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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. References used for treatment recommendations (CQ1–CQ35).

Table S1. Terminology for clinical features of mycosis fungoides/Sézary syndrome.

Table S2. Tumor, lymph nodes, metastasis, blood (TNMB) classification for mycosis fungoides/Sézary syndrome.

Table S3. TNMB staging for mycosis fungoides/Sézary syndrome (International Society for Cutaneous Lymphomas/ European Organization for Research and Treatment of Cancer, Cutaneous Lymphomas Task Force).

Table S4. Tumor–node–metastasis classification for primary cutaneous lymphomas other than mycosis fungoides/Sézary syndrome.

Table S5. Ann Arbor/Cotswold staging.

Figure S1. Clinicopathological features of mycosis fungoides/ Sézary syndrome.

Figure S2. Clinical features of anaplastic large cell lymphoma.

Figure S3. Clinical features of adult T-cell leukemia/lymphoma.

Figure S4. Clinicopathological features of subcutaneous panniculitis-like T-cell lymphoma.

Figure S5. Clinical features of extranodal natural killer/T-cell lymphoma, nasal type.

Figure S6. Clinicopathological features of hydroa vacciniforme-like lymphoma.

Figure S7. Clinical features of blastic plasmacytoid dendritic cell neoplasm.

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REVIEW ARTICLE

Review Series: Advances in Consensus, Pathogenesis and Treatment of Urticaria and Angioedema

Pathogenesis of Cholinergic Urticaria in Relation to Sweating

Toshinori Bito[1], Yu Sawada[2] and Yoshiki Tokura[3]

ABSTRACT

Cholinergic urticaria (CU) has clinically characteristic features, and has been frequently described in the literature. However, despite its comparatively old history, the pathogenesis and classification remains to be clarified. CU patients are occasionally complicated by anhidrosis and/or hypohidrosis. This reduced-sweat type should be included in the classification because the therapeutic approaches are different from the ordinary CU. It is also well-known that autologous sweat is involved in the occurrence of CU. More than half of CU patients may have sweat hypersensitivity. We attempt to classify CU and address the underlying mechanisms of CU based on the published data and our findings. The first step for classification of CU seems to discriminate the presence or absence of hypersensitivity to autologous sweat. The second step is proposed to determine whether the patients can sweat normally or not. With these data, the patients could be categorized into three subtypes: (1) CU with sweat hypersensitivity; (2) CU with acquired anhidrosis and/or hypohidrosis; (3) idiopathic CU. The pathogenesis of each subtype is also discussed in this review.

KEY WORDS

acetylcholine, acetylcholine receptor, anhidrosis, hypohidrosis, sweat hypersensitivity

INTRODUCTION

Cholinergic urticaria (CU) is a rare condition, but its incidence might be higher than that expected by general physicians. CU is clinically characterized by pinpoint-sized, highly pruritic wheals. Although the symptoms subside rapidly, commonly within one hour, CU may significantly impair the quality of life, especially sporting and sexual activities.[1] This unique disease was described by Duke in 1924,[2] however, despite its comparatively old history, the pathogenesis and classification remains to be clarified. CU is typically provoked by stimulation such as exercise, warmth, and emotional distress, which increases the body core temperature and promotes sweating.[3,4]

Since acetylcholine is known to induce both sweating and wheals when injected intradermally,[4] it has been considered that this sweating-associated, syringeal orifice-coincident wheal is mediated by acetylcholine. In fact, acetylcholine stimulation can elicit hives as seen in CU, suggesting that the etiology of CU includes certain events that are triggered by a cholinergic stimulus. A well-known hypothesis has been put forward to explain the pathogenesis of CU. The patients are hypersensitive to unknown substances in their sweats and develop wheals in response to sweat substance leaking from the syringeal ducts to the dermis possibly by obstruction of the ducts.[5,6] This "sweat hypersensitivity" hypothesis has been supported by the fact that not all but some patients with CU exhibit a positive reaction to intrademal injection of the patients' own diluted sweat as well as acetylcholine.[7] Based on the distinct responses to the autologous factors and clinical characteristics, Fukunaga *et al.* proposed two subtypes in the entity of CU, sweat hypersensitivity (non-follicular) type and follicular type.[7]

However, sweat hypersensitivity theory lacks suffi-

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cient evidence and can not encompass the whole etiology of CU. CU is occasionally associated with depressed sweating, as reported under the name of anhidrosis (complete lack of sweating) or hypohidrosis (incomplete lack of sweating).[8] There have been reported 29 patients with CU with anhidrosis and/or hypohidrosis in the literature,[8-32] and notably, 26 patients are Japanese. This type of CU may be caused by reduced expression of acetylcholine receptor but not by sweat hypersensitivity, as described below. In addition, two Japanese patients with CU had episodes of seizures upon occurrence of urticaria.[33,34] Given that acetylcholine mediates epileptic seizures,[35,36] seizures possibly occur when steroid therapy induces the re-expression of acetylcholine receptors in the brain.

By exploring the enigmatic relationship between wheal formation and sweating, we have addressed the general mechanism underlying CU.[37] In this review, we show the relationship between sweating and the pathogenesis of CU according to the published findings and propose the classification of CU based on the mechanisms.

CLINICAL SUBTYPES OF CU ACCORDING TO RESPONSES TO SWEAT

Since CU is not a homogeneous disease, its classification is necessary for the clinical use. However, few attempts have been performed to classify CU in the literature, and there is no solid consensus on the categorization. Horikawa *et al.* observed that a strong hypersensitivity to sweat was not observed in all CU patients and assumed other factors than sweat hypersensitivity are also involved in the pathogenesis of CU.[38] Some patients with chronic urticaria have autoantibodies to FcεRI or IgE, and the autologous serum skin test (ASST) has been used to detect the autoantibodies.[39,40] Horikawa *et al.* also found that 8 of 15 patients with CU showed responsiveness to ASST. While the follicular manifestation of wheal was mainly associated with positive ASST, the non-follicular hives tended to show strong hypersensitivity to sweat and were probably associated with sweat ducts. Based on this observation, they proposed to classify CU into two subtypes: (1) the sweat hypersensitivity type (non-follicular type) showing non-follicular hives, strong hypersensitivity to autologous sweat, development of satellite wheals following acetylcholine injection, and negative ASST; and (2) the follicular type showing follicular hives and positive ASST without hypersensitivity to autologous sweat or satellite wheals.[38] It remains unclear how ASST induces the follicular eruption. It is notable that some CU patients have hypersensitivity to both autologous sweat and serum.

In the above simple classification, it should be clarified whether the sweat and serum hypersensitivities can encompass the whole spectrum of CU. In addition, there are some important issues to be dissolved. CU patients are occasionally complicated by anhidrosis and/or hypohidrosis. This reduced-sweat type should be included in the classification because the therapeutic approaches are different from the ordinary CU. It has been thought that the anhidrosis and/or hypohidrosis are caused by obstruction of sweat orifice. However, the sweat reduction is not necessarily associated with poral obstruction. Nakamizo *et al.* proposed four subtypes of CU: (1) CU with poral occlusion; (2) CU with acquired generalized hypohidrosis; (3) CU with sweat allergy; and (4) idiopathic CU.[41] The incidence of overlapping of (1)-(3) is an important issue to elucidate their independence and concurrence.

One of the interesting observations in CU is that the sweat ducts are obstructed by lymphocytic inflammation around the ducts, and resultant retention and subsequent leakage of sweat from the damaged ducts induce wheals because of sweat hypersensitivity.[5,6] Given this mechanism, CU with anhidrosis and/or hypohidrosis might belong to all of "CU with poral occlusion", "CU with acquired generalized hypohidrosis", and "CU with sweat allergy". However, several observations concerning CU with anhidrosis and/or hypohidrosis are not in accordance with the conventional poral occlusion/sweat leakage theory. First, the symptoms are usually exacerbated in winter and resolved in summer, although it can be explained with the hypothesis that daily sweating in summer inhibits the formation of keratotic plugs to prevent the occurrence of CU.[6] However, the vast majority of this type of CU have been reported from Asia, whose climate is hot and humid, and the disease is rare in Europe, where it is cool and dry. This regional divergence in the occurrence of the disease is not consistent with the poral occlusion/sweat leakage theory. Second, in the literature, few of the patients with CU with anhidrosis or hypohidrosis showed positive results of the intradermally injected autologous sweat, suggesting that sweat hypersensitivity is not responsible for this form of CU. In addition, if sweat hypersensitivity is the mechanism, the anhidrotic area should be the predilection site for wheals, but wheals cannot occur on the anhidrotic area.[37] We found mosaic distribution of hypohidrotic or anhidrotic areas on the body surface, and the patients develop wheals on the hypohidrotic area. Therefore, we suggest that "CU with acquired generalized hypohidrosis" and "CU with poral occlusion" are mostly independent.

Considering all these findings, the first step for classification of CU seems to discriminate the presence or absence of hypersensitivity to autologous sweat. The second step is proposed to determine whether the patients can sweat normally or not. With these data, the patients could be categorized into three subtypes: (1) CU with sweat hypersensitivity; (2) CU with acquired anhidrosis and/or hypohidro-

Table 1 Clinical subtypes of CU according to responses to sweat

Subtype	Sweat allergy	Anhidrosis/ hypohidrosis	Intradermal test for acetylcholine	Pathology	Treatment
Sweat hypersensitivity	Positive	None	Positive	Infiltrate of lymphocytes around sweat glands	Desensitization
Anhidrosis or hypohidrosis	Mostly negative	Necessary, mosaic	Partial positive	Normal	Systemic steroid
Idiopathic	Negative	None	Negative	Normal	Antihistamines

sis; (3) idiopathic CU (Table 1). It is suggested that only a small percentage of the patients belong to the combination of (1) and (2).

ASSESSMENTS OF SWEAT HYPERSENSITIVITY

Several studies have suggested the involvement of autologous sweat in the occurrence of CU, as represented by the well-known finding that a considerable percentage of the patients show positive reactions to diluted own sweat by intradermal tests.[5,7,8,42] In this procedure, sweat is collected after exercise from the forearm of each patient, sterilized by using a 0.45-mm polyethersulfone membrane, and preserved at -80℃ until use. Sweat samples are diluted at 1 : 100 with physiological saline before the skin test. Serum is simultaneously obtained by centrifugation of venous blood. Samples of autologous diluted sweat (0.02 mL), autologous serum (0.05 mL), and 0.9% sterile saline as control (0.02 or 0.05 mL) are separately injected intradermally into the forearm of each patient at the time when they have no wheal. The diameters of wheals and erythema are measured 15 min after injection. Reactions are assessed as positive when the diameter of wheal induced by sweat and serum is 6 mm or more. The sterile saline, 0.05 and 0.02 mL, usually induces mild edema less than 4 mm and less than 2 mm, respectively. In our study, 11 (64.7%) of 16 patients with CU showed positive reactions to their own 1/100 diluted sweat by skin test.[7] Hide *et al.* have developed histamine release test (HRT) using leukocytes of patients,[42] which is more reliable than skin test. Results of skin test may be affected by both sweat constituents and skin conditions such as hypersensitivity, whereas HRT with standardized semi-purified sweat represents basophile conditions regardless of the constituent of sweat of individual subjects. This assay has been already commercialized. The collection of sweat is not an easy manipulation. HRT could be a potent practically beneficial tool to screen sweat hypersensitivity. Recently, they developed also histamine release-neutralization (HRN) assay by using the semi-purified and standardized sweat antigen, and showed high sensitivity (0.87) and specificity (0.522) to type I hypersensitivity against sweat in patients with AD and/or CU.[43]

