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Double-positive Immunoglobulin E+ and *Dermatophagoides farinae* antigen+ dendritic cells are observed in Skin Lesions of Older Adults with Atopic Dermatitis: An Immunohistological Study

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ABSTRACT

Background: The immunopathogenic role of common environmental allergens such as house dust mite (HDM) in the development of skin lesions in atopic dermatitis (AD) has not been precisely clarified.

Objectives: In the present study, we evaluated the localization of Der f1, the major antigen of *Dermatophagoides farinae*, with IgE-positive epidermal and dermal dendritic cells (DCs) in skin lesions of older patients with AD and allergic sensitization to HDMs.

Methods: Four biopsied specimens (uninvolved skin, n=1; atopy patch test-induced erythema, n=1; lichenified lesions, n=2) from patients with IgE-allergic AD were analyzed by single immunohistochemical staining, double immunofluorescence staining, and immunohistochemical re-staining.

Results: Few IgE-bearing inflammatory DCs (IgE+ CD11c+ cells) were observed in uninvolved skin. After 48-h challenge with *D. farinae* antigens, infiltrating IgE-bearing inflammatory dendritic epidermal cells (IDECs; IgE+ CD1a+ CD11c+ cells) formed a cluster in the spongiosis in atopy patch test-induced erythema. In the chronic active lichenified lesion, IgE-bearing IDECs were observed in the central area of the spongiosis. Additionally, IgE-bearing Langerhans cells (LCs) with Der f1 (IgE+ CD1a+ CD207+Der f1+ cells) were present in the peripheral area of the spongiosis associated with inflammatory infiltrating cells mainly comprising CD8+ cells. In the chronic lichenified lesion, IgE-bearing IDECs existed mostly in the lower epidermis, IgE-bearing LCs with Der f1 existed in the stratum malpighii including the area under tight junctions, and IgE-bearing IDEC-like dermal DCs with Der f1 (IgE+ CD1a- or CD1a+/- CD11+ Der f1+ cells) existed in the upper dermis.

Conclusion: These results suggest that HDM allergens such as Der f1 are involved in the immunopathogenesis of eczematous dermatitis of IgE-allergic AD, in which IgE-associated delayed-type hypersensitivity may develop with the collaboration of IgE-bearing DCs and T cells.

Keywords: Der f1, Dermal dendritic cells, Immunopathogenesis, Inflammatory dendritic epidermal cells, Langerhans cells, Spongiosis

INTRODUCTION

The pathology of atopic dermatitis (AD) is complex [1,2], and the immunopathogenic role of common environmental allergens such as house dust mite (HDM) in the development of skin lesions in AD has not been precisely clarified. Epidermal and dermal dendritic cells (DCs), e.g., Langerhans Cells (LCs), Inflammatory Dendritic Epidermal Cells (IDECs), and Inflammatory DCs, are thought to be the principal antigen-presenting cells in AD which initiate and sustain cellular infiltrates composed of allergen-specific

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T cells and other effector and regulatory cells. LCs are defined as epidermal DCs that contain Birbeck granules and mainly express cluster of differentiation (CD) 1a and CD207 (langerin) antigens. IDECs are defined as epidermal inflammatory DCs that do not contain Birbeck granules and mainly express CD1a, CD11b, CD11c, and CD206 antigens [3]. Both LCs and IDECs are classified as subsets of myeloid DCs. Inflammatory DCs are composed of two subsets, myeloid (CD11c+ CD123-) DCs and plasmacytoid (CD11c- CD123+) DCs [4,5]. LCs, IDECs, and inflammatory DCs have major histocompatibility complex class I and II molecules and can become immunoglobulin (Ig) E-bearing DCs with complexes of IgE and high-affinity receptor FcεRI in the major form of AD with IgE allergy (hereafter, IgE-allergic AD) [1-7]. It has been considered that IgE-bearing DCs can capture large amounts of allergens and may cause IgE-associated delayed-type hypersensitivity reactions in AD [8].

In the present study, we demonstrate the localization of Der f1 antigens, one of the major allergens of HDMs (*Dermatophagoides farinae*), is associated with IgE-bearing epidermal and dermal DCs including LCs and IDECs in skin lesions of older patients with AD and allergic sensitization to HDMs. We also discuss a possible role of HDM allergens in the pathomechanism of eczematous dermatitis in IgE-allergic AD.

SUBJECTS AND METHODS

Skin samples and Subjects

Skin biopsy specimens were obtained from chronic lichenified lesions of two patients (Cases 1 and 2), and atopy patch test (APT)-induced infiltrated erythema (48h after APT) and uninvolved skin on the upper back of Case 1. Brief clinical reports of Cases 1 and 2 have been described in our previous reports [9,10]. AD was diagnosed according to the clinical criteria of Hanifin and Rajka [11]. Based on positive findings of Der f1 antigens in our preliminary immunohistological studies performed in six patients (adult, 1; older, 5) with IgE-allergic AD, we selected skin samples of Cases 1 and 2 for detailed analyses.

A biopsy was performed on the lichenified lesions with therapy-free interval of topical corticosteroids for a few weeks in Case 1 and without therapy-free interval of topical corticosteroids (medium-class) in Case 2. As a control, a skin sample was obtained from an older patient with eosinophilic granulomatosis with polyangiitis.

AD patients and the control subject all provided written informed consent for every biopsy and research use of all specimens. The study protocols were approved by the Ethics Committee of the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology (No. R15-42).

Histological, immunohistochemical and double immunofluorescence staining

Single immunohistochemical staining was performed using the streptavidin-biotin method with an LSAB kit (Dako, Tokyo, Japan). Double immunofluorescence staining was performed as previously described [10]. The streptavidin-fluoresce in conjugates used were DyLight488 streptavidin (SA-5488; Vector, Burlingame, CA, USA) and DyLight594 streptavidin (SA-5594; Vector). Double immunofluorescence stained sections were viewed under fluorescence microscopy (BIOREVO BZ-9000; Keyence, Osaka, Japan). Double immunofluorescence staining was performed with anti-IgE and anti-CD11c monoclonal antibodies (mAbs), anti-IgE mAbs and anti-Derf1 polyclonal antibodies (pAbs), and anti-CD1a mAbs and anti-Derf1 pAbs. Immunohistochemical re-staining with anti-CD1a or anti-CD207 mAbs was carried out after image recording of double immunofluorescence staining. For specificity enhancement, immunohistochemical re-staining was carried out after deactivation of the secondary antibodies in double immunofluorescence staining by using a citric acid buffer in most sections.

The following primary mAbs and pAbs were applied: mouse mAbs against CD4 (helper/inducer T cells, #713181; Nichirei, Tokyo, Japan), CD8 (cytotoxic/suppressor T cells, #713201; Nichirei), CD68 (macrophages, N1576; Dako), CD1a (LCs/DCs, NCL-CD1a-235; Novocastra, Newcastle, UK), CD207 (LCs, ABIN1027332; antibodies-online.com, Atlanta, GA, USA), mast cell tryptase (mast cells, AA1; Abcam, Tokyo, Japan), and IgE ε-chain (IgE; MH25-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit mAbs to CD11c (dermal/epidermal DCs, EP1347Y; LSBio, Seattle, WA, USA); and rabbit pAbs to Der f1 (mite Der f1, LB-7111; Cosmo Bio, Tokyo, Japan). Dilution ratios for primary antibodies were as follows: 1:30 for anti-CD1a; 1:250 for anti-CD11c; 1:200 for anti-IgE; and 1:1000 for anti-Der f1. The other primary antibodies were applied as ready to use.

RESULTS

Case presentations

Case 1

An 84-year-old Japanese man had chronic eczema from his 50s and exteriorization of a pathological state of AD from his 60s. He showed lichenified eczema on the face, neck, upper trunk, and upper extremities. Laboratory data indicated the following: white blood cell count of 5,140/mm³ with 7% eosinophils, total IgE of 19,757 IU/ml, and specific IgEs for *D. farinae* as class 5 (120–159 lumi count) detected in multiple antigen simultaneous test (MAST)-33 (BML, Tokyo, Japan) [9]. APT using *D. farinae* allergen extracts (Torii, Tokyo, Japan) indicated positive results as erythema and infiltration (single positive using criteria for reading APT [12]) on the uninvolved skin of his upper back.

