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Article in *Journal of Investigative Dermatology* · April 2017

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PII: S0022-202X(17)31440-9

DOI: [10.1016/j.jid.2017.04.018](https://doi.org/10.1016/j.jid.2017.04.018)

Reference: JID 850

To appear in: *The Journal of Investigative Dermatology*

Received Date: 4 July 2016

Revised Date: 30 March 2017

Accepted Date: 3 April 2017

Please cite this article as: Kim KE, Jung MJ, Houh Y, Kim TS, Lee WJ, Yang Y, Bang SI, Kim CH, Kim H, Park HJ, Cho D, Erdr1 attenuates *Dermatophagoides farina* body extract-induced atopic dermatitis in NC/Nga mice, *The Journal of Investigative Dermatology* (2017), doi: 10.1016/j.jid.2017.04.018.

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Erdr1 attenuates *Dermatophagoides farina* body extract-induced atopic dermatitis in NC/Nga mice.

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SHORT TITLE

Erdr1 exerts preventive effects on AD

ABBREVIATIONS USED:

AD: Atopic dermatitis

Erdr1: Erythroid differentiation regulator 1

CCL17: Chemokine (C-C motif) ligand 17

CCL22: Chemokine (C-C motif) ligand 22

TSLP: Thymic stromal lymphopoietin

VEGF: Vascular endothelial growth factor

TO THE EDITOR:

Atopic Dermatitis (AD) is a multifactorial inflammatory skin disease characterized by a defective skin barrier and dysregulation of the immune system in terms of Th1 and Th2 cell imbalance (Peng and Novak, 2015). Th2 cytokines, such as interleukin (IL)-4, IL-13, and thymic stromal lymphopoietin (TSLP), disrupt skin barrier and immune system function and induce inflammation in the lesional skin. TSLP is significantly increased and activates dendritic cells (DCs) to produce Th2 chemoattractants, including chemokine (C-C motif) ligands CCL17 and CCL22, resulting in an increased Th2-skewed inflammatory responses in the lesional skin (Soumelis et al. , 2002).

Previously, erythroid differentiation regulator 1 (Erdr1) was shown to have a negative correlation with IL-18, which is closely associated with AD severity (Kim et al. , 2016a, Trzeciak et al. , 2011). Erdr1 is first discovered in WEHI-3 cell line and released under stress conditions, modulating growth and survival of cells (Dormer et al. , 2004a, Dormer et al. , 2004b). Although functions and mechanisms of action remain unclear, recent studies show that Erdr1 displays anti-inflammatory and anti-cancer properties on various skin diseases, including psoriasis, rosacea, and melanoma (Jung et al. , 2011, Kim, Houh, 2016a, Kim et al. , 2016b, Kim et al. , 2015). Therefore, we hypothesized that Erdr1 would exert preventive effects on AD through its anti-inflammatory functions, similar to its effects on psoriasis and rosacea.

Here, we compared the expression level of Erdr1 between AD lesional skin and normal skin from humans and mice. To determine Erdr1 expression in AD lesional skin from human subjects, skin tissues were collected from normal control (n = 5) and AD (n = 11) patients. This experiment using human subjects was approved by the ethical committee of the Catholic University of Korea, and all human subjects provided written informed consent. Also, all

experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Sookmyung Women's University [SM-IACUC-2013-0726-022]. As shown in Fig S1a and S1b, Erdr1 expression was significantly lower in AD lesional skin than normal skin, and effective treatments restored expression, indicating that Erdr1 may play a critical role in AD. To investigate the role of Erdr1 in AD, *Dermatophagoides farina* body (Dfb) ointment was topically applied to the ears of NC/Nga mice to induce AD-like skin inflammation. Then, 10 µg/kg of recombinant Erdr1 (rErdr1) or 2 mg/kg of dexamethasone as a positive control were administered by intraperitoneal injection. As shown in Fig 1a, rErdr1 significantly improved the external symptoms of AD-like skin inflammation compared with the group injected with the vehicle, PBS. Histological analysis also showed that rErdr1 administration resulted in decreased inflammatory cell infiltration, such as eosinophils and mast cells, and hyper-proliferation in the epidermis.

To determine the severity of AD, symptoms of redness, edema, scaling, and excoriation were scored, and the overall score was evaluated by the sum of each category. The scores were considerably decreased by rErdr1 administration compared to those in the vehicle-injected group (Fig 1b and S2). In addition, ear thickness and ear weight indicative of edema were also examined. rErdr1 treatment markedly reduced both ear thickness and weight, showing decreased hyper-proliferation in the epidermis and edema in the ear skin (Fig 1c and d). The effects of rErdr1 on AD were further confirmed using 2,4-dinitrochlorobenzene (DNCB)-induced AD in BABL/C mice. rErdr1 alleviated epidermis thickness and mast cell infiltration in DNCB-induced AD lesional skin (Fig S3a and S3b).