ASSESSMENTS OF SWEATING CONDITION

CU is occasionally accompanied by anhidrosis or hypohidrosis. The sweating condition can be evaluated by exercise-induced sweating, which is assessed by iodine-starch test. Provocation is performed by exercise using an ergometer. The areas of hypohidrosis and anhidrosis are identified with iodine-starch staining.[6] In this assessment, normal hidrotic areas are changed from white to dark blue, whereas the anhidrotic skin remains white. To clarify the area of anhidrosis, photographs of both areas are taken, and skin biopsy specimens can be taken from both anhidrotic and hypohidrotic areas for further histological examination. In CU patients with anhidrosis or hypohidrosis, we have clearly demonstrated that the depressed-sweat areas are divided into the two areas, anhidrotic and hypohidrotic ones, and there is no normal sweating area in the patients.[37] Although sweating is reduced in the hypohidrotic area, a substantial degree of sweating is still observed, as compared to the anhidrotic area, where complete lack of sweating is seen. As judged from the four patients, anhidrotic areas are seen in a large mosaic pattern, and the four limbs and face may be the predilection sites.[37]

ROLE OF ACETYLCHOLINE IN CU

Acetylcholine is known to induce degranulation in rat mast cells.[44,45] Subcutaneous injection of a cholinergic agent, carbaminoylcholine, induced sweating and numerous pin-point hives that are similar to CU.[42] Thus, it is believed that acetylcholine plays an essential role in the development of CU. On the other hand, there is a skeptical opinion for acetylcholine, as it remains unclear whether acetylcholine stimulates degranulation from human mast cells. To address the issue, we used LAD2 cells, a human mast cell line, for *in vitro* assay, and found that acetylcholine dose-dependently induced the degranulation of the mast cells,[37] suggesting an essential role of acetylcholine in the development of CU.

PATHOGENESIS OF CU WITH SWEAT HYPERSENSITIVITY

Tanaka *et al.* found that the sweat-induced release of histamine from basophils is mediated by specific IgE for the partially purified antigen present in the sweat of patients with atopic dermatitis.[46] The sweat hyper-

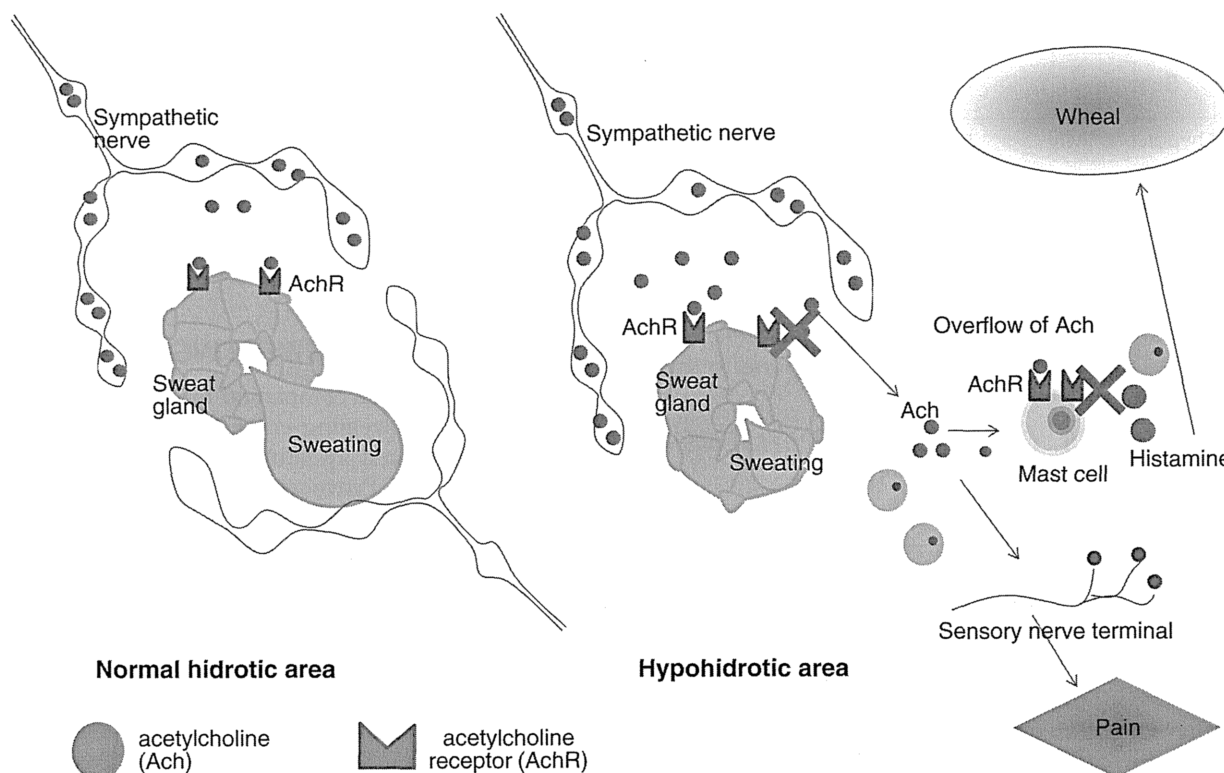

Fig. 1 A schematic model of the induction of wheals and pain in CU with hypohidrosis. In the normal hidrotic area, acetylcholine released from nerves upon exercise is completely trapped by acetylcholine receptor of eccrine glands and normally induces sweating. In the hypohidrotic area, acetylcholine released from nerves upon exercise cannot be completely trapped by acetylcholine receptor of eccrine glands and overflows to the adjacent mast cells. Subsequently, degranulation of the mast cells induces wheals and pain.

sensitivities of CU and atopic dermatitis seem to be virtually the same, and therefore, the sweat-induced histamine release from basophils may also be mediated by a specific IgE for sweat in atopic dermatitis as well as CU. The response depends on the concentration of sweat to some extent, which is compatible with the clinical observation that few patients with CU develop urticaria with small amount of sweating. Takahagi *et al.* recently reported that 23 (65.7%) of 35 CU patients associated with atopic diathesis showed basophil histamine release with semi-purified sweat antigen.[47] More than half of CU patients may have sweat hypersensitivity. Based on the observation that wheals are coincident with perspiration points of sweating, it is assumed that sweating causes pin-point wheals at sweat ducts that might allow sweat to leak.[38] However, there has been no strong evidence for the sweat leak to the dermis.

PATHOGENESIS OF CU WITH ANHIDROSIS AND/OR HYPOHIDROSIS

Various causes of anhidrosis or hypohidrosis have been reported, including absence of sweat glands, impaired function of sweat glands, poral occlusion,[6] or dysfunction of sympathetic nerves in neuropathies.[48] The causes are a matter of controversies. We address the mechanism based on our recent study.[37] As described in the clinical subtypes chapter, the sweat-depressed areas can be divided into two distinct areas; the hypohidrotic area, where a substantial degree of sweating is still observed, and the anhidrotic area, where the skin completely lacks sweating. By examining the sweating condition on the whole body surface, it was revealed that anhidrosis and hypohidrosis comorbid in the patients with CU.[37] Of note is the observation that the patients develop wheals in the hypohidrotic but not anhidrotic area, demonstrating that the occurrence of wheals is associated with sweating. In consistent with the result of the exercise induction test, wheals are provoked by the intradermal acetylcholine injection in the hypohidrotic but not anhidrotic area.

We performed the morphological and functional analyses of sweat glands in CU patients and found the involvement of acetylcholine receptor for sweating. In the anhidrotic and hypohidrotic areas, there is the gradient disturbance of the expression of cholinergic receptor muscarin 3 (CHRM3). CHRM3 is not expressed in the anhidrosis area, but its expression is retained to some extent in the hypohidrotic area. His-

tologically, there is an infiltrate of $CD4^+$ and $CD8^+$ T cells around the eccrine glands in the anhidrotic area, suggesting the inflammation-attenuated expression of the acetylcholine receptor. The expression pattern of CHRM3 on mast cells is similar to the eccrine gland epithelial cells in the anhidrotic and hypohidrotic areas. Acetylcholine receptor mediates wheal development,[48-50] and acetylcholine can induce degranulation of mast cells, as shown in our study,[37] and the past studies.[44,45] The data lead to the theory that CHRM3 expression is responsible for both sweating and wheal development. In the hypohidrotic area, we are tempting to speculate that acetylcholine released from nerves upon exercise cannot be completely trapped by acetylcholine receptor of eccrine glands and overflows to the adjacent mast cells (Fig. 1). In this scenario, it is assumed that mast cells can produce histamine in response to acetylcholine, because mast cells in the hypohidrotic area express some degree of CHRM3.[37]

IDIOPATHIC CU

CU is generally categorized into two major subtypes, CU associated with or without sweat hypersensitivity. We propose that the majority of CU patients with anhidrosis and/or hypohidrosis belong to CU associated without sweat hypersensitivity. However, there still exist unclassified patients with CU despite full examinations. Such patients without any clue to diagnosis are categorized into idiopathic CU. We need to further clarify the causative agents and underlying mechanism in this type of CU.

TREATMENT OF CU

Desensitization with autologous sweat has recently been attempted in CU with sweat hypersensitivity. Tanaka *et al.* demonstrated that desensitization using partially purified sweat antigen was effective in a patient with CU.[51] Kozaru *et al.* performed rapid desensitization with autologous sweat in 6 CU patients with sweat hypersensitivity, and succeeded in 5 of the 6 patients.[52] Desensitization therapy may become a familiar choice of treatment for CU patients with sweat hypersensitivity. A severe CU patient with sweat allergy has been successfully treated with an anti-IgE antibody, omalizumab,[53] suggesting that an IgE-mediated response is involved in the pathogenesis of CU. One of the first line treatments of CU with anhidrosis and/or hypohidrosis is the pulse therapy with a high dose of corticosteroid. The treatment may decrease the lymphocytic infiltrate around the sweat glands and allow acetylcholine receptor to re-express, resulting in the improvement of sweating and CU. Anti-histamines should be combined with other treatments owing to the limited effect. Various therapeutic variations including scopolamine,[54] danazol,[55,56] β2-adrenergic stimulants[57] and β2-adrenergic blockers[58] have been reported, however, those therapies are the second line treatments and recommended to be used as combination therapies.

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REVIEW ARTICLE

Clinical and histopathological differential diagnosis of eosinophilic pustular folliculitis

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ABSTRACT

Eosinophilic pustular folliculitis (EPF) is an inflammatory disease characterized by repeated pruritic follicular papules and pustules arranged in arcuate plaques, and folliculotropic infiltration of eosinophils. The diagnosis of EPF is occasionally difficult and problematic because EPF may share the clinical appearance and histological findings with other diseases. Moreover, EPF has several clinical subtypes, including the classical type, infantile type and immunosuppression-associated type. Because the therapies of EPF are relatively specific as compared to eczematous disorders, accurate diagnosis is essential for the management of EPF. Clinical differential diagnoses include tinea, acne, rosacea, eczematous dermatitis, granuloma faciale, autoimmune annular erythema, infestations and pustular dermatosis. Histologically, cutaneous diseases with eosinophilic infiltrates can be differentially diagnosed. Follicular mucinosis, mycosis fungoides and other cutaneous T-cell lymphomas are the most important differential diagnoses both clinically and histopathologically. It should be kept in mind particularly that the initial lesions of cutaneous T-cell lymphoma resemble EPF.