Case 2

A 71-year-old Japanese man who had a 10-year history of AD was referred to our department. He had a history of classic childhood AD until 5 years of age. He presented with atopic red face and diffuses lichenified eczema on the trunk and extremities. Laboratory data showed the following: white blood cell count of 7,130/mm³ with 14% eosinophils, total IgE of 2,413 IU/ml, and specific IgEs for *D. farinae* detected inMAST-26 (BML) as class 3 (20.1–99.9 lumi count) [10].

Control case

A 78-year-old Japanese man who had purpuric skin lesions on the legs and respiratory manifestations of eosinophilic granulomatosis with polyangiitis was referred to our department. He showed an elevated serum total IgE level of 3,007 IU/ml and weak positivity for specific IgEs for *D. farinae*.

Immunohistological analyses

Results of immunohistochemical and double immunofluorescence studies are summarized in **table 1**.

Table 1. Summary of the results of immunohistological studies

Case No.	Clinical and histological features of skin samples	Findings of single immunohistochemical staining, double immunofluorescence staining, and immunohistochemical re-staining by using paraffin and/or frozen sections				
		Histopathology and immunohistopathology	Double immunofluorescence staining with anti-IgE and anti-CD11c mAbs	Double immunofluorescence staining with anti-IgE mAbs and anti-Der f1 [†] pAbs	Double immunofluorescence staining with anti-CD1a mAbs and anti-Der f1 pAbs	Immunohistochemical re-staining with anti-CD1a mAbs or anti-CD207 mAbs
1	Uninvolved skin	Almost normal histology with no obvious inflammatory infiltration: the distribution of IgE+ cells in the upper dermis was similar to that of tryptase+ mast cells	Small numbers of single-positive IgE+ cells with dendritic morphology and regular distributions were observed in the epidermis Single-positive IgE+ cells were observed in the upper dermis	Double-positive IgE+ Der f1+ cells were not observed.	Double-positive CD1a+ Der f1+ cells were not observed	IgE+ cells in the epidermis were positive for CD1a antigens The numbers of epidermal IgE+ CD1a+ cells (i.e., IgE-bearing LCs) were much lower than those of IgE-CD1a+ cells (i.e., LCs without IgE expression)
1	Atopy patch test-positive acute lesion	Mild spongiotic dermatitis: aggregated IgE+ CD11c+ CD1+ cells (i.e., IgE-bearing IDECs) were observed in the central area of the spongiosis and in the subcorneal	A few infiltrating double-positive IgE+ CD11c+ cells were observed in the upper dermis and small numbers of aggregated IgE+ CD11c+/- cells were seen in the spongiotic epidermis	Small numbers of double-positive IgE+ Der f1+ cells with dendritic morphology were observed in the spongiotic epidermis and the upper dermis	Double-positive CD1a+ Der f1+ cells were observed in the spongiotic epidermis	Double-positive IgE+ CD11c+ cells in the upper dermis and aggregated IgE+ CD11c+/- cells in the spongiosis were positive for CD1a antigens Double-positive IgE+ Der f1+ cells in the spongiotic epidermis were mostly positive for

		epidermis of the spongiosis				CD1a and CD207 antigens A few IgE+ CD207+ Der f1+ cells (i.e., IgE-bearing LCs with Der f1) existed adjacent to the spongiosis, but not in the central area of the spongiosis
2	Chronic active lichenified lesion	Chronic active lesion of eczematous dermatitis; acanthosis with focal spongiosis in the epidermis and inflammatory cell infiltrations in the upper dermis Cellular infiltrations mainly composed of CD8+ cells were observed in the lower to middle areas of the spongiosis	Double-positive IgE+ CD11c+ cells with dendritic morphology infiltrated to the central area of the spongiosis Infiltrating double-positive IgE+ CD11c+ cells were mainly observed in the upper dermis in areas without the spongiotic epidermis	Double-positive IgE+ Der f1+ cells were observed in the lower to middle areas of the epidermis, especially in the peripheral area of a spongiotic formation, but not in the central area of the spongiosis	Double-positive CD1a+ Der f1+ cells were also observed in the peripheral area of the spongiotic formation and some double-positive CD1a+ Der f1+ cells co-localized with IgE+ Der f1+ cells	Double-positive IgE+ Der f1+ cells in the epidermis were positive for CD1a antigens Double-positive CD1a+ Der f1+ cells that co-localized with IgE+ Der f1+ cells in the peripheral area of the spongiotic formation were positive for CD207 antigens (i.e., IgE-bearing LCs with Der f1 were present in the peripheral area of the spongiosis) Double-positive IgE+ CD11c+ cells in the central area of the spongiosis were positive for CD1a antigens (i.e., IgE-bearing IDECs were present in the central area of the spongiosis) The majority of infiltrating double-positive IgE+ CD11c+ cells in the upper dermis were negative for CD1a antigens
1	Chronic lichenified lesion	Chronic eczematous reaction with no focal spongiotic	Double-positive IgE+ CD11c+ cells were mainly observed in	Small numbers of double-positive IgE+ Der f1+ cells were observed	Small numbers of double-positive CD1a+ Der f1+ cells were observed	Infiltrating double-positive IgE+ CD11c+ cells in the upper dermis were mostly negative for

		change	the upper dermis Double-positive IgE+ CD11c+ cells were infrequently observed in the lower epidermis	both in the epidermis and upper dermis The majority of double-positive IgE+ Der f1+ cells co-localized with double-positive IgE+ CD11c+ cells in the upper dermis (i.e., IgE-bearing inflammatory dermal DCs with Der f1)	both in the epidermis and upper dermis	CD1a antigens Most double-positive IgE+ Der f1+ cells in the epidermis were positive for CD1a and CD207 antigens (i.e., IgE-bearing LCs with Der f1), whereas most double-positive IgE+ Der f1+ cells in the upper dermis were negative for CD1a and CD207 antigens Some IgE+ CD207+ Der f1+ cells (i.e., IgE-bearing LCs with Der f1) that existed in the area under the TJs had elongated dendrites into the TJs
Control	Cutaneous vasculitis	Leukocytoclastic vasculitis: IgE+ cells in the upper dermis demonstrated similar distribution to tryptase+ mast cells	A few single-positive IgE+ cells with dendritic morphology were observed in the epidermis Single-positive IgE+ cells were observed in the upper dermis	Double-positive IgE+ Der f1+ cells were not observed	NT	IgE+ cells in the epidermis were positive for CD1a antigens

Abbreviations: CD, cluster of differentiation; DCs, dendritic cells; IDECs, inflammatory dendritic epidermal cells; IgE, immunoglobulin E; LCs, Langerhans cells; mAbs, monoclonal antibodies; NT, not tested; pAbs, polyclonal antibodies; and TJs, tight junctions † Der f1 antigens: one of the major allergens of *Dermatophagoides farinae*

Uninvolved skin in Case 1

Paraffin sections: A biopsy specimen of the uninvolved skin of the older AD patient demonstrated almost normal histology with no obvious inflammatory infiltration. Immunostaining of serial sections showed IgE+ cells, with a similar distribution to tryptase+ mast cells, evenly distributed throughout the dermis. Few IgE+ cells were observed in the epidermis in paraffin sections.

Frozen sections: In double immunofluorescence stained sections with anti-IgE and anti-CD11c mAbs, few double-positive IgE+ CD11c+ cells were observed in the uninvolved skin. IgE+ cells were observed both in the epidermis and the upper dermis, however, few CD11c+ cells were seen in the upper dermis. Single-positive IgE+ cells in the epidermis showed dendritic morphology and even distribution (**Figure 1a**). Immunohistochemical re-staining indicated that single-positive IgE+ cells in the epidermis were strongly positive for CD1a antigens, however, the numbers of IgE+CD1a+

cells were much fewer than those of IgE-CD1a+ cells (**Figure 1a, 1b**). In sections stained with anti-IgE mAbs and anti-Derf1 pAbs and anti-CD1a mAbs and anti-Derf1 pAbs, neither double-positive IgE+ Derf1+ cells nor double-

positive CD1a+ Derf1+ cells were observed in the epidermis and dermis in the uninvolved skin.