Although AD pathogenesis remains unclear, general characteristics of AD include Th2 polarization and high levels of serum IgE (Peng and Novak, 2015). To determine whether rErdr1 administration could suppress IgE and IL-4 production in the AD mouse model, levels

of IgE and IL-4 were measured. Serum IgE levels were significantly increased in mice induced to have AD that were injected with vehicle (Fig 1e). Administration of rErdr1 considerably reduced serum IgE level in the AD-induced mice, and the reduction range was similar to the dexamethasone-injected group, showing that this dose of Erdr1 has similar preventive potential to dexamethasone. Expression of IL-4 both in the lesional skin and splenocytes also increased upon AD induction, and this increase in expression was significantly smaller in the rErdr1-injected group, implying that the distribution of Th2 cells was reduced in rErdr1-injected mice (Fig 1f).

During AD pathogenesis, Th2 chemoattractants, CCL17 and CCL22, are increased to recruit the Th2 cells to AD lesional skin (Gros et al. , 2009). As shown in Fig 2a, *Tslp* mRNA expression in AD lesional skin was significantly decreased in the rErdr1-injected group compared with the vehicle-injected group. In accordance with the decreased level of *Tslp*, both the mRNA and protein levels of CCL17 and CCL22 were also downregulated by rErdr1 injection in the AD lesional skin. Keratinocytes and activated DC are known to be the main producers of CCL17 and CCL22, respectively, in AD (Soumelis, Reche, 2002, Vulcano et al. , 2001). In the vehicle-injected group, CCL17 and CCL22 were expressed primarily in the epidermis and dermis, respectively, and expression was decreased by rErdr1 injection (Fig 2b).

Next, we determined the effect of Erdr1 on angiogenesis. It has been reported that a representative angiogenic factor VEGF expression is significantly higher in the stratum corneum of AD than normal stratum corneum, suggesting that keratinocytes in AD lesional skin produce greater amounts of VEGF. Also, VEGF levels are highly correlated with AD scores, especially redness and edema (Amarbayasgalan et al. , 2012). Fig 2c shows that expression of VEGF in the lesional skin, especially keratinocytes in the epidermis, was

significantly inhibited by rErdr1 injection. CD31 immunofluorescence staining data indicated that angiogenesis was induced in the vehicle-injected group but was significantly decreased by rErdr1 injection (Fig 2d).

This study suggests that rErdr1 has preventive effects on AD. Th2 chemoattractants and IL-4 expression were decreased by rErdr1 in AD-induced mouse model. Erdr1 was previously reported to also have a therapeutic effect on psoriasis, which is a Th1-dominant skin disease (Kim, Houh, 2016b). These studies imply that rErdr1 might regulate both Th1 and Th2 responses. Recent studies have shown that several therapeutic agents improve the symptoms of AD by increasing generation of Treg. The treatment significantly increases the number of Treg cells, leading to suppression of Th1 and Th2 responses along with reduction of serum IgE in AD (Haitz and Anandasabapathy, 2015, Han et al. , 2015, Li et al. , 2016). Therefore, further investigation is needed to determine whether Erdr1 suppresses both Th1 and Th2 responses by enhancing generation of Treg.

CONFLICT OF INTREST DISCLOSURE

The authors state no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2013R1A1A2062797) and Creative Materials Discovery Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT

and Future Planning (2016M3D1A1021387).

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FIGURE LEGENDS

Figure 1. Erdr1 alleviated the characteristic clinical symptoms of atopic dermatitis (AD).

(a) Representative lesions on the ear and the back of the head from each group (N=6 /group) are shown. The sections from mouse ear tissues were stained with hematoxylin and eosin to examine histopathological features of the skin lesions 21 days after AD induction. Black arrows indicate the infiltration of eosinophils. Toluidine blue staining was used to determine the infiltration of mast cells in the lesional skin. Red arrows indicate mast cells. (Scale bar = 60 μ m)

(b) The severity of AD symptoms was evaluated based on 3-point scoring system. The AD score was graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) based on the sum of the scores of clinical symptoms, such as redness, edema, scaling and excoriation.

(c and d) Ear thickness was measured with a caliper twice a week. On day 21 after AD induction, a 0.8-cm diameter punch hole was excised from both ears of each mouse (total 12 tissues) and then weighed by using an electronic scale.

(e) On day 21, sera were collected, and the concentration of IgE was determined by an ELISA.

(f) IL-4 mRNA levels were determined using RT-PCR. To quantify the intensity of bands for IL-4 expression, the densitometry program, TotalLab100, was used. Densitometry data shows the ratio of nucleic acid intensity to the loading control. All values were normalized relative to the normal. Data shows the average of IL-4 mRNA expression from three representatives of each group (N=6 /group). To determine systemic IL-4 production, spleen was isolated from each mouse. Spleen size and percentage of CD3⁺CD4⁺ T cells within an equal number of splenocytes were assessed as shown in Fig S4A and S4B. Treatment with rErdr1 or Dex

suppressed enlargement of the spleen, however, there was no difference in T cell percentage between the groups. An equal number of cells (5×10^6) from each group was used to determine cytokine levels. The concentration of IL-4 in splenocyte-conditioned media was measured by an ELISA. Each bar represents the mean $\pm$ SD (*P < 0.05, **P < 0.01, ***P < 0.001). A representative experiment of three independent experiments is shown.