Key words: **cutaneous T-cell lymphoma, eosinophil, eosinophilic pustular folliculitis, Ofuji disease, pustular dermatosis.**

INTRODUCTION

Eosinophilic pustular folliculitis (EPF), also known as Ofuji disease, is an inflammatory disease clinically characterized by repeated pruritic follicular papules and sterile pustules arranged in arcuate plaques with central healing and peripheral spread.[1] Histopathologically, EPF lesions exhibit folliculotropic infiltration of eosinophils, which is a critical diagnostic hallmark.[1] More than 300 cases of EPF have been reported the English-language published work. Among them, over one-third of the patients are Japanese, and far more cases have been documented in Japanese published work.[2] Thus, the incidence of EPF is higher in Japan than in Western countries. However, it has been shown that EPF develops in association with various conditions, such as HIV infection,[3] and in those cases, patients are not confined to Japanese.

The diagnosis of EPF is made based on clinical features and pathological findings. In some cases, however, these features are hardly distinguishable from other diseases. Moreover, there are several clinical subtypes of EPF, which might have been historically diagnosed as other diseases. The therapies of EPF are relatively specific as compared to eczematous disorders. For example, oral indomethacin has shown a complete response rate of 70% or more for the classical type of EPF.[4,5] Therefore, accurate diagnosis is required for efficacious treatment of EPF. In this review, we describe the clinical and histopathological differential diagnoses of EPF to better understand the diagnostic criteria and pitfalls of EPF. We focus especially on cutaneous T-cell lymphoma, because EPF occasionally shares the clinical and histopathological features with this life-threatening disease.

TYPES AND SKIN MANIFESTATIONS OF EPF

On the basis of the eruption type, cause or etiology, and patient's age, EPF is divided into several clinical subtypes, including the classical type,[1,6] infantile type (I-EPF)[7] and immunosuppression-associated type (IS-EPF). IS-EPF may be further subdivided into HIV-associated type[3] and malignancy-associated type.[8] In the classical type of EPF, which was first described by Ofuji *et al.* as a new clinical entity in 1970,[1] young adults are usually affected. The infantile type is seen mostly in infants less than 1 year of age. In any type of EPF, men are affected more frequently than women.

The classical type involves seborrheic areas, and the face is the predilection site, where the initial lesion occurs in most of the cases.[2] The trunk and the upper outer arms are also frequently affected. The typical skin eruption is recurrent crops of intensely pruritic grouped follicular pustules or papulopustules developing in an explosive fashion. Furthermore, there are erythematous patches with superimposed coalescent pustules with central clearing and centrifugal extension.[1,9] Thus, most of

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the skin lesions show annular configuration on the face (Fig. 1a). In approximately 20% of the cases, pustular lesions are additionally observed on the palms or soles.[10] Lesions on the scalp or legs have been also reported.[11,12] The EPF lesions last at least 7–10 days, involute and relapse in an average interval of every 3–4 weeks.[1,9] The patients usually have no accompanying systemic symptoms.[9]

In I-EPF, the scalp is frequently affected and severe pruritic crusted papulopustules are the characteristic features. Approximately 70% of patients with I-EPF present the initial eruption by 6 months of age, and the lesions resolve by 3 years of age in over 80% of cases.[13]

Immunosuppression-associated EPF is represented by the HIV-associated type, which is characterized by perifollicular erythematous papules and pustules with intense itching, usually distributed on the head and neck, upper limbs and upper trunk. Thus, unlike the classical type, the HIV-associated type lacks annular arrangement and face predilection.

Elevated serum immunoglobulin E levels and peripheral eosinophilia may be present in any type of EPF.[13,14]

HISTOPATHOLOGICAL FINDINGS OF EPF

The histopathology is crucial for the precise diagnosis of classical type EPF, and it is important to obtain an entire papule or pustule with associated follicles for histological examination and diagnosis.[9] The involved follicles may show spongiosis with exocytosis of lymphocytes and eosinophils extending from the sebaceous gland and its duct to the infundibular zone.[4,6,12] The epidermis around the affected follicles often contains eosinophils and lymphocytes. In the dermis, perifollicular and perivascular infiltration of eosinophils and lymphocytes can be observed (Fig. 1b,c). Neutrophils are also present in more inflamed lesions. Micropustular aggregation develops, followed by the hallmark findings, namely, infundibular eosinophilic pustules. Infiltration of neutrophils and basophils are also reported.[9,15]

In the HIV-associated cases, degranulating eosinophils and mast cells are observed in the perifollicular area.[16,17] Immunohistochemically, $CD8^+$ lymphocytes are predominant in the HIV-associated type.[9,17]

Infantile EPF does not show folliculitis in all cases, but eosinophils may spread within the dermis in a perifollicular, interfollicular and/or periadnexal array.[13] Occasionally, flame figure, known as a sign of Wells' syndrome, can be observed.

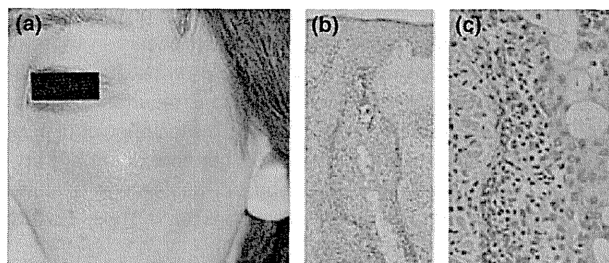

Figure 1. (a) Clinical and (b,c) histological findings of eosinophilic pustular folliculitis (hematoxylin–eosin, original magnifications: [b] ×100; [c] ×200).

CLINICAL DIFFERENTIAL DIAGNOSES

The clinical differential diagnosis from the viewpoint of the eruption morphology includes infectious diseases, infestations, inflammatory diseases and even lymphocytic neoplasms.

Follicular mucinosis and mycosis fungoides (MF)

Differentiation of EPF from follicular mucinosis is important not only clinically but also histologically as mentioned below. Follicular mucinosis is boggy cutaneous plaques showing follicular prominence (Fig. 2a). In this condition, follicular papules and plaques are often associated with severe pruritus and predilection for the face and scalp. When the central healing is unremarkable in EPF cases, it is sometimes difficult to differentiate from follicular mucinosis. Similarly, folliculotropic MF, which often follows follicular mucinosis,[18] can be a differential diagnosis.

Tinea faciei

Tinea faciei usually manifests scale, annular configuration, and pustules on the border (Fig. 2b).[19] When presenting multiple follicular pustules with intense inflammation, it is called tinea barbae,[20] a condition remarkably resembling EPF. Tinea incognito, which results from inappropriate topical use of corticosteroids, may share the clinical features with the classical type of EPF.[21,22]

Acne

Acne shows follicular papules and pustules on the face, and upper chest and back, with occasional pruritus.[23] The pustulopapules of acne may be larger than those of EPF, and acne lesions do not exhibit central healing, peripheral spread or annular configuration (Fig. 2c). Nevertheless, it is notable that

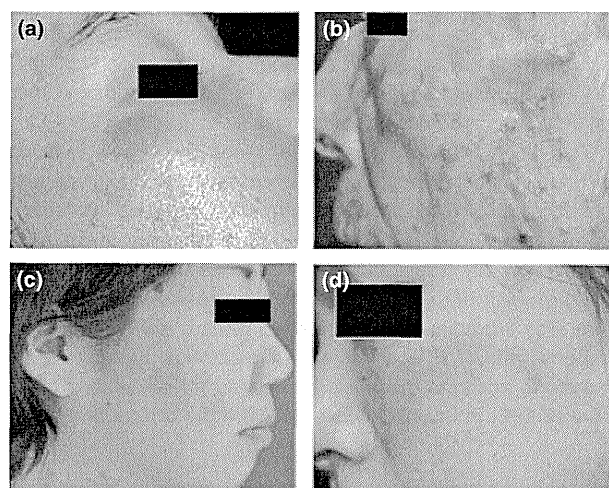

Figure 2. (a) Follicular mucinosis. (b) Tinea faciei. (c) Acne. (d) Annular erythema associated with Sjögren's syndrome.

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some patients with EPF are misdiagnosed as having acne and extensively treated with acne therapies.

Rosacea and lupus miliaris disseminatus faciei (LMDF)

Rosacea affects the face with a vascular change serving as the initial lesion. Advanced cases show follicular and non-follicular papules and pustules without comedones, and ultimately present a *peau d'orange* appearance and phymas. There is resemblance between EPF and the erythematous eruption of rosacea, but rosacea is often accompanied by a burning sensation, not by severe pruritus.

Lupus miliaris disseminatus faciei is considered to be a granulomatous and self-limiting variant of rosacea and is seen mainly in young adults and adolescents. The predilection sites include the central face, the chin, forehead, cheeks and eyelids.[24] LMDF presents multiple, monomorphic, symmetrical, red to yellowish-brown papules, which may occasionally become pustular.[25] Diascopy of the larger lesions often reveals an apple-jelly nodule-like appearance, indicating granulomatous histology.

Eczematous dermatitis

Seborrheic dermatitis in adults shows sharply demarcated, symmetrical scaly erythema or thin plaques involving seborrheic areas, namely, the scalp, face and upper trunk. Follicular papules are observed in some cases, although pustules and severe pruritus are very rare in this disease.

Atopic dermatitis (AD) presents pruritic erythema, plaques and papules, and some adult AD patients have a prominent head and neck distribution, although follicular pustules are not usually observed. The clinical course of AD is different from that of EPF.

Granuloma faciale

This disease is characterized by a single or multiple solitary asymptomatic smooth red-brown to violaceous plaques on the face. Granuloma faciale typically follows a chronic course. Histopathologically, a mixed inflammatory infiltrate with predominance of neutrophils and eosinophils in the dermis are characteristic, and Grenz zone is present. Leukocytoclastic vasculitis may be noted.

Autoimmune annular erythema

Annular erythema associated with Sjögren's syndrome usually affects young female adults. The distribution is often photosensitive areas and limited to the face (Fig. 2d). Although it is annular, the eruption of Sjögren's syndrome does not have pustulopapules.

Infestations

Demodex, scabies and insect bites may present with similar skin lesions to EPF. *Demodex* folliculitis usually causes acneiform follicular papules and pustules on the face, often with a background of diffuse erythema. Scabies usually shows pruritic papules, and the individual lesions may be accompanied by secondary bacterial infection, forming pustules. However, it does not involve the face in most of the cases. Examination of scale with KOH is helpful for the differential diagnosis.

Subcorneal pustular dermatosis (SPD)

Subcorneal pustular dermatosis shows annular polycyclic lesions with sterile pustules, usually commencing in the flexures.[26,27] Because SPD almost never affects the face, only the trunk lesions of EPF should be differentiated from SPD. In addition, the pustules of SPD are very superficial and rupture easily.[26,28]

Palmoplantar pustulosis (PPP)

As EPF sometimes involves the palms and soles,[9,10] PPP should be a differential diagnosis. In 8% of EPF cases, the skin lesions appear first in the palmoplantar region with an average period of 26 months until the appearance of the main eruptions of EPF.[29] The histological findings and good therapeutic response to the indomethacin[29,30] will help the diagnosis.

HISTOLOGICAL DIFFERENTIAL DIAGNOSIS

The most critical hallmark of EPF is spongiosis of the hair follicle epithelium with infiltration of lymphocytes and eosinophils, forming eosinophilic follicular pustules.[9] However, there are two major notes to make the diagnosis of EPF with these histological criteria. One is that skin biopsy specimens of EPF do not necessarily contain follicular pustules, and in these case, dermatologists should make the diagnosis collectively with clinical appearance and histological findings. The other note is the existence of many diseases other than EPF that show eosinophilic spongiosis or pustules in the follicles.