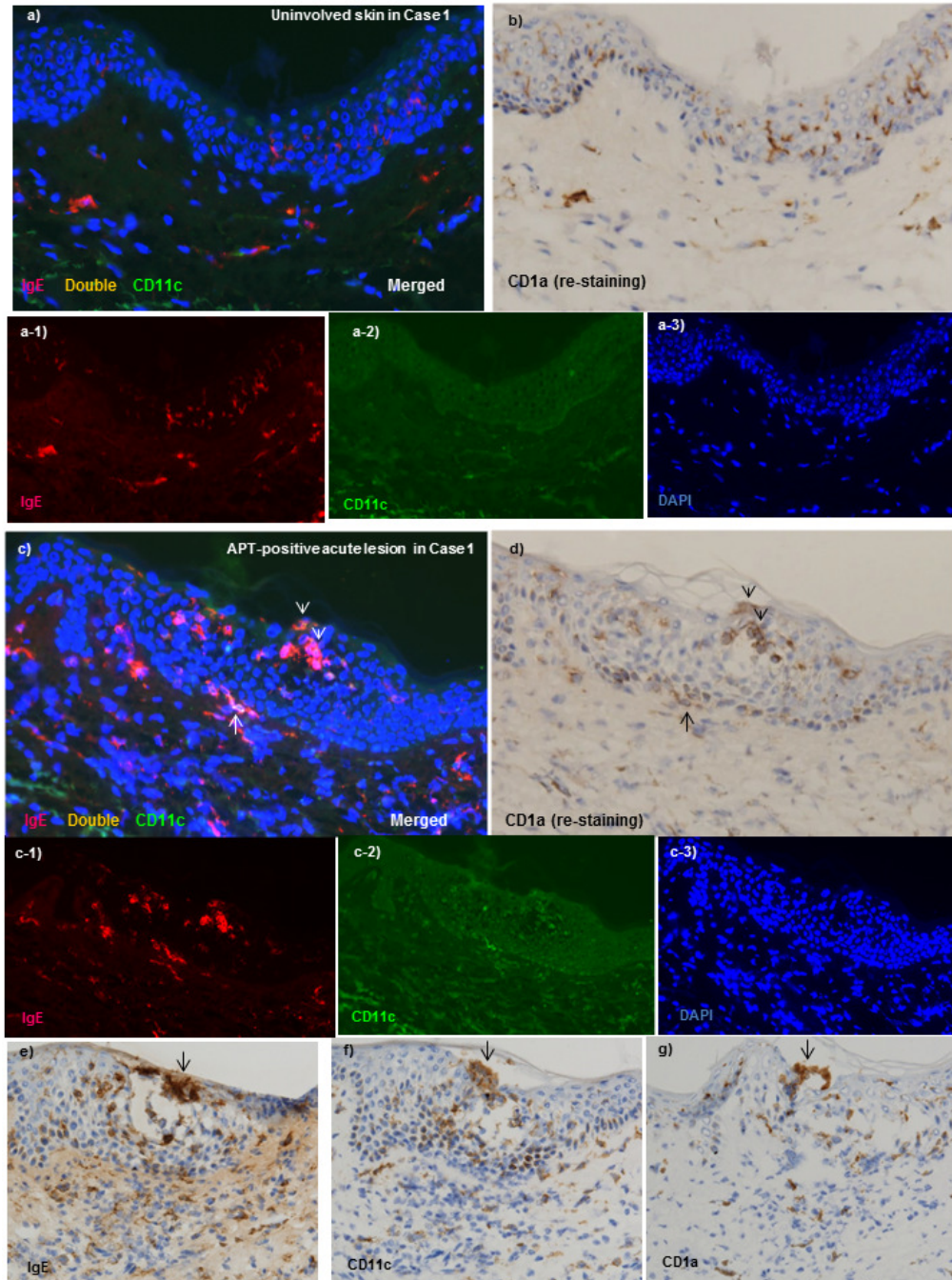


Figure 1. Uninvolved skin and atopy patch test (APT)-positive acute lesion in Case 1.

Double immunofluorescence staining: **a** and **c**; immunohistochemical re-staining: **b** and **d**; and single immunohistochemical staining: **e-g**. In immunohistochemical staining, sets of figures (**e-g**) represent serial sections.

- a)** Few double-positive IgE+ CD11c+ cells are present in the uninvolved skin. Single-positive IgE+ cells in the epidermis exhibit dendritic morphology and regular distributions.
- b)** Single-positive IgE+ cells in the epidermis are strongly positive for CD1a antigens.

- c) A double-positive IgE+ CD11c+ cell (yellow image) in the upper dermis (arrow) and aggregated IgE+ CD11c+/- (weak) cells (arrowheads) in the spongiosis in the APT-positive acute lesion.
- d) The double-positive IgE+ CD11c+ cell in the upper dermis (arrow) and aggregated IgE+ CD11c+/- (weak) cells (arrowheads) in the spongiosis are strongly positive for CD1a antigens.
- e) Numerous IgE+ cells in inflammatory infiltrating cells in the spongiotic epidermis and the upper dermis in the APT-positive acute lesion. A cluster of aggregated IgE+ cells is observed in the upper area of the spongiosis (arrow).
- f) Infiltrating CD11c+ cells are observed in the spongiotic epidermis and the upper dermis in the APT-positive acute lesion. A cluster of aggregated CD11c+ cells is also observed in the upper area of the spongiosis (arrow).
- g) A cluster of aggregated CD1a+ cells is also observed in the upper area of the spongiosis (arrow) with sporadic distribution of CD1a+ cells in the spongiotic epidermis and the upper dermis in the APT-positive acute lesion.

Note, in **e-g**, clusters of IgE+ cells, CD11c+ cells, and CD1a+ cells in the upper area of the spongiosis show the same morphology and localization (arrows).

Nuclei are labeled with 4', 6-diamidino-2-phenylindole (DAPI, blue images). Original magnification: 200×, **a-g**.

APT-positive acute lesion in Case 1

Paraffin sections: A biopsy specimen of the APT-positive acute lesion of the older AD patient showed a histopathology of mild spongiotic dermatitis. Immunostaining showed inflammatory cell infiltrations with IgE+ cells in the upper dermis. The majority of IgE+ cells co-localized with tryptase+ mast cells in serial sections. Small numbers of IgE+ cells, CD4+ cells and CD8+ cells were observed in the lower epidermis in association with a spongiotic formation.

Frozen sections: In sections stained with anti-IgE and anti-CD11c mAbs, a few infiltrating double-positive IgE+ CD11c+ cells were observed in the upper dermis, and small numbers of IgE+ CD11c+/- (weak) cells, which showed lower immunofluorescence intensity for CD11c antigens, were seen in the spongiotic epidermis (**Figure 1c**). Immunohistochemical re-staining indicated that double-positive IgE+ CD11c+ cells in the upper dermis and IgE+ CD11c+/- cells in the spongiotic epidermis were positive for CD1a antigens (**Figure 1d**), and IgE+ CD11c+/- CD1a+ cells aggregated in the spongiosis (**Figure 1c,d**). Additionally, comparative analysis of single immunohistochemical stained serial sections revealed that infiltrating IgE+ cells in the spongiosis showed the same morphology and distribution of CD11c+ cells and CD1a+ cells as aggregated cells in the spongiosis. Moreover, the aggregation of IgE+ CD11c+ CD1a+ cells observed in the subcorneal epidermis might indicate the possibility of transepidermal elimination of those cells (**Figure 1e-1g**).

In sections stained with anti-IgE mAbs and anti-Derf1 pAbs, small numbers of double-positive IgE+ Derf1+ cells with dendritic morphology were infrequently observed in the spongiotic epidermis and the upper dermis. Immunohistochemical re-staining demonstrated that double-positive IgE+ Derf1+ cells in the epidermis were mostly positive for CD1a and CD207 antigens (**Figure 2a-2d**). A few IgE+ CD207+Derf1+ cells were assembled focally or existed adjacent to the spongiosis in the epidermis, however, no IgE+ CD207+ Der f1+ cells were seen in the central area of the spongiosis (**Figure 2a-2d**). Only a few IgE+ CD207-

Derf1+ cells were observed in the epidermis. In addition, double immunofluorescence staining with anti-CD1a mAbs and anti-Derf1 pAbs also revealed the presence of double-positive CD1a+ Derf1+ cells in the spongiotic epidermis.

Taking together, the aforementioned results suggest that IgE+ cells in the spongiotic epidermis, which respond to *D. farinae* antigens, are chiefly composed of two types of DCs: IgE+CD11c+CD1a+ cells, i.e., IgE-bearing IDECs, and IgE+CD1a+ CD207+ cells, i.e., IgE-bearing LCs that mostly present with Der f1. From the different localization patterns of the two types of DCs, i.e., IgE-bearing IDECs that mainly infiltrated in the central area of the spongiosis and IgE-bearing LCs with Der f1 that existed adjacent to the spongiosis, these DCs might play different roles in the development of spongiotic dermatitis in the APT-positive acute lesion in Case 1.

