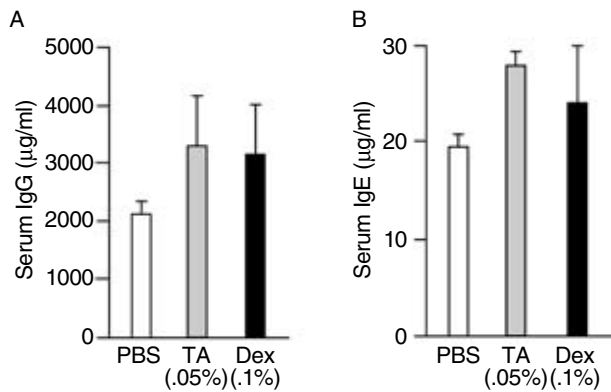


Fig. 5 Effects of Terreic Acid and Dexamethasone on IgG and IgE in Der f/SEB-treated Mice. Serum IgG (A) or IgE (B) levels were not reduced in mice treated with terreic acid or dexamethasone (see Fig. 4C).

during the same period (Fig. 2B). By contrast, treatment with terreic acid or dexamethasone reversed the infiltration of Mac-1⁺ monocytes/macrophages and neutrophils to baseline, although high mast cell levels were sustained (Fig. 2B). Interestingly, serum IgE levels as well as IgG levels in terreic acid- and dexamethasone-treated mice were only slightly lower than those before the treatment, but much higher than those in mice before dermatitis induction (Fig. 5). The IgE results are consistent with the notion that IgE is not skin-pathogenic in NC/Nga mice, which was supported by a previous study showing that Stat6^{-/-} NC/Nga mice develop skin lesions without increased serum IgE.³⁰ The histopathological results indicate that infiltrates of skin lesions in our AD model become monocyte/macrophage- and neutrophil-dominant after 14 days without exposure to allergen and SEB, and that terreic acid and dexamethasone suppress the accumulation of these cells. Similar patterns of leukocyte infiltration in terreic acid- and dexamethasone-treated skin lesions (Fig. 2B) may suggest that these agents target identical or overlapping cell signaling pathways in the same cell types.

As bacterial infection is considered to be a precipitating or exacerbating factor in human AD and terreic acid has an antibiotic activity, we tested effects of ampicillin application on skin lesions. Ampicillin also exhibited some, albeit statistically insignificant ($p = 0.07$), effects on improving clinical scores (Fig. 4 D).

DISCUSSION

In this study, we established a new protocol that can induce AD-like skin lesions in dermatitis-prone NC/Nga mice with 100% efficiency. Half of the mice showed clinical scores of 9 or higher. Induced skin lesions were basically identical to spontaneously developed skin lesions in NC/Nga mice, characterized by infiltrations of immune cells (CD4⁺ T cells, eosino-

phils, mast cells, monocytes/macrophages, and neutrophils) and high serum IgE levels, mimicking clinical findings in human AD patients. Consistent with roles of infiltrated immune cells in the development of skin lesions, terreic acid and dexamethasone were both efficacious in treating induced skin lesions. Therefore, this AD model seems useful to study pathogenetic processes of AD and to evaluate the efficacy of drug candidates.

Based on similarities discussed between induced or spontaneously developed dermatitis in NC/Nga mice and human AD, it would be fair to say that both our NC/Nga model and other NC/Nga models mimic limited attributes of human AD: for instance, food sensitization is often found to be a trigger in human patients, while it is not clear whether food allergen plays any role in NC/Nga mice. Pruritis is a common clinical manifestation in human AD. In our model, however, the back skin was treated with Der f/SEB and occluded to prevent confounding effects of scratching the skin area.

Terreic acid was originally isolated as an antibiotic in 1942, but has not been used clinically probably because of its toxicity to the kidney. Our previous study identified it as an inhibitor of Tec family kinases.²⁶ Tec family kinases, such as Btk, Itk and Tec, act downstream of Src and Syk family kinases, phosphorylate phospholipase C- γ , and regulate Ca²⁺ mobilization.²⁹ Our present results suggest that compounds, like terreic acid, which have antibiotic as well as Tec inhibitory activities, may be particularly useful in treating AD skin lesions. Another advantage of targeting Tec family kinases therapeutically is that these kinases are signal amplifiers in immune cell activation, but not an all-or-none switch like Syk.^{29,31} Therefore, some immune cell functions will remain intact with impaired activity of Tec family kinases. Thus, a topical approach of skin treatment with terreic acid and other Tec family inhibitors limits potential systemic side effects. Although the mice treated with up to 1% terreic acid stayed healthy and active with good appetite, the long-term toxicity of TA to the kidney and other organs remains to be carefully evaluated.

AD patients are known to be highly susceptible to viral infections.³² For example, vaccination against smallpox by scarification with vaccinia virus occasionally causes serious complications termed eczema vaccinatum.³³ Herpes simplex virus infection also causes severe infection in AD patients termed eczema herpeticum.³² Therefore, our AD model may also be useful to study pathogenetic mechanisms of these viral infections.

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