Cutaneous diseases with eosinophilic infiltrate

There are a large number of skin diseases showing dermal infiltrate of eosinophils, including arthropod assault, AD, allergic contact dermatitis, drug eruption, bullous pemphigoid, pemphigus vulgaris, pemphigus vegetans, papuloerythroderma, parasitic infestations, scabies, pruritic papular eruption of HIV, angiolymphoid hyperplasia with eosinophilia, Kimura's disease, Churg–Strauss syndrome, urticaria, urticarial vasculitis, pruritic urticarial papules and plaques of pregnancy, and Wells' syndrome. Although follicular pustule formation is rare in these disorders, some show eosinophilic spongiosis or even pustules, as represented by pemphigus vegetans, which exhibits eosinophilic spongiosis and eosinophilic microabscesses in the epidermis.[31]

Cutaneous diseases with lymphocytic and eosinophilic infiltrate in hair follicle epithelium

Follicular mucinosis is one of the most important diseases that should be differentiated from EPF, because eosinophils often infiltrate in and around the hair follicles of follicular mucinosis. To dissect them, it is notable that T lymphocytes predominantly infiltrate in follicular mucinosis,[32,33] while eosinophils markedly infiltrate in EPF. This discernible eosinophilic infiltration may be caused by the different pathomechanisms between EPF and follicular mucinosis. In the pathogenesis of

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EPF, it was shown that prostaglandin D_2 induces eotaxin-3 production by sebocytes, thereby accumulating eosinophils in the pilosebaceous unit.[34] Instead of such an eosinophil chemokine, T-cell chemokines, such as CXCR3, CCR4, CCR10 and CCL27, are produced by epidermal keratinocytes in MF,[35,36] a related disorder to follicular mucinosis. In addition, the mucinous change, which is a possible T-cell-provoking phenomenon toward skin constituents, is more prominent in follicular mucinosis.

Variable follicular eosinophilic infiltrate may be observed in *Malassezia* folliculitis (Fig. 3a), *Demodex* folliculitis, and folliculitis evoked by administration of inhibitors of epidermal growth factor receptors (Fig. 3b). In these diseases, however, the majority of the inflammatory cells are neutrophils and lymphocytes, and eosinophils are a minor population.

Cutaneous T-cell lymphoma

It should be stressed that cutaneous T-cell lymphoma is an important differential diagnosis for EPF in two respects. First, cutaneous lymphoma and pseudolymphoma, especially MF and $CD30^+$ lymphoproliferative disorders, can show eosinophils intermingled with lymphocytes in the upper dermis.[37] Intriguingly, some of the cases of follicular mucinosis progress into folliculotropic MF, which also shows spongiosis of hair follicle epithelium, mucinous degeneration of follicles, and eosinophil infiltrate in both the epidermis and dermis,[38] sharing the features with EPF (Fig. 3c).

Second, the EPF-like histological changes may be an initial sign of cutaneous T-cell lymphoma. MF and Sézary syndrome (SS) are well known to have erythematous patches and plaques resembling eczematous dermatitis at the early stage. In some patients with MF or SS, therefore, the skin lesions may be misdiagnosed as AD.[39] Blood and tissue eosinophilia may further mislead the diagnosis.

In relation to the resemblance between EPF and cutaneous T-cell lymphoma, EPF is a possible initial eruption of MF and other lymphomas. We experienced a case of primary cutaneous anaplastic large cell lymphoma (ALCL), whose patches/plaques were at first diagnosed as EPF and evolved into tumors. On our initial examination, this 70-year-old man had erythematous lesions on the face and trunk (Fig. 4a). Histologically, the lesions exhibited eosinophilic follicular pustules, indistinguishable features of EPF (Fig. 4b). Eight years later, however, the lesions progressed into tumors (Fig. 4c) with massive infiltration of $CD30^+$ large atypical cells, diagnosed as primary cutaneous ALCL (Fig. 4d). Notably, the clinical and histological EPF morphology may be a sign of cutaneous T-cell lymphoma. In this regard, it is interesting that, in the HIV-associated type, the immunosuppressive condition may allow expansion of a certain population of T cells and resultant occurrence of EPF.

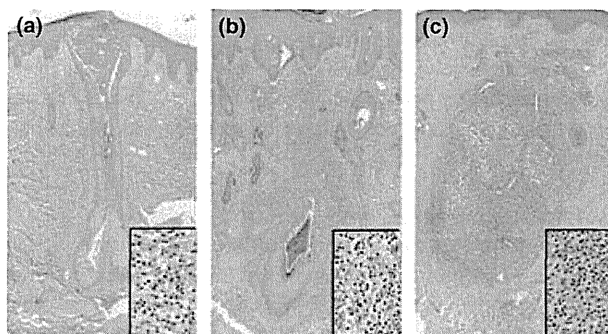

Figure 3. (a) *Malassezia* folliculitis with eosinophilic infiltration around the follicle. (b) Folliculitis associated with inhibitors of epidermal growth factor receptors. (c) Folliculotropic cutaneous T-cell lymphoma (hematoxylin–eosin, original magnifications: [a–c] ×100; insets, ×400).

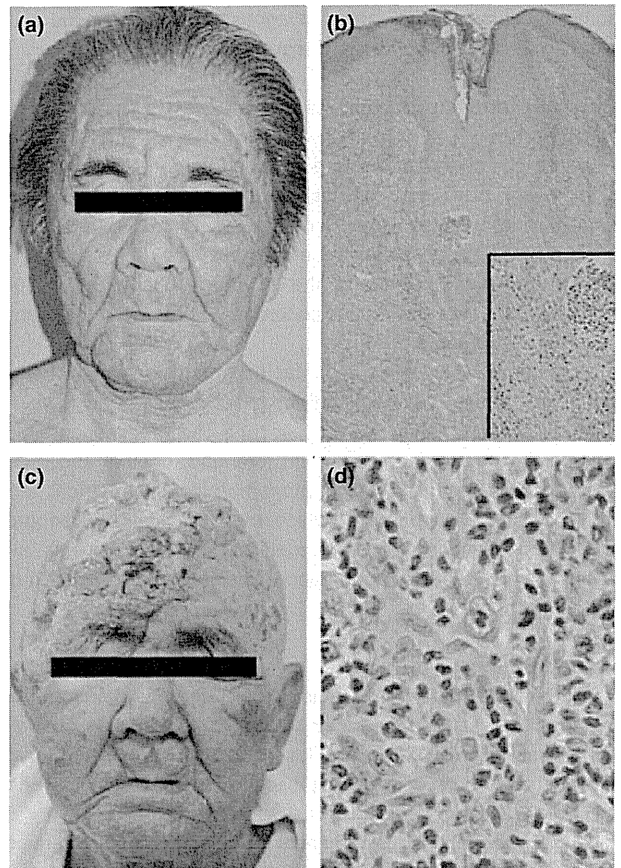

Figure 4. (a) Initial clinical and (b) histological findings of a case of primary cutaneous anaplastic large cell lymphoma. (c) Eight years later, the lesions progressed into tumors (d) with massive large atypical cells (hematoxylin–eosin, original magnifications: [b] ×100; inset, ×200; [d] ×400).

CONCLUSIONS

Clinical and pathological differential diagnoses of EPF include infectious diseases, infestations, inflammatory diseases and lymphomas. It should be kept in mind that the initial lesions of cutaneous T-cell lymphoma share the histological findings with EPF.

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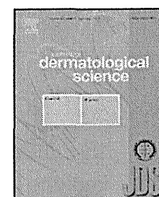

CXCL10 produced from hair follicles induces Th1 and Tc1 cell infiltration in the acute phase of alopecia areata followed by sustained Tc1 accumulation in the chronic phase

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ABSTRACT

Background: Alopecia areata (AA) is an organ-specific and cell-mediated autoimmune disease. T lymphocytes densely surround lesional hair bulbs, which is histologically referred to as "swarm of bees". However, pathomechanisms of "swarm of bees" are still uncertain.
Objective: We investigated the pathological mechanisms of "swarm of bees", focusing on T-cell chemotaxis so that inhibition of chemotaxis may be strong candidate of novel treatments for AA.
Methods: We investigate the expression of chemokine receptors on T cells obtained from peripheral blood mononuclear cells (PBMCs) and skin infiltrating cells in AA patients. In addition, real-time chemotaxis assay was also demonstrated.
Results: In PBMCs, the frequency of $CXCR3^+CD4^+$ T cells (Th1) was significantly higher in acute-phase AA than in chronic-phase AA or healthy control, while $CXCR3^+CD8^+$ T cells (Tc1) were significantly increased in chronic-phase AA. In the skin lesions of acute-phase AA, $CXCR3^+CD4^+$ and $CXCR3^+CD8^+$ T cells infiltrated in the juxta-follicular area. In chronic-phase AA, $CXCR3^+CD8^+$ T cells dominated the infiltrate around hair bulbs, possibly contributing to the prolonged state of hair loss. Lymphocytes obtained from a lesional skin of acute-phase AA contained $CXCR3^+CD4^+$ and $CXCR3^+CD8^+$ T cells at higher percentages than those of PBMCs, suggesting preferential emigration from the blood. Immunohistochemical and real-time RT-PCR studies demonstrated that hair follicles of acute-phase AA expressed a high level of Th1-associated chemokine CXCL10. By chemotaxis assay, freshly isolated PBMCs from acute-phase AA patients had a strong velocity of chemotaxis toward CXCL10 with increased expression of F-actin.
Conclusions: These results suggest that the increased production of CXCL10 from hair follicles induces preferential infiltrates of highly chemoattracted Th1 and Tc1 cells in the acute phase of AA, and Tc1 infiltration remains prolonged in the chronic phase.
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1. Introduction

Alopecia areata (AA) has recently been shown to be a tissue-specific autoimmune disease [1–3]. A T-cell-mediated immune reaction may induce hair loss based on autoimmune etiopathogenesis [4]. The hair follicle (HF) autoantigen remains unfully identified, but melanocyte-related protein has been suggested as a strong candidate [5–7]. Melanocyte-associated T-cell epitopes are capable of functioning as autoantigens to induce AA in the human scalp graft/severe combined immunodeficiency (SCID) mouse model [8,9]. Both $CD4^+$ and $CD8^+$ T cells were required to induce AA-like hair loss. The high frequency of vitiligo in AA patients also implicates the autoimmune reaction against melanocytes in AA [10], although overlapping is most likely due to shared risk alleles of genes in autoimmunity.

In the physiological condition, autoantigens generated by anagen HFs should be protected from recognition by autoreactive T cells. Therefore, anagen hair bulbs may maintain an immuno-privileged milieu at the proximal outer root sheath (ORS) and hair matrix. This milieu creates immunotolerance such that excess immune reactions are avoided [11–16]. HF immune privilege (HF-IP) is characterized by the low or absent expression of major histocompatibility complex (MHC) class I, the substantial expression of immunosuppressants, and the rare distribution of immune

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cells [13–15,17–21]. When HF-IP is collapsed by stressors, autoantigens are revealed, resulting in an autoimmune reaction by T cells that causes the unique pathological feature called "swarm of bees" in the acute phase of AA [22]. The mononuclear cells that accumulate in and around the lesional hair bulb consist of 60–80% $CD4^+$ T cells, 20–40% $CD8^+$ T cells, and some natural killer cells [15,23]. Th1 cytokines, as represented by interferon (IFN)-γ, are dominantly detected in AA lesions and may induce the collapse of HF-IP, including the up-regulation of MHC class I [24]. Autoimmune hair loss similar to AA can be induced in C3H/HeJ mice by the intravenous injection of IFN-γ, which induces up-regulation of the follicular MHC class I and II expression [25].