Chronic active lichenified lesion in Case 2

Paraffin sections: The lichenified skin of Case 2 showed a chronic active lesion of eczematous dermatitis, which had acanthosis with focal spongiosis in the epidermis and inflammatory cell infiltrations in the upper dermis. Single immunohistochemical staining revealed dermal infiltration of inflammatory cells, mainly comprising CD4+ cells, CD8+ cells, CD68+ cells, tryptase+ mast cells, and IgE+ cells. Comparative analysis of serial sections revealed that many infiltrating IgE+ cells displayed the same localization pattern as tryptase+ mast cells, however, the numbers of IgE+ cells were more numerous. IgE+ cells were not observed in the epidermis of this case in paraffin sections [10]. In the epidermis, epidermal CD1a+ cells were observed focally in the central area of the spongiosis and scattered throughout the stratum spinosum of the epidermis. Cellular infiltrations mainly composed of CD8+ cells were observed in the lower to middle areas of the spongiosis (**Figure 3a-3d**).

Frozen sections: In double immunofluorescence stained sections with anti-IgE and anti-Derf1 mAbs, a few double-positive IgE+ Der f1+ cells were observed in the lower to middle areas of the epidermis, especially in the peripheral

area of a spongiotic formation, but not in the central area of the spongiosis (**Figure 4a**).

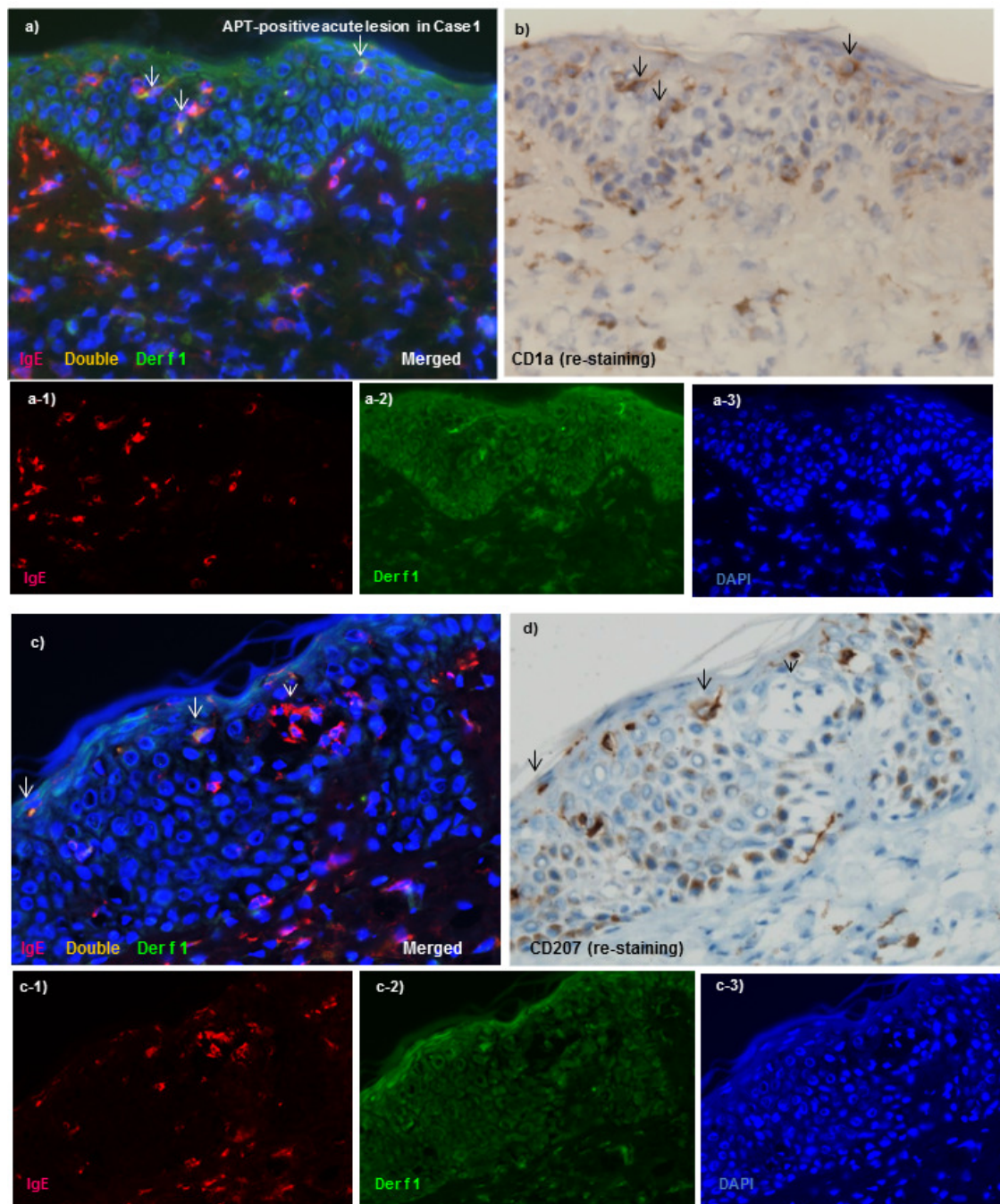


Figure 2. Atopy patch test (APT)-positive acute lesion in Case 1.

Double immunofluorescence staining: **a** and **c**; and immunohistochemical re-staining: **b** and **d**.

a) Double-positive IgE+Der f1+ cells (yellow images) in the epidermis (arrows).

b) Double-positive IgE+ Der f1+ cells (arrows) in the epidermis are strongly positive for CD1a antigens.

c) Double-positive IgE+ Der f1+ cells (yellow images) in the epidermis (arrows) next to the spongiosis.

d) Double-positive IgE+ Der f1+ cells (arrows) in the epidermis are also positive for CD207 antigens.

Note, in **c** and **d**, clusters of IgE+ Der f1- cells (arrowheads) in the central area of spongiosis are negative for CD207 antigens.

Nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI, blue images). Original magnification: 400×, **a** and **b**; 200×, **c** and **d**.