Figure 2. Erdr1 inhibited Th2 chemoattractants and angiogenesis in atopic dermatitis (AD)-induced mouse ear tissue.

(a) The relative mRNA expression levels of *Tslp*, *Ccl17*, and *Ccl22* were measured by quantitative RT-PCR analysis on mouse ear tissue from three representatives of each group (N=6 /group). Each bar represents the mean $\pm$ SD.

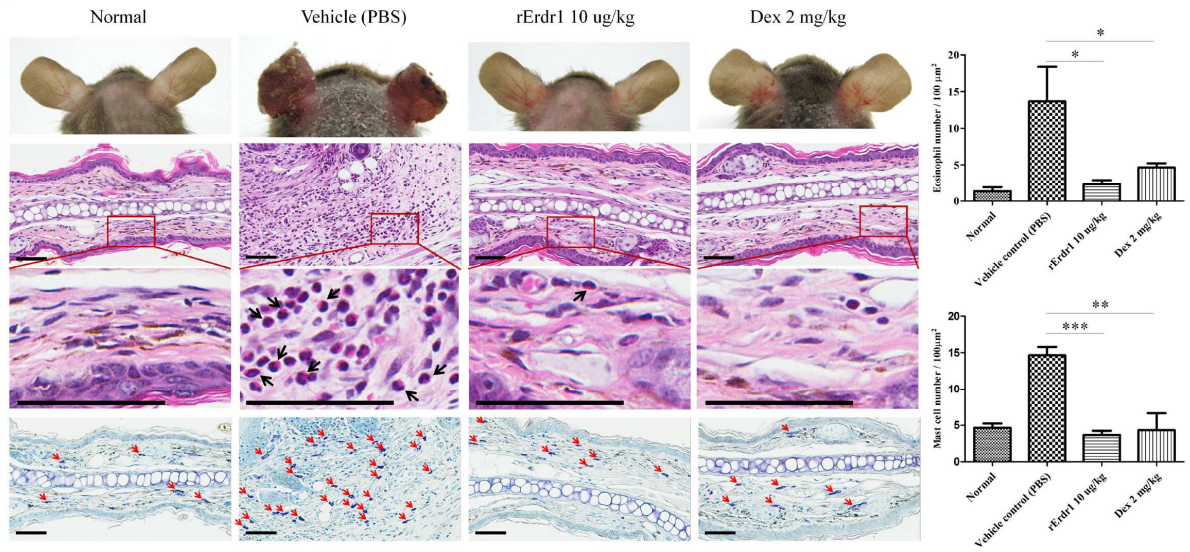
(b) Protein expression of the Th2 chemoattractants CCL17 and CCL22 were detected through immunohistochemistry (IHC). Paraffin-embedded sections were stained with rabbit anti-mouse CCL17 and CCL22 antibodies (1:1000 dilution). Rabbit IgG was used as an isotype control. CCL17 expression was highly dominant in the epidermis, and CCL22 expression was detected in the dermis (red arrows). (Scale bar = 60 μ m)

(c) The expression of VEGF was detected through IHC staining. Paraffin-embedded sections were stained with rabbit anti-mouse VEGF antibody (1:200 dilution). VEGF expression was highly dominant in the epidermis. (Scale bar = 60 μ m) In addition, the relative mRNA expression level of *veg*f was measured by quantitative RT-PCR analysis on mouse ear tissue from three representatives of each group (N=6 /group). Data are presented as the mean $\pm$ SD.

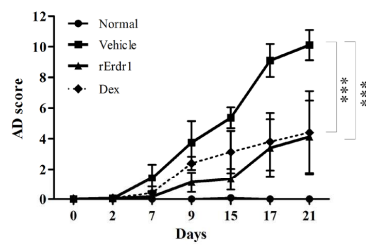
(d) Immunofluorescence determination of CD31 expression. Paraffin-embedded ear tissue sections from representatives of normal, vehicle (PBS) control, rErdr1 10 μ g/kg, and Dex (2

mg/kg) groups were stained with goat anti-mouse CD31 antibody (red) and DAPI (blue). White arrows indicate CD31⁺ expression. A representative experiment of three independent experiments is shown.

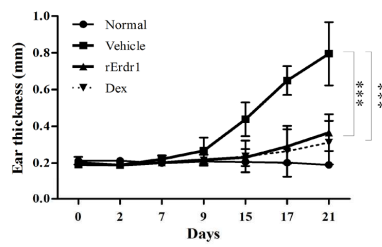
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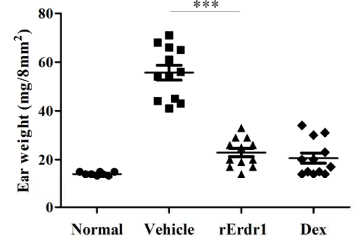
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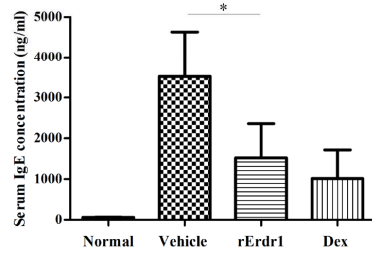
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