There have been several studies on the expression of chemokines and their receptors in AA lesions [8,26]. For example, IFN-γ-inducible expression of Th1 chemokines, CXCL9/MIG and CXCL10/IP-10, were detected in the lesions of AA patients [8,27]. CXCL9 was elevated in human AA, and its level correlated with disease activity [28]. Transcriptional profiling was also demonstrated and CXCL10 was highly up-regulated in AA lesion compared to non-lesional skin [29]. However, the mechanisms of immune cell accumulation in and around the hair bulbs of AA remain unelucidated. In the present study, we addressed the pathological mechanisms underlying "swarm of bees" by examining the expression of chemokines in the tissue of AA, and the chemokine receptor expression and chemotaxis of patients' T cells.

2. Materials and methods

2.1. Patients and samples

Peripheral blood mononuclear cells (PBMCs) were taken from 7 acute-phase AA (hair loss within 6 months) patients, 7 chronic-phase AA (hair loss longer than 6 months of alopecia universalis) patients, and 7 healthy volunteers after informed consent. Not only the disease duration but also the alopecia areas were different between the two groups. While the acute-phase AA exhibited 1 to 20 alopecia lesions (Severity of Alopecia Tool Score [SALT] score: S1–S3), the chronic-phase AA was represented by alopecia universalis (SALT score: S5 and B1/B2) [30]. All of the patients were treated only with topical corticosteroids or untreated, and had never been applied with systemic immunosuppressants such as corticosteroids and cyclosporine. PBMCs were isolated from heparinized venous blood by centrifugation with Ficoll-Paque PLUS in LeucoSep (Greiner Bio-One, Frickenhausen, Germany). Skin samples were obtained from 3 acute-phase and 3 chronic-phase AA patients and 3 normal subjects. Skin specimens used for cryosections were snap-frozen in liquid nitrogen and stored at −80 °C until used. One of the skin samples was used for isolation of skin infiltrating cells. The study was approved by the ethical committee of the Hamamatsu University School of Medicine, and conducted according to the declaration of Helsinki principles.

2.2. Isolation of skin-infiltrating cells

Skin-infiltrating cells were isolated as described before [31]. Briefly, a 3-mm skin biopsy was cut into 4 pieces and cultured in 10 ml RPMI-1640 containing IL-2 (50 U/ml) and 10 μl anti-CD3/CD28 antibody-coated microbeads (Dynal Biotech, Oslo, Norway) in a 25 ml culture bottle for 7 days. On day 8, the cells were harvested and the bound microbeads were detached with a magnetic device (Dynal). The cells were studied by flow cytometric analysis.

2.3. Flow cytometric analysis

Cell surface expression of CD4, CD8, CXCR3 and CCR4 on PBMCs was determined by flow cytometric analysis. PBMCs were collected from 7 acute-phase AA patients, 7 chronic-phase AA patients and 7 healthy subjects. In addition, skin-infiltrating cells were obtained from one of the acute-phase AA patients for flow cytometric analysis. Briefly, FITC-conjugated, mouse anti-human CD4 antibody (1:100 in PBS) and PE-conjugated mouse anti-human CXCR3 monoclonal antibody (1:100 in PBS) (PharMingen BD) or PE-conjugated mouse anti-human CCR4 monoclonal antibody (1:100 in PBS) were incubated with PBMCs for 30 min. After washing the cells in PBS for 5 min three times, the cells were subjected to flow cytometric analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ).

2.4. Histopathology and immunohistochemical staining

Frozen skin specimens were embedded and processed for transversal cryosections (8 μm) as described previously [14,32]. Cryosections of normal human scalp skin and AA lesions were immunostained with goat polyclonal anti-human CXCL10/IP-10 antibody (1:100 in PBS) (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA); goat polyclonal anti-human CCL17/TARC (N-20) antibody (C-19) (1:100 in PBS) (Santa Cruz Biotechnology); monoclonal mouse anti-human CXCR3 antibody (1:100 in PBS) (abcam, Cambridge, U.K.); monoclonal mouse anti-human CCR4 antibody (1:100 in PBS) (abcam); monoclonal mouse anti-human CD4 antibody (1:100 in PBS) (DAKO, Glostrup, Denmark) and monoclonal mouse anti-human CD8 antibody (1:100 in PBS) (DAKO). Before staining with the first antibodies, cryosections were immersed in 3% H_2O_2 and 100% methanol for 10 min to quench endogenous peroxidase. After washing in PBS 3 times, the cryosections were incubated with 10% normal goat serum (DAKO) for 20 min, followed by staining with the first antibodies against CD4, CD8, CXCR3 or CCR4 for overnight at 4 °C. After washing in PBS 3 times, biotin-conjugated polyclonal goat anti-mouse immunoglobulins (DAKO) were applied for 40 min at room temperature. Then, the cryosections were incubated with peroxidase-labeled streptoavidine (Nichireibioscience, Tokyo, Japan), followed by AEC staining (Nichirei). For staining of CXCL10 and CCL17, the cryosections were incubated with 10% normal rabbit serum (DAKO) for 20 min, followed by staining with the first antibodies against CXCL10 or CCL17 for overnight at 4 °C. After washing in PBS 3 times, biotin-conjugated polyclonal rabbit anti-goat immunoglobulins (DAKO) were applied for 40 min at room temperature. Then, the cryosections were incubated with peroxidase-labeled streptoavidine (Nichireibioscience, Tokyo, Japan), followed by AEC staining (Nichirei).

2.5. RT-PCR and real-time RT-PCR

The expression of mRNA for CXCL10 in AA lesions was determined by semiquantitative RT-PCR and real-time RT-PCR. Total RNA was extracted from AA lesions using an RNeasy kit (Qiagen, Hilden, Germany). The extracted RNA was reverse-transcribed with random hexamer primers and reverse transcriptase provided in the 1st Strand cDNA Synthesis kit for RT-PCR (Boehringer, Mannheim, Germany), and the subsequent PCR amplification was performed on the UNO-Thermoblock (Biometra, Göttingen, Germany). The RT-PCR primers for CXCL10 were 5′-CCTGCTTCAAATATTTCCCT-3′ (sense) and 5′-CCTTCCTGTATGTGTTTGGA-3′ (antisense) (GenBank accession number: NM001565) (Sigma ARK, Steinheim, Germany) (229 bp). The primers for β-actin were 5′-CGACAACGGCTCCGGCATGTGC-3′ (sense) and 5′-CGTCACCGGAGTCCATCACGATGC-3′ (antisense) (GenBank accession number: NM001101) (420 bp) (Sigma ARK). Following initial denaturation at 94 °C for 5 min, the profile of 30 PCR cycles was denaturation for 30 s at 94 °C, annealing for 1 min at 57 °C (CXCL10) or 55 °C (β-actin), and extension for 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. The PCR products

were electrophoresed on a 2% agarose gel that was stained with 0.02 mg/ml ethidium bromide and visualized with an ultraviolet transilluminator. Real-time RT-PCR was performed on an ABI PRISM 7000 sequence Detection System (Applied Biosystems, Foster City, CA) as described previously [33]. TaqMan probes were obtained from Applied Biosystems.

2.6. T-ell isolation and real-time horizontal chemotaxis assay

$CD4^+$ T cells were isolated from PBMCs with a $CD4^+$ and $CD8^+$ T-Cell Isolation Kit II by negative selection (Miltenyi Biotec, Auburn, CA). In detail, non-$CD4^+$ T cells, i.e., $CD8^+$ T cells, γ/δ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes, and erythroid cells, were labeled by using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (glycophorin A) (Miltenyi Biotec). These cells were subsequently magnetically labeled with anti-biotin microbeads (Miltenyi Biotec) for depletion. Then, these cells were injected into MS Column on the MiniMACS™ Separator (Miltenyi Biotec). Afterwards, non-$CD4^+$ T cells were magnetically separated and non labeled $CD4^+$ T cells went through the columns and dripped into the tubes under the columns. Non-$CD8^+$ cells, i.e., $CD4^+$ T cells, monocytes, neutrophils, eosinophils, B cells, stem cells, dendritic cells, NK cells, granulocytes, γ/δ T cells, or erythroid cells were labeled by using a cocktail of biotin-conjugated antibodies (Miltenyi Biotec). The cocktail contains antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR γ/δ, and CD235a (Glycophorin A) (Miltenyi Biotec). Subsequently, non-$CD8^+$ cells were magnetically labeled with the $CD8^+$ T Cell microbead cocktail. Then, these cells were injected into MS Column on the MiniMACS™ Separator (Miltenyi Biotec). Afterwards, non-$CD8^+$ T cells were magnetically separated and non-labeled $CD8^+$ T cells went through the columns and dripped into the tubes under the columns.

Time-lapse images of cell migration during chemotaxis were observed directly with an optically accessible horizontal chemotaxis apparatus EZ-TAXIScan (Effector Cell Institute, Kanagawa, Japan) via a CCD camera (GE Healthcare, Tokyo, Japan) as described previously [34,35]. The chemoattracted extent of T cells was plotted and analyzed by Image J software (National Institutes of Health). The apparatus consisted of front and back chambers connected by a microchannel. A 1-μl suspension of $CD4^+$ or $CD8^+$ T cells (5×10^6 cells/ml) from patients with acute-type AA or chronic-type AA was placed in the front chamber, and 1 μl of either recombinant human CXCL10 (10 μg/ml) (R&D, MN, U.S.A.) or recombinant human CCL17 (25 μg/ml) (R&D) was injected into the back chamber to initiate chemotaxis under the concentration gradient in the channel. The length of the terrace between the 2 microchannels was 260 μm. Data were analyzed with Image J software (National Institutes of Health) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie, Orsay, France).

2.7. F-actin polymerization

Phalloidin binds to polymeric and oligomeric forms of actin, but it does not bind to monomeric actin. Therefore, the level of polymerized actin was determined by staining cells with phalloidin as described previously [35]. Cells were permeabilized in a Cytofix/Cytoperm solution for 20 min, washed in Perm/Wash buffer for 10 min, and incubated with 5 μg/ml FITC-conjugated phalloidin for 30 min. All procedures were performed at 4 °C. Cells were analyzed with a FACSCalibur, and the level of actin polymerization was expressed as mean fluorescence intensity (MFI). Because MFI varied among each cell type, the percentage of fluctuation was calculated as the ratio before and after treatment with chemokines as follows: %MFI = MFI of treated cells/MFI of control, non-treated cells.

2.8. Statistical analysis

All values were expressed as mean ± standard deviation (SD). Data were analyzed with the one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. High frequencies of circulating $CXCR3^+CD4^+$ T cells in acute-phase AA and $CXCR3^+CD8^+$ T cells in chronic-phase AA

The acute and chronic phases of AA were defined as the hair loss conditions within 6 months and longer than 6 months, respectively [36]. Not only the disease duration but also the alopecia areas were different between the two groups. While the acute-phase AA exhibited 1–20 alopecia lesions, the chronic-phase AA was represented by alopecia universalis. All of the patients were treated only with topical corticosteroids or untreated, and had never been applied with systemic immunosuppressants such as corticosteroids and cyclosporine.