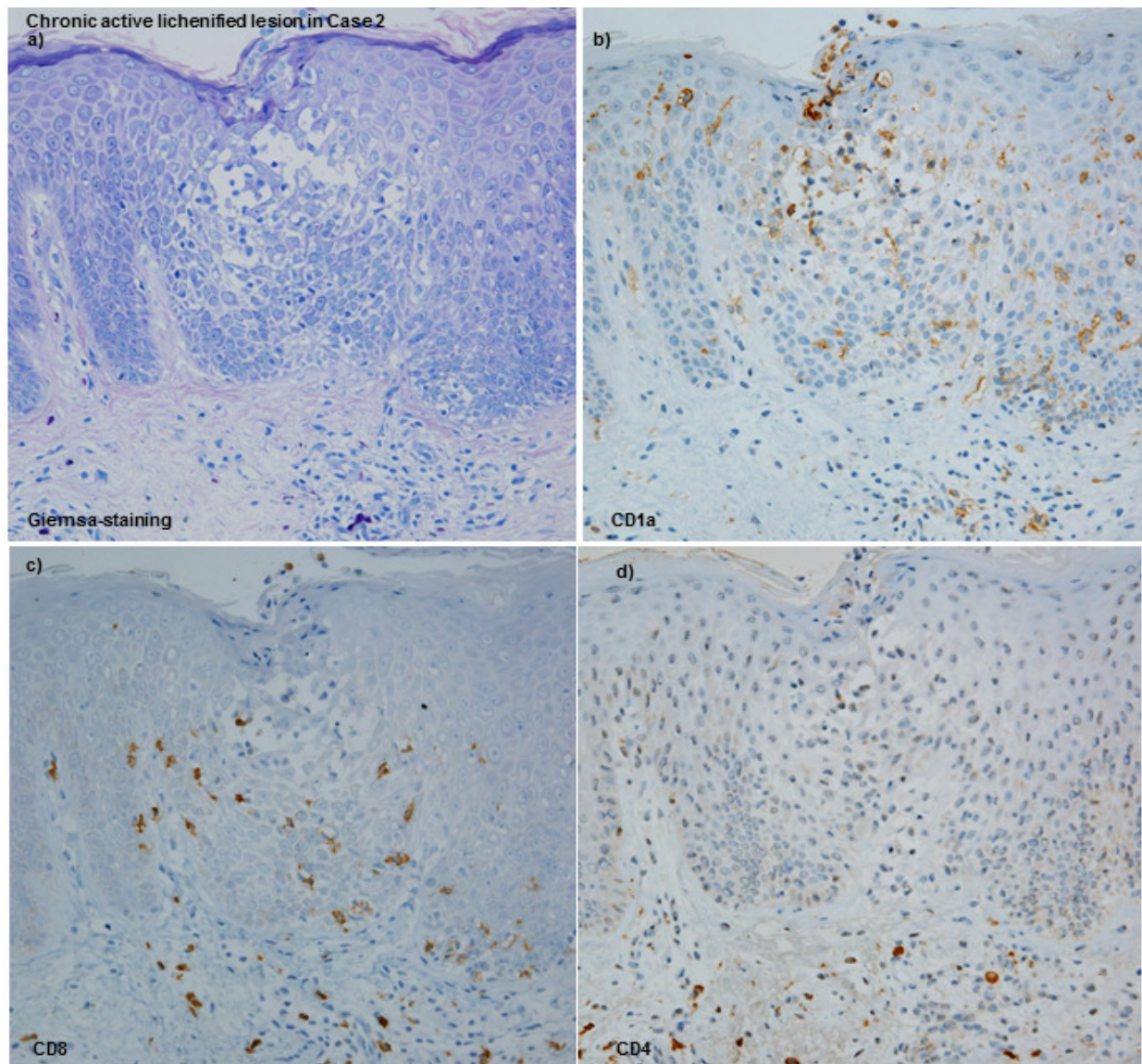


Figure 3. Chronic active lichenified lesion in Case 2.

Single immunohistochemical staining using serial paraffin sections: **a-d**.

- a)** A spongiotic formation in a chronic active lichenified lesion. Densely stained mast cells by Giemsa staining in the upper dermis seem to be unrelated to the spongiotic formation.
- b)** Epidermal CD1a+ cells are observed focally in the central area of the spongiosis and scattered throughout the stratum spinosum of the epidermis.
- c)** Infiltrating CD8+ cells are observed in the lower to middle areas of the spongiosis.
- d)** Few CD4+ cells are observed in the spongiosis in the paraffin section.

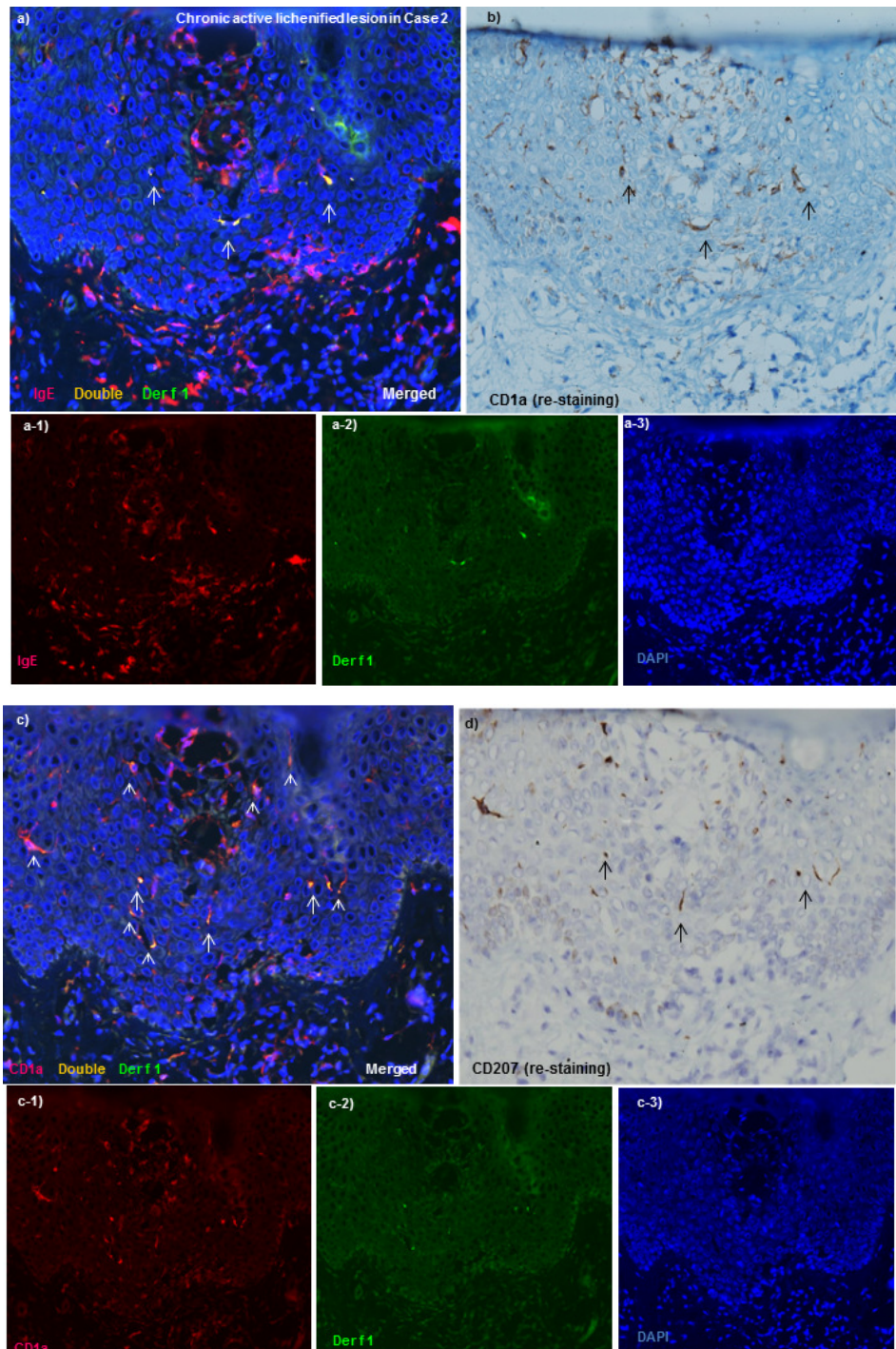
Original magnification: 200×, **a-d**.

Immunohistochemical re-staining indicated that double-positive IgE+ Der f1+ cells were strongly positive for CD1a antigens (**Figure 4b**). The majority of IgE+ Der f1- cells in the central area of the spongiosis were also positive for CD1a antigens (**Figure 4a,4b**). In addition, double

immunofluorescence staining with anti-CD1a mAbs and anti-Derf1 pAbs demonstrated that some double-positive CD1a+ Der f1+ cells co-localized with IgE+ Der f1+ cells (**Figure 4a,4c**). Furthermore, immunohistochemical re-staining indicated that double-positive CD1a+ Der f1+ cells were strongly positive for CD207 antigens (**Figure 4c,4d**).

Therefore, it was evident that IgE+ CD1a+ CD207+Der f1+ cells, i.e., IgE-bearing LCs with Der f1antigens, existed in the peripheral area of the spongiosis (**Figure 4a-4d**). The approximate numbers of double-positive cells per microscopic field (200×) in areas with the highest numbers of positive cells among four consecutively evaluated fields were determined as follows. In the area with spongiosis, 4

IgE+ Der f1+ cells in the epidermis and 0 in the upper dermis were observed and 11 CD1a+ Der f1+ cells in the epidermis and 1 in the upper dermis were observed. In the area without spongiosis, 2 IgE+ Der f1+ cells in the epidermis and 0 in the upper dermis were observed and 3 CD1a+ Der f1+ cells in the epidermis and 1 in the upper dermis were observed.