We first examined the frequencies of $CXCR3^+CD4^+$, $CCR4^+CD4^+$, $CXCR3^+CD8^+$ and $CCR4^+CD8^+$ T cell subsets, representing Th1, Th2, Tc1 and Tc2 cells, respectively [37,38], in PBMCs of 7 acute-phase and 7 chronic-phase AA patients, and 7 healthy volunteers. The percentage of $CXCR3^+CD4^+$ T cells was significantly higher in acute-phase AA patients than in chronic-phase AA patients or healthy control (Fig. 1A). On the other hand, the frequencies of $CXCR3^+CD8^+$ T cells were significantly higher in chronic-phase AA than in acute-phase AA or health control (Fig. 1B). $CCR4^+CD4^+$ cells were significantly increased in both acute and chronic-phases of AA compared to healthy control, and there was no statistically significant difference between the acute and chronic groups

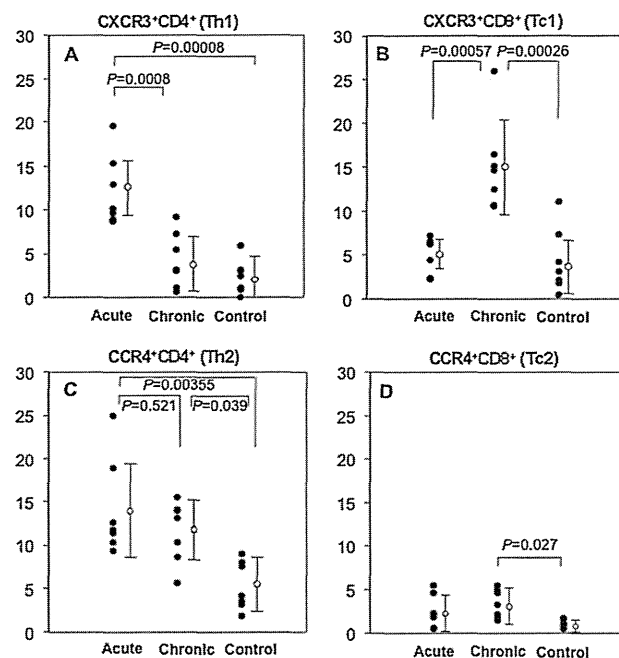

Fig. 1. Frequencies of $CXCR3^+CD4^+$, $CXCR3^+CD8^+$, $CCR4^+CD4^+$, and $CCR4^+CD8^+$ T cells in peripheral blood of acute-phase and chronic-phase AA patients. PBMCs were taken from acute-phase ($n = 7$) and chronic-phase ($n = 7$) AA patients and normal control subjects ($n = 7$). The percentages of $CXCR3^+CD4^+$ (A), $CXCR3^+CD8^+$ (B), $CCR4^+CD4^+$ (C), and $CCR4^+CD8^+$ (D) T cells were analyzed by flow cytometry. Open circles and vertical bars represent mean ± SD.

(Fig. 1C). The frequencies of CCR4$^+$CD8$^+$ T cells were very low in all three groups (Fig. 1D). Thus, the acute-phase and chronic-phase are characterized by the increased frequencies of circulating Th1 and Tc1 cells, respectively.

3.2. Mean intensity of CXCR3 and CCR4 is significantly higher in acute-phase AA patients

The mean intensity (M.I.) of CXCR3 and CCR4 in PBMCs was also studied. The M.I. of CXCR3 in acute AA (66.2 ± 16.4) was significantly higher than that in healthy controls (23.74 ± 5.87) (P = 0.019) but not significantly higher as compared to chronic AA (26.9 ± 6.32) (P = 0.16) (Fig. 2A). Representative data are shown in Fig. 2B. The M.I. of CCR4 in acute AA (101.5 ± 15) was also significantly higher than in healthy controls (38.9 ± 8.7) (P = 0.013) but not significantly higher as compared to chronic AA (64.6 ± 31.2) (P = 0.23) (Fig. 2C). Representative data are shown in Fig. 2D.

3.3. Dominant infiltration of CXCR3-bearing CD4$^+$ and CD8$^+$ T cells around HFs in acute-phase AA and sustained infiltration of CD8$^+$ T cells in chronic-phase AA

It is well known that T cells attack HFs in AA lesions. Biopsy specimens were taken from 3 acute-phase and 3 chronic-phase AA patients and 3 healthy control subjects, and they were immunohistochemically stained with antibodies to CD4, CD8, CXCR3 and CCR4. As shown by a representative case of acute-phase AA, both CD4$^+$ T cells (Fig. 3A) and CD8$^+$ T cells (Fig. 3B) infiltrated around HFs with invasion into the outer root sheath. CXCR3$^+$ T cells markedly infiltrated in and around hair bulbs (Fig. 3C) compared to CCR4$^+$ T cells (Fig. 3D). In chronic-phase AA, while CD4$^+$ T cells around hair follicles were decreased in number (Fig. 3E), CD8$^+$ T cells remained continuously infiltrating (Fig. 3F). In this phase, CXCR3$^+$ T cells were more densely present (Fig. 3G) than CCR4$^+$ T cells (Fig. 2H). In healthy controls, CD4$^+$, CD8$^+$, CXCR3$^+$ and CCR4$^+$ cells were sparsely observed around anagen hair follicles (Fig. 3I–L).

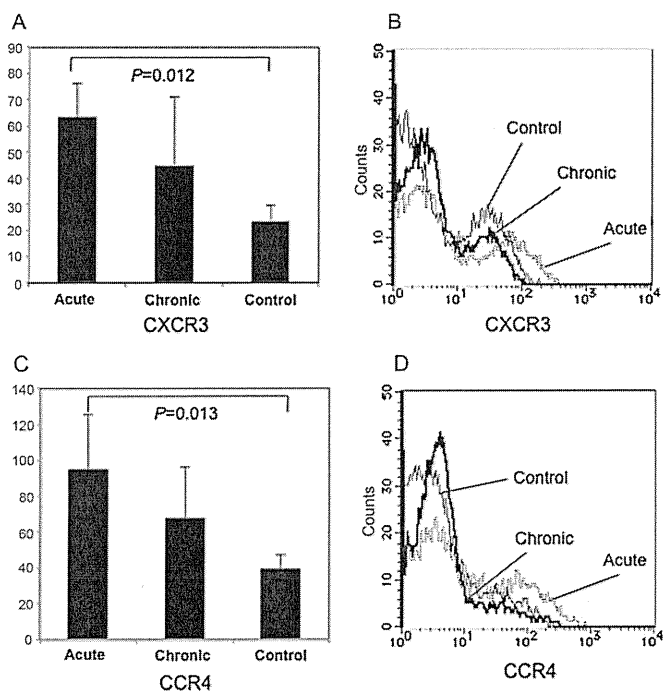

Fig. 2. Mean intensity of CXCR3 and CCR4 is significantly higher in acute-phase AA patients. PBMCs were taken from acute-phase (n = 7) and chronic-phase (n = 7) AA patients and normal control subjects (n = 7). The M.I. of CXCR3 (A) in PBMCs was studied by flowcytometric analysis. (B) Data shown is representative result of CXCR3 M.I. The M.I. of CCR4 (C) in PBMCs was studied by flowcytometric analysis. (D) Data shown is representative result of CCR4 M.I. (means ± SD).

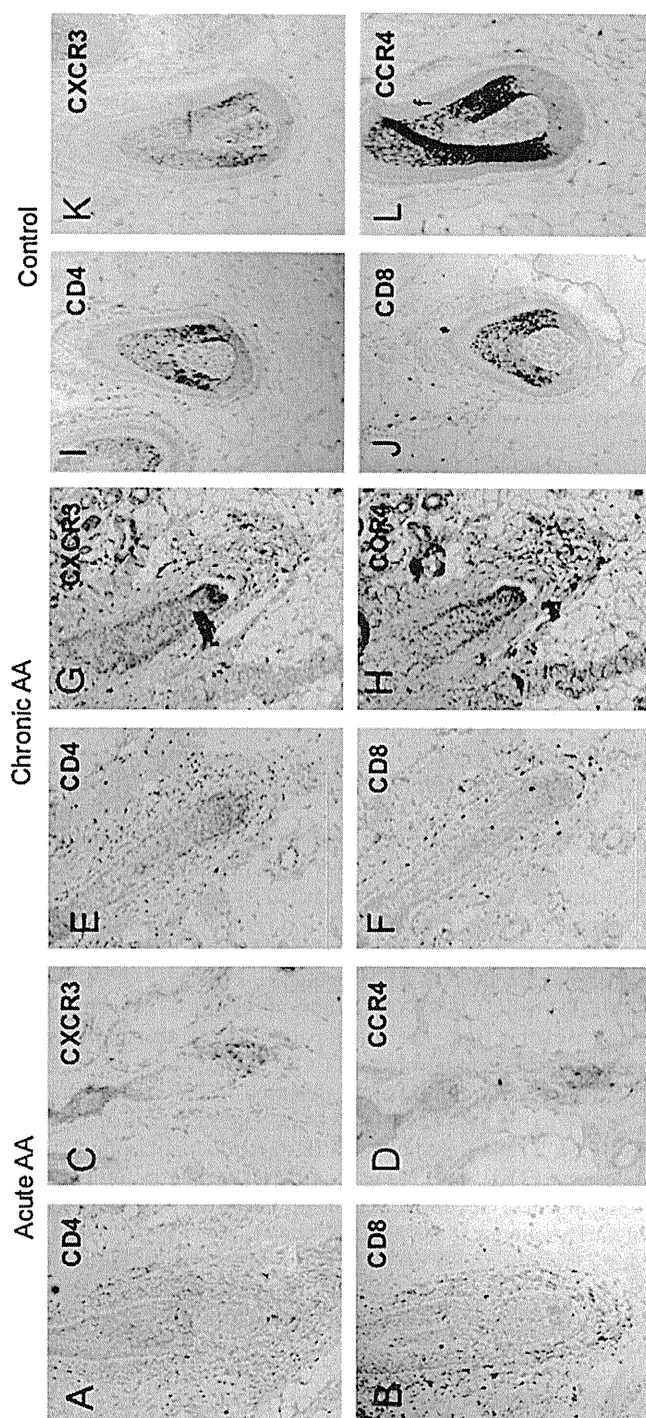

Fig. 3. Immunohistochemical stainings for CD4, CD8, CXCR3, and CCR4 around hair bulbs in acute-phase and chronic-phase AA and normal control. Frozen sections were immunohistochemically stained for CD4 (A), CD8 (B), CXCR3 (C), and CCR4 (D) in acute phase AA, and stained for CD4 (E), CD8 (F), CXCR3 (G), and CCR4 (H) in chronic phase AA, and stained for CD4 (I), CD8 (J), CXCR3 (K), and CCR4 (L) in control.