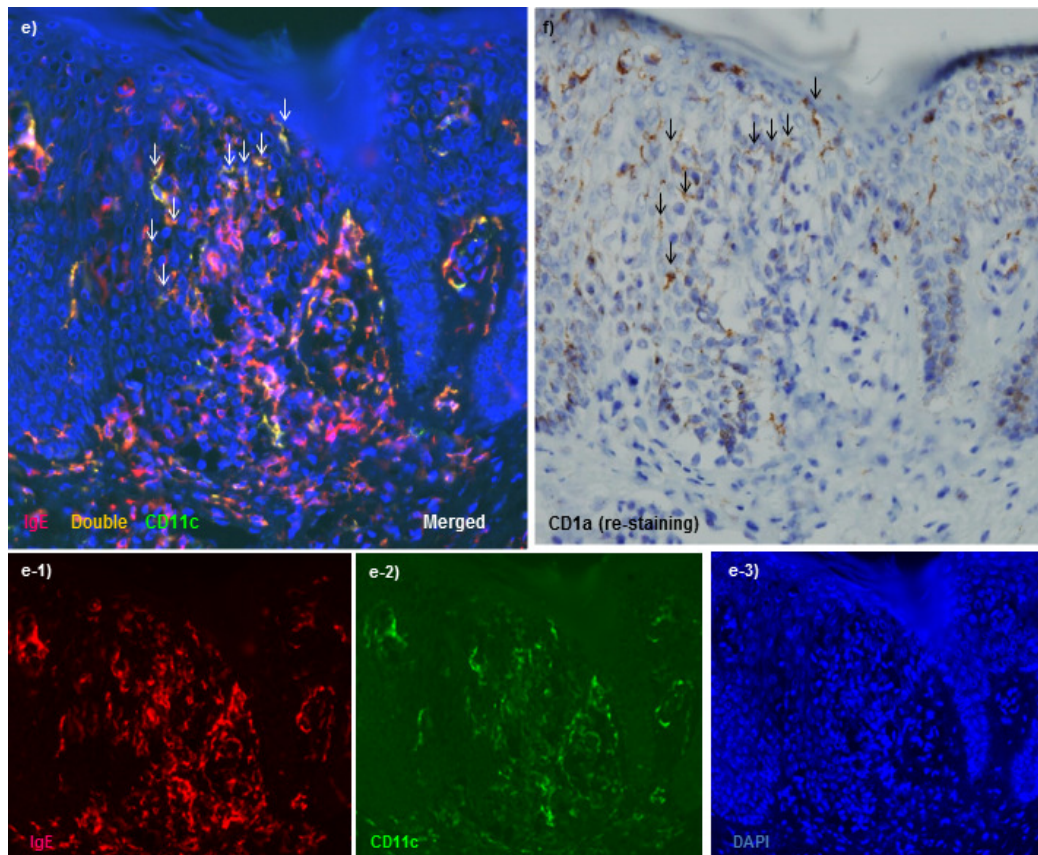


Figure 4. Chronic active lichenified lesion in Case 2.

Double immunofluorescence staining using serial frozen sections: **a**, **c**, and **e**; and immunohistochemical re-staining: **b**, **d**, and **f**. Sets of figures (**a** and **c**; and **b** and **d**) represent serial sections.

- a)** Double-positive IgE+ Der f1+ cells (yellow images) are observed in the peripheral area of a spongiotic formation (arrows).
b) Double-positive IgE+ Der f1+ cells in the epidermis are strongly positive for CD1a antigens (arrows).
c) Double-positive CD1a+ Der f1+ cells (yellow images) are also observed in the peripheral area of the spongiotic formation (arrows and arrowheads).
d) Double-positive CD1a+ Der f1+ cells that co-localize with IgE+ Der f1+ cells in the peripheral area of the spongiotic formation are strongly positive for CD207 antigens (arrows).
e) Double-positive IgE+ CD11c+ cells with dendritic morphology (yellow images) infiltrate to the central area of the same spongiosis (arrows).

f) Double-positive IgE+ CD11c+ cells in the central area of the spongiosis are positive for CD1a antigens (arrows). However, the majority of infiltrating double-positive IgE+ CD11c+ cells in the upper dermis are negative for CD1a antigens.

Note, in sections **a** and **c**, some CD1a+ Der f1+ cells co-localize with IgE+ Der f1+ cells (arrows). In sections **a-d**, the horny layer is slightly bent by artifact.

Nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI, blue images). Original magnification: 200×, **a-f**.

Double immunofluorescence staining with anti-IgE and anti-CD11c mAbs revealed the following results. Infiltrating double-positive IgE+ CD11c+ cells with dendritic morphology were prominently observed in the central area of the spongiosis (**Figure 4e**). Infiltrating double-positive IgE+ CD11c+ cells were also observed in the parakeratotic horny layer above the spongiosis. In areas without a spongiotic epidermis, a few IgE+ CD11c+ cells and IgE+ CD11c- cells, which exhibited dendritic morphology, were

also observed in the lower epidermis, although infiltrating double-positive IgE+ CD11c+ cells were mainly observed in the upper dermis in those areas [10]. Immunohistochemical re-staining indicated that double-positive IgE+ CD11c+ cells and single-positive IgE+ cells in the epidermis were positive or weakly positive for CD1a antigens (**Figure 4e, 4f**). The majority of infiltrating double-positive IgE+ CD11c+ cells in the upper dermis was negative for CD1a antigens, although a few double-positive IgE+ CD11c+ cells positive for CD1a antigens certainly existed. Many IgE- CD11c-

CD1a+ DCs, which showed strong staining for CD1a antigens, were present in the upper epidermis. Consequently, infiltrating IgE+ CD1a+ CD11c+ cells into the spongiotic epidermis in the chronic active lichenified lesion of the AD patient were thought to be IgE-bearing IDECs. Single immunohistochemical stained serial sections revealed cellular infiltration of CD8+ and CD4+ cells into the area with spongiosis.

Taken together, the aforementioned results demonstrate that in the spongiotic epidermis of the chronic active lichenified lesion in Case 2, IgE-bearing IDECs mostly infiltrated the central area of the spongiosis. IgE-bearing LCs with Der f1 antigens were mainly present in the peripheral area of the spongiosis, in which cellular infiltrations mainly composed of CD8+ cells were also observed.

Chronic lichenified lesion in Case 1

Paraffin sections: The lichenified skin of Case 1 showed a chronic eczematous reaction with no focal spongiotic change. Single immunohistochemical staining with a comparative analysis of serial sections revealed the characteristics of inflammatory infiltrating cells as similar to those observed in the chronic lichenified lesion in Case 2.

Frozen sections: The findings of double immunofluorescence staining with anti-IgE and anti-CD11c mAbs and immunohistochemical re-staining with CD1a mAbs were similar to the findings of areas without spongiotic formation in Case 2 (**Figure 5a,5b**). IgE+CD11c+ cells were infrequently observed in the lower (especially basal) epidermis. In double immunofluorescence sections stained with anti-IgE mAbs and anti-Derf1 pAbs, small numbers of double-positive IgE+ Der f1+ cells were observed both in the epidermis and upper dermis (**Figure 5c**). Immunohistochemical re-staining indicated that double-positive IgE+ Derf1+ cells in the epidermis were strongly positive for CD1a antigens, whereas most double-positive IgE+ Derf1+ cells in the upper dermis were negative for CD1a antigens (**Figure 5c,5d**). In addition, most double-positive IgE+ Derf1+ cells in the stratum malpighii, but not in the upper dermis, were also positive for CD207 antigens. Some IgE+ CD207+Derf1+ cells that existed in the area under the tight junctions (TJs; physical barriers at the stratum granulosum layer of the epidermis) had elongated dendrites into the TJs (**Figure 5e,5f**). Double immunofluorescence staining with anti-CD1a mAbs and anti-Derf1 pAbs revealed that small numbers of double-positive CD1a+ Derf1+ cells were also observed both in the epidermis and in the upper dermis. The approximate numbers of double-positive cells per microscopic field (200×) in areas with the highest numbers of positive cells among four consecutively evaluated fields were as follows: 4 IgE+ Derf1+ cells in the epidermis and 12 in the upper dermis, and 6 CD1a+ Derf1+ cells in the epidermis and 6 in the upper dermis. Comparative analysis of double immunofluorescence stained serial sections with anti-IgE

and anti-CD11c mAbs and anti-IgE mAbs and anti-Derf1 pAbs demonstrated that the majority of double-positive IgE+ Derf1+ cells co-localized with double-positive IgE+ CD11c+ cells in the upper dermis (**Figure 5g, 5h**).

Taken into consideration the aforementioned results, in the chronic lichenified lesion in Case 1, double-positive IgE+Der f1+ cells in the epidermis are mostly composed of IgE+CD1a+CD207+ cells, i.e., IgE-bearing LCs. Regarding double-positive IgE+ Der f1+ cells in the upper dermis, the precise phenotype was incomplete, however, the majority of infiltrating IgE+ Der f1+ cells in the upper dermis might be IgE+ CD1a- CD11c+cells, i.e., a subset of IgE-bearing inflammatory DCs that might differ from IgE-bearing IDECs [5].