The average numbers of CD4$^+$, CD8$^+$, CXCR3$^+$, and CCR4$^+$ cells infiltrating around hair bulbs (per 1×10^4 μm^2) were calculated from 5 different areas in 3 patients or control subjects of each group (Fig. 4). The number of CD4$^+$ cells was significantly higher in acute-phase AA than in chronic-phase AA or healthy control. Not only acute-phase but also chronic-phase AA showed significantly high frequencies of CD8$^+$ cells than control. Thus, prolonged

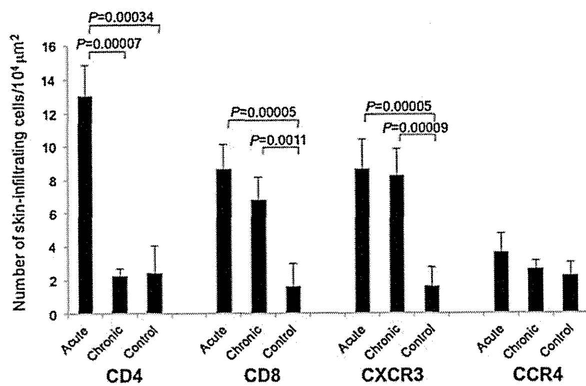

Fig. 4. Prominent number of CD4$^+$, CD8$^+$ and CXCR3$^+$ T cells around hair bulbs in acute-phase AA. Frozen skin sections of acute-phase ($n = 3$) and chronic-phase ($n = 3$) AA and normal control skin ($n = 3$) were stained as shown in Fig. 2. The numbers of CD4$^+$, CD8$^+$, CXCR3$^+$, and CCR4$^+$ T cells infiltrating around hair bulbs were calculated in 5 areas each section and expressed as cell number per 10^4 mm^2. Error bars represent SD.

accumulation of CD8$^+$ T cells even in chronic phase of AA was apparent. CXCR3$^+$ T cells infiltrated in both acute-phase and chronic-phase AA at higher frequencies than control. The number of CCR4$^+$ T cells was lower than that of CXCR3$^+$ T cells in both groups and slightly increased in acute-phase AA.

3.4. Phenotype of T cells isolated from a skin specimen of acute-phase AA in a comparison with PBMC phenotype

To further characterize the phenotype of locally accumulating T cells, skin-infiltrating cells were obtained from a biopsied specimen of an acute-phase AA patient by culturing the specimen in complete RPMI medium for 7 days. The lymphocytes that migrated and emerged in the medium were subjected to flow cytometric analysis in the frequencies of CXCR3$^+$CD4$^+$ cells, CCR4$^+$CD4$^+$ cells, CXCR3$^+$CD8$^+$ cells, and CCR4$^+$CD8$^+$ cells. PBMCs freshly isolated from the same patient were also monitored in parallel. The percentage of CXCR3$^+$CD4$^+$ T cells in the AA skin lesion (21.5%; Fig. 5A) preferentially infiltrated in the skin lesion when compared to the patient's PBMCs (9.2%, Fig. 5B). On the other hand, CCR4$^+$CD4$^+$ T cells were not elevated in number but rather scarce in AA lesions (2.6%, Fig. 5C) as compared to the patient's PBMCs (14.2%, Fig. 5D). CXCR3$^+$CD8$^+$ cells infiltrated in the lesional skin at a higher frequency (38.7%, Fig. 5E) than that of the patient's PBMCs (24.2%, Fig. 5F). CCR4$^+$CD8$^+$ T cells were scarce in both the skin lesion (2.7%, Fig. 5G) and the blood (2.9%, Fig. 5H). These data confirmed the preferential migration of Th1 and Tc1 cells in acute-phase AA lesions.

3.5. High expression of CXCL10 in hair follicle epithelial cells and interstitial cells of acute-phase AA lesions

We next investigated the expression of CXCR3 ligand CXCL10 in acute AA lesions. Immunohistochemical study demonstrated that there was strong CXCL10 immunostaining in HFs of acute-phase AA lesions compared to normal skin (Fig. 6A). The CXCL10 immunoreactivity was found in the outer and inner root sheath epithelial cells, dermal papilla cells, and juxta-follicular interstitial cells, while normal HFs showed slight CXCL10 staining in the outer root sheath. By RT-PCR, mRNA expression of CXCL10 (229 bp) was observed in the lesional skin of acute-phase AA but not in a normal skin (Fig. 6B). The expression of Th2 chemokine CCL17 was very weak in AA lesions (data not shown). Real-time RT-PCR analysis of AA lesions in 3 patients with acute-phase AA showed significantly higher levels of CXCL10 mRNA expression than healthy control (Fig. 6C).

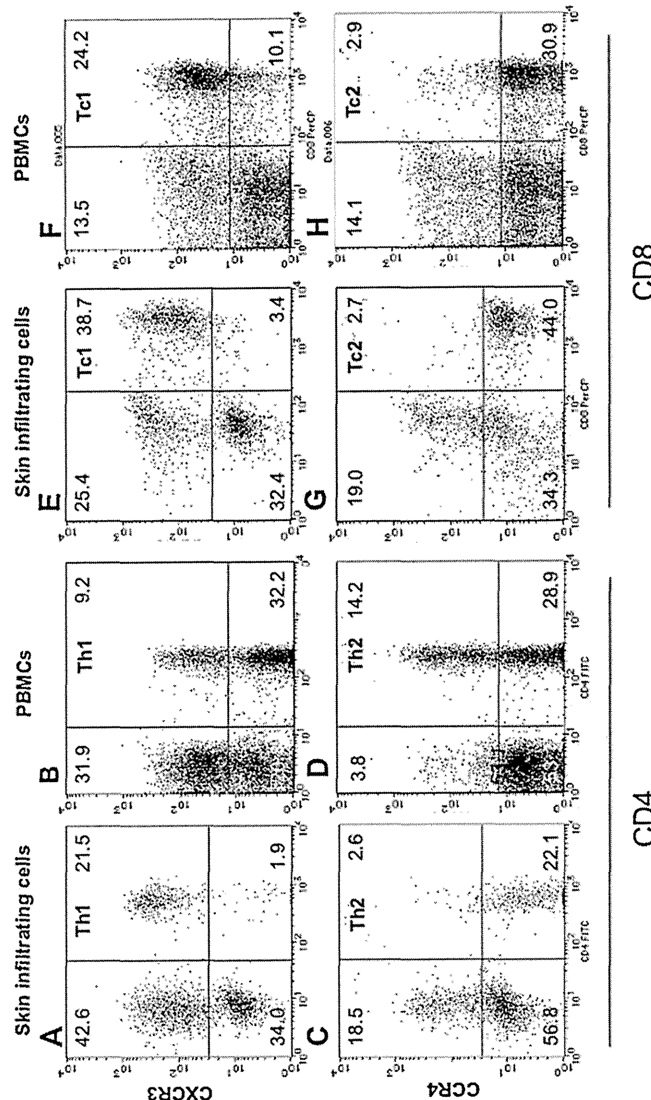

Fig. 5. Phenotypes of lymphocytes isolated from AA lesion and PBMCs in an acute-phase AA patient. A biopsied skin specimen was taken from acute-phase AA lesion and cultured in complete RPMI-1640. Lymphocytes that emigrated into the medium were subjected to double-staining and subsequent flow cytometric analysis to see the percentages of CXCR3$^+$CD4$^+$ (A), CCR4$^+$CD4$^+$ (C), CXCR3$^+$CD8$^+$ (E), and CCR4$^+$CD8$^+$ (G) cells. PBMCs freshly isolated from the same patient were also monitored for CXCR3$^+$CD4$^+$ (B), CCR4$^+$CD4$^+$ (D), CXCR3$^+$CD8$^+$ (F), and CCR4$^+$CD8$^+$ (H) cells.

3.6. High migratory ability of circulating CD4$^+$ and CD8$^+$ T cells toward CXCL10 in acute-phase AA patients

The functional ability of circulating T cells to migrate toward chemokines was examined by direct chemotaxis assessment in acute-phase and chronic-phase AA patients. Migrating cells were photo-documented by EZ-TAXIScan, and the chemoattracted extent of T cells was plotted and analyzed by Image J software. CD4$^+$ T cells were isolated from PBMCs of AA patients and healthy individuals and subjected to the chemotaxis assay. CD4$^+$ T cells from a patient with acute-phase AA migrated roughly straightly to the CXCL10 top chamber (Fig. 7A top left), while those from a normal control subject reached the CXCL10 chamber with wondering movement (Fig. 7A bottom left). The migration of AA patient's CD4$^+$ T cells to CCL17 was not accelerated (Fig. 7A right). The migration traces of CD4$^+$ and CD8$^+$ T cells are exhibited in the functions of time and distance (Fig. 7B and C left), and the velocity levels were calculated in 5 acute-phase AA patients, 5 chronic

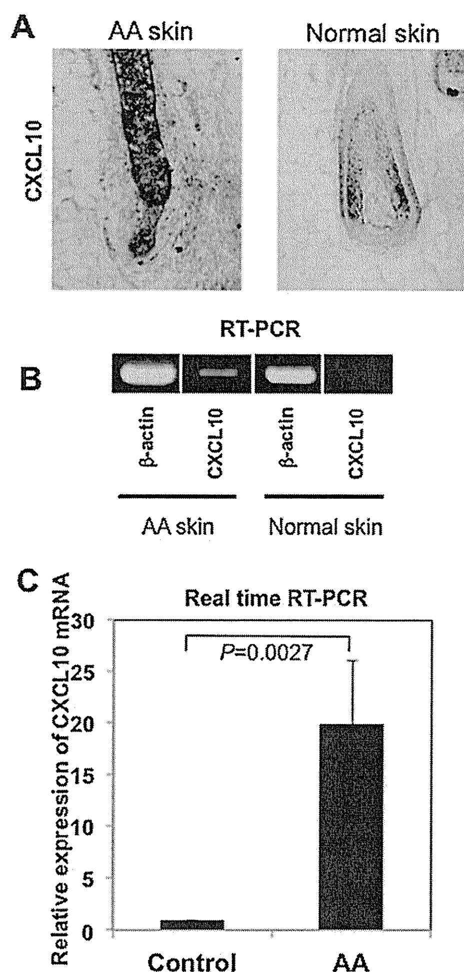

Fig. 6. Immunohistochemical staining and mRNA expression of CXCL10 in acute-phase AA lesions. (A) Frozen sections of an acute-phase AA specimen and normal skin were immunohistochemically stained for CXCL10. (B) Biopsied specimens from an acute-phase AA lesion and normal skin were analyzed by RT-PCR for mRNA expression of CXCL10 and (C) Skin specimens from acute-phase AA lesions ($n = 3$) and normal skin ($n = 3$) were analyzed by real-time RT-PCR for mRNA expression of CXCL10. Error bars represent SD.

phase AA patients, and 5 normal control subjects (Fig. 7B and C right). The velocity of $CD4^+$ T cells toward CXCL10 was significantly higher in acute-phase AA than in chronic-phase AA or control (Fig. 7C right). Similarly, $CD8^+$ T cells of acute-phase AA effectively migrated to CXCL10 as compared to those of chronic-phase AA and healthy control (Fig. 7C right).

F-actin plays an important role in T-cell chemotaxis [39–41]. FACS analysis revealed that the expression of F-actin in $CD4^+$ T cells was elevated in acute-phase AA patients (Fig. 7D left). The MFIs of 3 acute-phase and 3 chronic-phase AA patients was higher than those of healthy control (Fig. 7D right). Thus, the higher expression of F-actin may induce strong velocity of $CXCR3^+$ T cells to CXCL10 in acute-phase AA patients.

4. Discussion

In this study, we demonstrated that the frequency of $CXCR3^+CD4^+$ T cells in the peripheral blood was significantly higher in acute-phase AA patients than normal subjects, suggesting that the immunological state of circulating T cells is polarized to Th1 cells in acute-phase AA. This Th1 skewing is more accentuated in the skin lesions of acute-phase AA, as CXCR3-bearing $CD4^+$ and $CD8^+$ T cells preferentially infiltrated in the lesional skin. In the healthy human scalp skin, both $CXCR3^+$ and

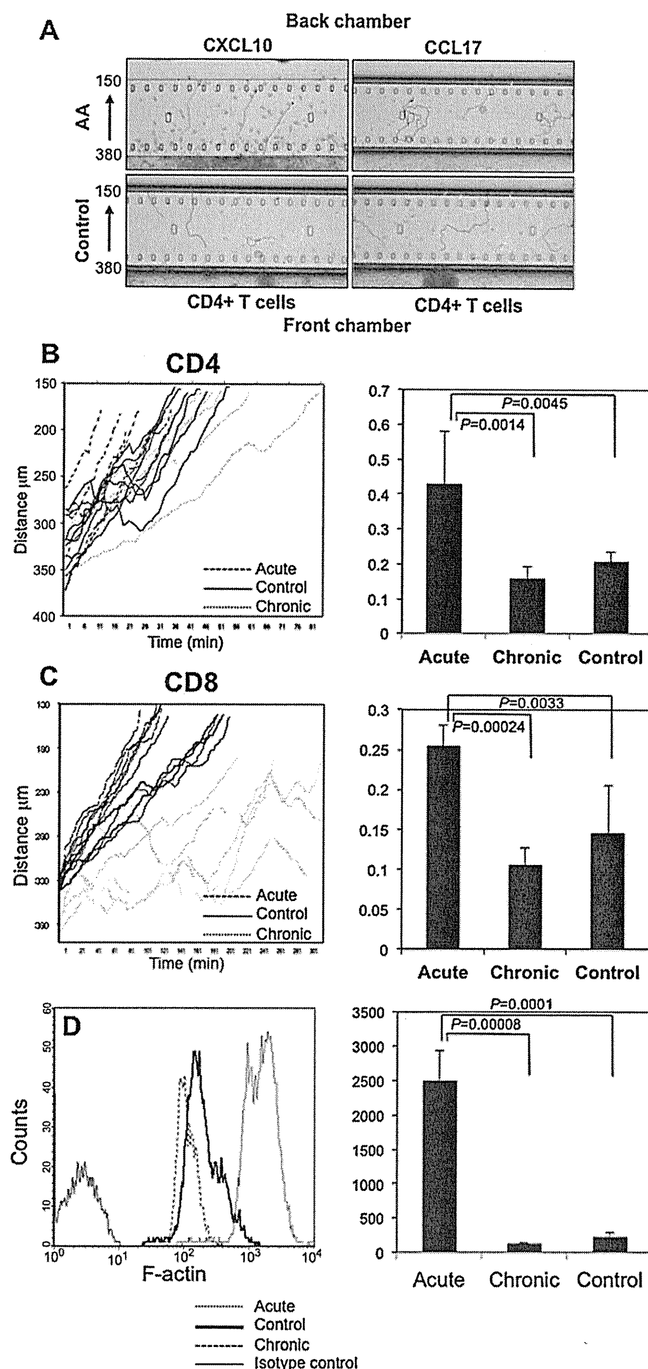

Fig. 7. Migration to CXCL10 of circulating $CD4^+$ and $CD8^+$ T cells from acute-phase and chronic-phase AA patients. (A) $CD4^+$ and $CD8^+$ T cells were purified from PBMCs of patients with acute-phase or chronic-phase AA. Their chemotactic activities toward CXCL10 and CCL17 were analyzed by EZ-TAXIScan. The top and bottom chambers contained chemokine (CXCL10 or CCL17) and T cells ($CD4^+$ or $CD8^+$ T cells), respectively. The trace lines show the movement of T cells from the front chamber to the back chamber. The migrating $CD4^+$ (B) and $CD8^+$ (C) T cells were plotted in the function of time (left panel). The velocity levels of $CD4^+$ (B) and $CD8^+$ (C) T cells toward CXCL10 were calculated (right panel) in acute-phase ($n = 5$) and chronic-phase ($n = 5$) AA patients and normal control subjects ($n = 5$). Error bars represent SD. (D) F-actin expression in acute-phase AA and healthy control was analyzed by flow cytometric analysis. The MFI of F-actin expression was higher in an acute-phase AA patient (2503 ± 426.9) than in chronic phase-AA (133.9 ± 18.2) and a healthy control (233.4 ± 65.5) (left). The mean ± SD of F-actin expression levels were measured in acute-phase ($n = 3$), chronic-phase ($n = 3$) AA patient and healthy control subjects ($n = 3$).

CCR4$^+$ T cells were scarcely detected around hair bulbs [32]. We isolated T cells from the acute-phase AA skin and examined their phenotype by flow cytometry. CXCR3$^+$CD4$^+$ and CXCR3$^+$CD8$^+$ T cells were condensed in the lesional skin when compared with the peripheral blood lymphocytes from the same patient. Recent study also reported increased expression of CXCR3 and its ligands, CXCL9 and CXCL10, during the development of alopecia areata in the mouse [42]. Surely, not all of the CD4$^+$CXCR3$^+$ T cells are autoreactive CD4$^+$ T cells. However, some of these accumulated CD4$^+$ T cells may be autoreactive or helper T cells in the pathogenesis of AA. In chronic-phase AA, T cells infiltrating around hair bulbs have been thought to decrease in number, and thus, characterization of their phenotype has been ignored. Recent study also regarded CD8$^+$ T cells as important in pathomechanisms of AA [43]. Our study revealed sustained accumulation of CXCR3$^+$CD8$^+$ Tc1 cells in chronic-phase AA. It is suggested that Tc1 cells continuously attack hair follicles even after the acute phase, resulting in persistent hair loss in chronic-phase AA, as typically seen in alopecia universalis.

Both CXCR3 ligand CXCL10 and CCR4 ligand CCL17 were faintly expressed around anagen HFs in healthy control. In acute-phase AA lesions, CXCL10, but not CCL17, was highly expressed in the hair follicles and surrounding interstitial cells, although not all of CXCL10 is produced in only hair follicles. It seems that CXCL10 contributes to the accumulation of CXCR3$^+$ Th1 and Tc1 cells, leading to a dense perifollicular lymphocytic infiltrate called "swarm of bees" because main source of CXCL10 is likely from hair follicles (Fig. 6A). The chemotactic velocities of circulating CD4$^+$ and CD8$^+$ T cells to CXCL10 in acute-phase AA were higher than chronic-phase AA and healthy control, suggesting that Th1 and Tc1 are activated in the peripheral blood of acute-phase AA patients. The increased level of F-actin and the high expression of CXCR3 may be involved in the mechanism of activated chemotaxis in acute-phase AA. Since the chemotaxis of those T cell subsets was not enhanced in chronic-phase AA, the continuous accumulation of Tc1 cells in chronic-phase is not attributable to their chemotactic activity. The interaction between Tc1 and relieved expression of MHC class I may allow the prolonged infiltration of Tc1 cells. Such a long-lasting CD8$^+$ T cell infiltrate in the skin has been reported in skin disorders with lichenoid tissue reaction [44,45].

Several hypotheses for the mechanisms of AA have been put forward [46,47]. The T-cell-mediated autoimmune theory with the collapse of HF-IP is most likely and develops under a genetic background that includes specific HLA alleles [3,4]. Environmental factors, including viral infection, emotional stress, physical stress and diet may initiate not only the production of IFN-γ but also Th1 chemokines CCXCL10, CCL5, and CCL3, which induce the accumulation of CXCR3$^+$ Tc1 cells. AA may be induced by heredity and environmental factors, such as virus infection [48,49], and emotional and physical stresses [50]. These factors may induce IFN-γ production in and around the HF-IP site, which leads to the collapse of immunotolerance [13,14,51]. Subsequently, CD8$^+$ T cells stampede toward the hair bulb and recognize HF autoantigens, resulting in the initiation of AA. HF autoantigens are recognized by autoreactive Tc1 cells via upregulated MHC class I molecules. Our study showed the strong velocity of chemotaxis toward CXCL10 in freshly isolated T cells from AA patients. This strong velocity of T cells may stem from their activated state. The results from F-actin and M.I. level of chemokine receptor indicated that activated T cells showed higher actin-polymerization and expression of chemokine receptors in AA patients. Considering that the tissue accumulation of lymphocytes known as "swarm of bees" is especially observed in the acute-phase of AA, our velocity results seem to coincide with the histopathological findings in the acute-phase AA.

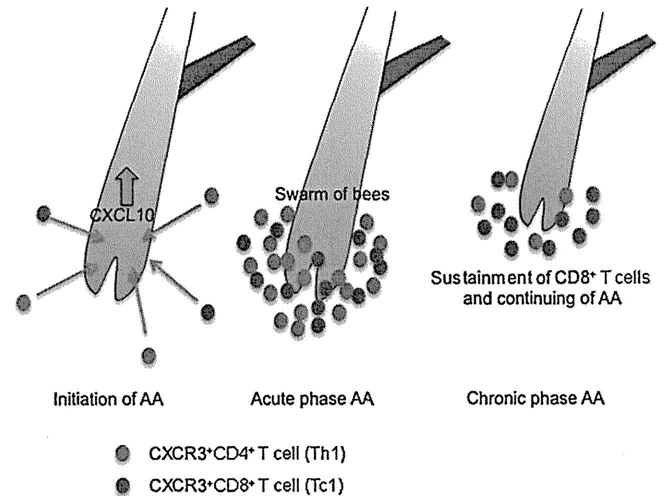

Fig. 8. Summary of immunological changes in alopecia areata. Some stressors may initiate AA with up-regulation of CXCL10 that results in accumulation of CXCR3$^+$CD4$^+$ and CXCR3$^+$CD8$^+$ T cells in and around hair bulbs. CD8$^+$ T cells sustaining around hair bulbs may contribute to continuing of hair loss in chronic phase of AA.

Serum chemokine profiles in AA with atopic dermatitis have been previously reported [28]. The levels of CXCL9/MIG, CCL5, IL-8 and CCL5/eotaxin were increased in AA patients. Furthermore, the increased levels of serum CXCL9 and CCL5 significantly correlated with the disease activity. Another study showed a strong mRNA expression of CXCL9, a moderate expression of CCL2/MCP-1, and a weak expression of CXCL10 [26]. These studies, together with our study, suggest the important role of IFN-γ-related chemokines in the pathogenesis of AA.

In summary, some physical/emotional stressor may initiate AA with up-regulation of CXCL10 that results in accumulation of CXCR3$^+$CD4$^+$ and CXCR3$^+$CD8$^+$ T cells in and around hair bulbs. In acute phase of AA, CD4$^+$ T cells dominantly constitute swarm of bees. Then, CD8$^+$ T cells sustaining around hair bulbs may contribute to continuing of hair loss in chronic phase of AA (Fig. 8).

No novel treatments for AA have been developed within the past several decades. Therapies that are used and modified by dermatologists include contact immunotherapy, ultraviolet light therapy, and corticosteroids (topical, oral or intradermal) [52]. On the basis of the present finding, CXCL10 and CXCR3 are logical therapeutic targets for intervening in chemotaxis using antibody to CXCL10 or CXCR3. In another autoimmune disease, IFN-induced T-cell-attracting chemokines, CXCL10, CXCL9, and CXCL11/I-TAC are predominantly expressed in ductal epithelial cells of Sjögren's syndrome patients [53,54]. When a mouse model for this disease was treated with an antagonist against CXCL10, periductal mononuclear cell infiltration and parenchymal destruction were alleviated [55]. Our study suggests that blockade of Th1 or Tc1 chemokines/chemokine receptors would be a new therapeutic approach for the treatment of AA.

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