Control case of eosinophilic granulomatosis with polyangiitis

Paraffin sections: A biopsy specimen of a purpuric skin lesion of an older patient with eosinophilic granulomatosis with polyangiitis showed leukocytoclastic vasculitis. Immunostaining of serial sections showed moderate numbers of IgE+ cells, with similar distribution to tryptase+ mast cells, in the upper dermis.

Frozen sections: Double immunofluorescence sections stained with anti-IgE and anti-CD11c mAbs and subsequent immunohistochemical re-staining with anti-CD1a mAbs revealed the following results. Single-positive IgE+ cells were observed mainly in the upper dermis and slightly in the epidermis, however, few CD11c+ cells were seen in the upper dermis. Single-positive IgE+ cells in the epidermis showed dendritic morphology and were positive for CD1a antigens. In double immunofluorescence sections stained for anti-IgE mAbs and anti-Derf1 pAbs, no double-positive IgE+ Der f1+ cells were observed in the vasculitis skin lesion.

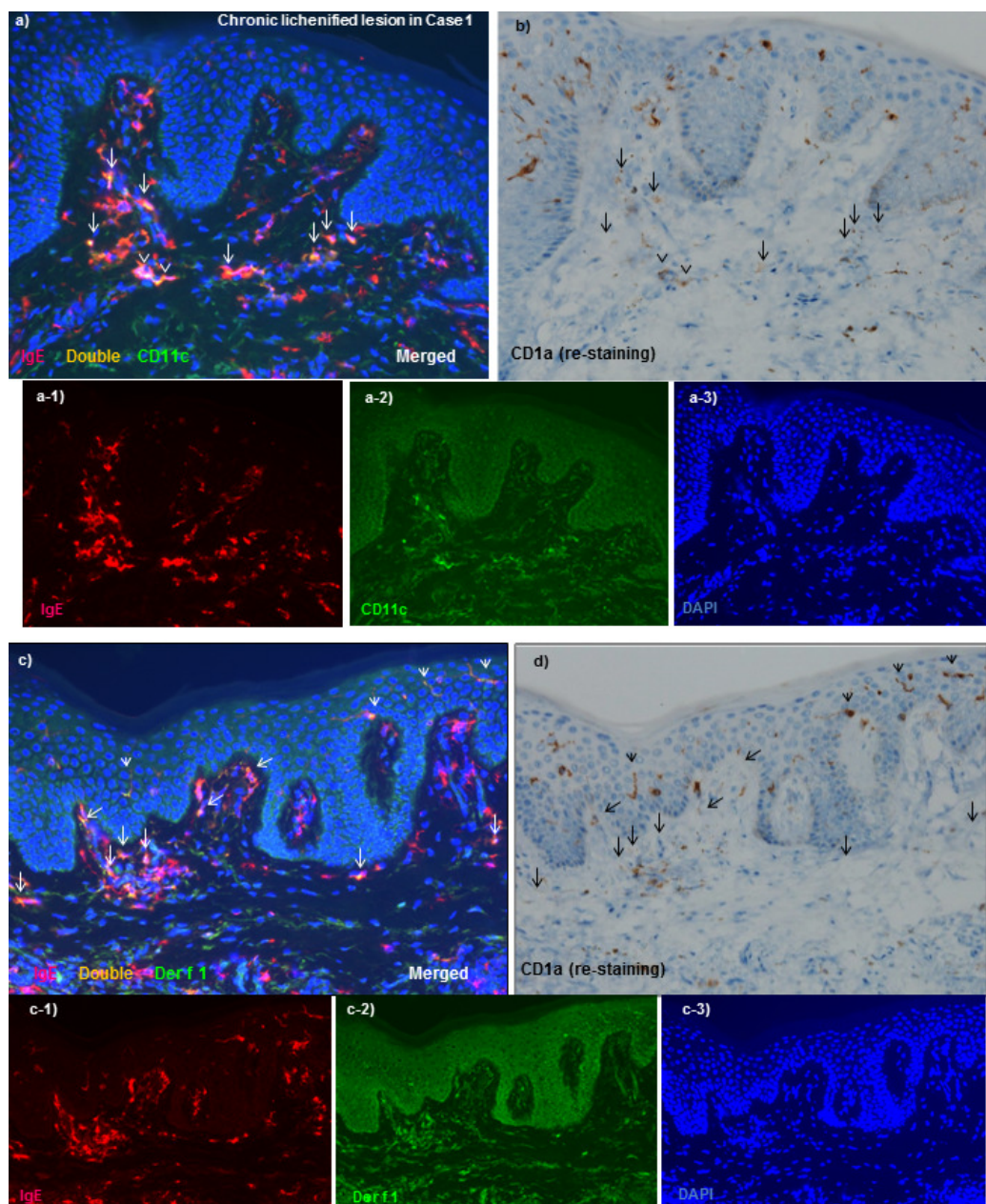
Some of the results of the control case were previously reported in our other studies [13,14].

DISCUSSION

In the present immunohistological studies, we analyzed the localization of Der f1 antigens with IgE-bearing myeloid DCs in skin lesions from older patients with AD and allergic sensitization to HDMs (*D. farinae*). The immunohistopathological analyses demonstrated some interesting findings. (1) Few IgE-bearing inflammatory DCs (IgE+ CD11c+ cells) [10], possibly associated with the lack of IgE+ Derf1+ cells and CD1a+ Der f1+cells, were observed in the uninvolved skin of the patient with IgE-allergic AD (Case 1). (2) After 48-h allergen challenge with *D. farinae*, IgE-bearing inflammatory DCs (IgE+ CD1a+ CD11c+ cells; “dermal” IDECs) appeared in the upper dermis. These IgE-bearing IDECs infiltrated the epidermis of the APT-positive acute lesion in the uninvolved skin and aggregated in clusters in the spongiosis, which appeared to

exhibit potential transepidermal elimination (Case 1). (3) In the chronic active lichenified lesion of patients with IgE-allergic AD, IgE-bearing IDECs were observed in the central area of the spongiosis and in the parakeratotic horny layer above the spongiosis. Additionally, IgE-bearing LCs with Der flantigens (IgE+ CD1a+ CD207+Der fl+ cells) were present in the peripheral area of the spongiosis and associated with inflammatory infiltration mainly comprising CD8+ cells (Case 2). (4) In the chronic lichenified lesion of patients with IgE-allergic AD, IgE-bearing IDECs were mostly located in the lower epidermis, and IgE-bearing LCs

with Der flantigens (IgE+ CD1a+ CD207+ Der fl+cells) were present in the stratum malpighii. IgE+ Der fl+ cells in the upper dermis were thought to be composed mainly of IgE+ CD1a- CD11+ Der fl+ cells, i.e., a subset of IgE-bearing inflammatory “dermal” DCs [5,15] that might differ from IgE-bearing IDECs (Case 1). (5) In the control case of eosinophilic granulomatosis with polyangiitis, neither double-positive IgE+ CD11c+ cells nor double-positive IgE+ Der fl+ cells were observed in the epidermis and upper dermis of the cutaneous vasculitis.



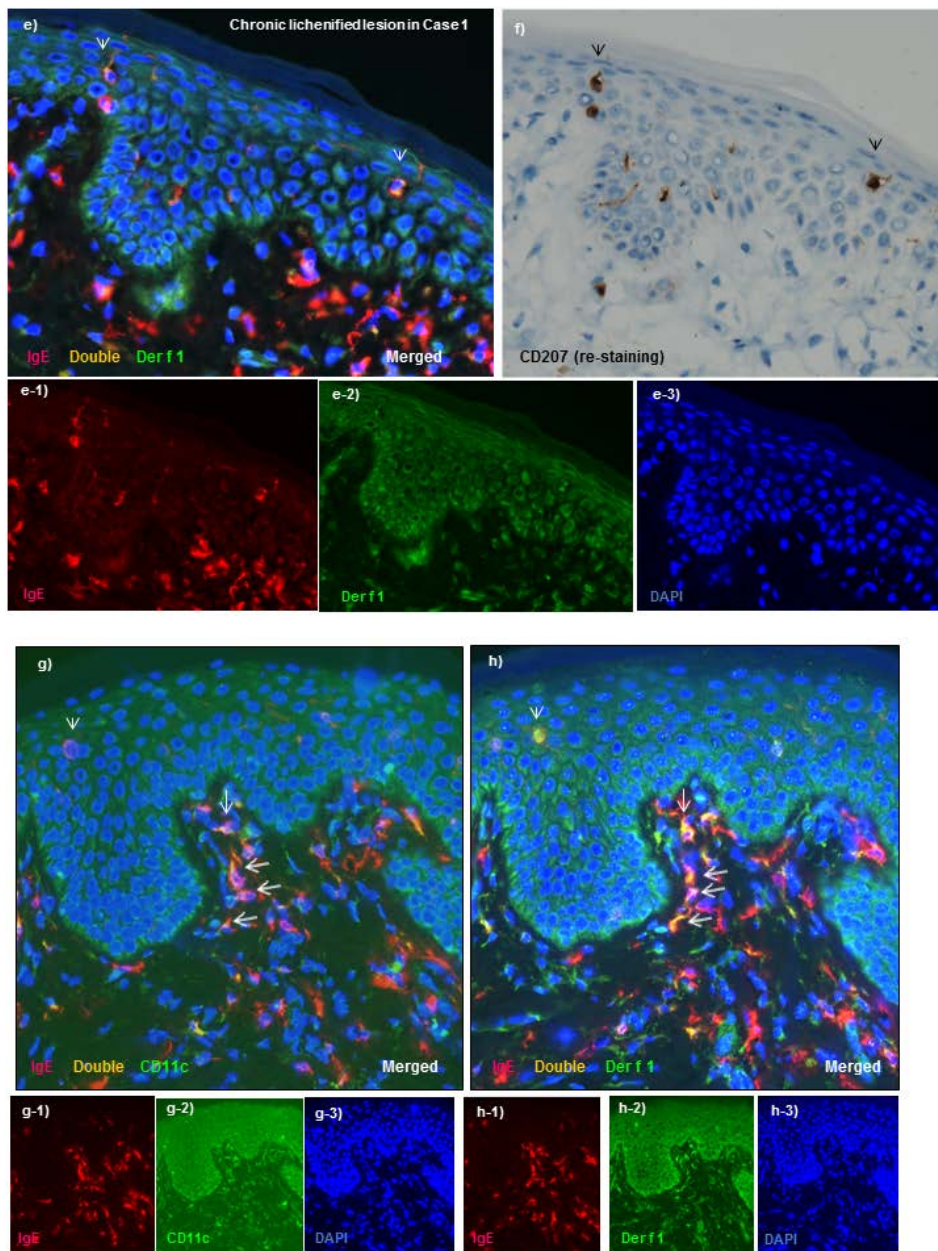


Figure 5. Chronic lichenified lesion in Case 1.

Double immunofluorescence staining using frozen sections: **a**, **c**, **e**, **g**, and **h**; and immunohistochemical re-staining: **b**, **d**, and **f**. Sets of figures (**g** and **h**) represent serial sections.

a) Infiltrating double-positive IgE+ CD11c+ cells (yellow images) are observed in the upper dermis of the chronic lichenified lesion (arrows and arrowheads).

b) Most double-positive IgE+ CD11c+ cells in the upper dermis show negative or vestigial findings for CD1a antigens (arrows). A few double-positive IgE+ CD11c+ cells are weakly positive for CD1a antigens (arrowheads).

c) Double-positive IgE+ Der f1+ cells (yellow images) are observed both in the epidermis and upper dermis of the chronic lichenified lesion.

d) The presence of epidermal CD1a+ dendritic cells coinciding with the morphology and localization of IgE+ Der f1+ cells in **c** are confirmed in the epidermis of the chronic lichenified lesion by immunohistochemical re-staining (arrowheads). Note, in **c** and **d**, the majority of IgE+ Der f1+ cells in the upper dermis do not co-localize with CD1a+ cells (arrows).

e) Double-positive IgE+ Der f1+ cells (yellow images) with elongated dendrites into the tight junction exist in the area under the tight junction (arrowheads).

f) Immunohistochemical re-staining indicated that double-positive IgE+ Der f1+ cells in the epidermis are strongly positive for CD207 antigens (arrowheads).

g) Double-positive IgE+ CD11c+ cells (yellow images) are observed in the upper dermis of the chronic lichenified lesion.

h) Double-positive IgE+ Der f1+ cells (yellow images) are also observed in the upper dermis of the chronic lichenified lesion.

Note, in sections **g** and **h**, the majority of IgE+ CD11c+ cells and IgE+ Der f1+ cells in the papillary dermis show the same morphology and localization (arrows). However, co-localization of an IgE+ CD11c- cell and an IgE+ Der f1+ cell in the epidermis is also observed (arrowheads).

Nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI, blue images). Original magnification: 200×, **a-h**.

The validity of the present results may be supported by several previous studies. Past experiments using APT as a model for early active lesion of AD have shown that the uninvolved skin of patients with AD does not contain relevant numbers of IDECs. However, a rapid influx of IDECs in the epidermis occurs within 72h after allergen application [16] and forms focal aggregates of epidermal DCs clusters mainly composed of IDECs with intermingled Foxp3+ T regulatory cells [17]. We consider that the results of the APT-positive acute lesion in the uninvolved skin in Case 1 showed similar findings, although an analysis of Foxp3+ T regulatory cells was not performed in our study. Additionally, we speculate that naturally occurring spongiosis of the chronic active lichenified lesion in Case 2 might demonstrate a similar pathomechanism as the APT-induced acute lesion in Case 1 because immunohistopathology of both lesions demonstrated the presence of IgE-bearing IDECs in the central area of the spongiosis, surrounded by IgE-bearing LCs with Der f1 antigens. Furthermore, based on the distributions of IgE-bearing IDECs and IgE-bearing LCs with Der f1 antigens, we speculate that, similar to inflammatory DCs against parasitic infection [18,19], IgE-bearing IDECs act as an initial defense against invading antigens (i.e., Der f1 antigens). IgE-bearing IDECs then eliminate the antigens with tissue damage (e.g., keratinocyte apoptosis) via proinflammatory cytokines (e.g., tumor necrosis factor- α [20]) in cooperation with IgE-bearing LCs that cross-present the specific antigens (i.e., Der f1 antigen) to interferon- γ -producing effector (CD8+) T cells. This may explain the characteristic spongiosis observed in skin lesions of patients with IgE-allergic AD and HDM sensitization [21-24]. However, the precise roles of epidermal LCs and IDECs with or without specific IgEs in the skin lesions of patients with IgE-allergic AD remain unclear.

In a recent study of adult patients with AD, distinct behavior and localization patterns of LCs and IDECs in the lesional skin were revealed. Activated LCs increased in the area underneath the TJs and extended their dendrites through the TJs, likely to capture antigens from outside the TJ barrier, while IDECs localized to the lower area of the epidermis [25]. In the present study, we confirmed the presence of IgE-bearing LCs that captured Der f1 antigens underneath the

TJs, and IgE-bearing IDECs mostly localized to the lower epidermis in the chronic lichenified lesion of a patient with IgE-allergic AD and HDM sensitization (Case 1).

In previous studies, the existence of HDM antigens (*D. farinae* and *D. pteronyssinus*) in the epidermis and dermis coincident with DCs in naturally occurring AD lesions [26] and IgE+ DCs in the site of ATP for HDMs [27] was demonstrated in adult patients with IgE-allergic AD and HDM sensitization. In naturally occurring AD lesions, HDM antigens were found in 61.3% (19 of 31) of patients with IgE-allergic AD and HDM sensitization, and most HDM antigens were seen on the surface of CD1a (OKT6)-positive DCs in the dermis [26]. In the present study, we also observed CD1a+ DCs with Der f1 antigens in the upper dermis in the chronic lichenified lesion of a patient with IgE-allergic AD (Case 1). However, the majority of IgE+ Der f1+ cells in the upper dermis were CD1a-. It has been suggested that dermal CD11c+ myeloid DCs may have remarkable plasticity [5], therefore, we speculate that the majority of IgE+ Der f1+ cells in the upper dermis might be a subset of IgE-bearing IDEC-like dermal DCs (IgE+ CD1a-CD11+ cells or IgE+ CD1a+/- (weak) CD11+ cells). Another cell phenotype that may be responsible for IgE+ CD1a- Der f1+ cells in the upper dermis might be IgE-bearing plasmacytoid DCs [4], however, this subset was not analyzed in the present study.

There are some limitations associated with this study, including the inherent technical limitations of immunohistological analyses and the small sample size. Nevertheless, the results of the present study suggested that HDM allergens such as Der f1 are involved in the immunopathogenesis of eczematous dermatitis of IgE-allergic AD, in which IgE-associated delayed-type hypersensitivity may develop with the collaboration of IgE-bearing DCs (i.e., LCs, IDECs, and IDEC-like DCs) and specific T cells.

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