Letter to the Editor

Impaired inhibitory function of circulating $CD4^+CD25^+$ regulatory T cells in alopecia areata

Alopecia areata (AA) presents as patches of hair loss, and progresses to include the entire scalp (alopecia totalis; AT) or all body regions (alopecia universaris; AU) in 7% of affected individuals. Ophiasis is distinct pattern of AA with extension of alopecia along the scalp margin. These three forms tend to be recalcitrant and have poor prognostic significance. AA is a chronic, organ-specific, and T cell-mediated autoimmune disease affecting the hair follicles. Impaired immune privilege in the hair follicles likely plays a key role in the primary pathogenesis [1,2]. Loss of immune privilege may be mediated by increased major histocompatibility complex class I under the influence of interferon-gamma, neuropeptides, and immunogenetics, and insufficient activity of regulatory T (Treg) cells [2,3].

Treg cells, a $CD4^+$ T cell subset that acts to suppress immune responses and maintain self-tolerance, have a central role in protecting against autoimmunity. Recent studies suggest that impaired Treg cell function is associated with susceptibility to several autoimmune and inflammatory diseases [4]. C3H/HeJ mice with AA exhibit a reduced number of $CD4^+CD25^+$ Treg cells and impaired Treg cell function [5]. Analysis of peripheral blood mononuclear cells (PBMCs) obtained from AA patients indicates that AA-associated changes in leukocytes are reflected at the systemic level, not localized to the skin [6].

Here we investigated the functional defects of circulating $CD4^+CD25^+$ Treg cells in common clinical types of AA. After obtaining written informed consent, 26 patients with AA who had stopped using immunotherapy, phototherapy, and oral corticosteroids for their AA at least 8 weeks prior to this study, and 8 age-matched healthy controls with no history of autoimmune disease were enrolled. The Ethics Committee of Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, approved the study. Cases were classified according to clinical type: 8 patients with focal AA, 7 patients with ophiasis, and 11 patients with AT/AU (Table 1).

To evaluate Treg cell number, defined as $CD4^+CD25^+Foxp3^+/CD4^+$ cells, PBMCs were stained with antibodies against CD4 and CD25 (DAKO, Carpinteria, CA), and Foxp3 (eBioscience, San Diego, CA), CD127 (Biolegend, San Diego, CA) and subjected to fluorescence-activated cell sorting analysis. To evaluate Treg function,

Table 1
Study population.

No.	Age	Gender	Type	Duration (year)	Lesion area (%)	Previous treatment
1	40	F	Universalis	5	100	Topical and injection steroid/SADBE
2	36	F	Universalis	10	100	Oral steroid/phototherapy/DPCP
3	6	F	Universalis	4	100	Oral steroid/SADBE
4	40	F	Universalis	20	100	None
5	52	F	Universalis	6	100	Phototherapy/SADBE
6	39	M	Universalis	2	100	Cryotherapy
7	34	M	Universalis	0.33	100	None
8	42	M	Universalis	0.41	100	Cryotherapy
9	55	M	Universalis	0.58	100	None
10	25	F	Totalis	6	100	Phototherapy/cryotherapy
11	46	F	Totalis	0.41	100	Cryotherapy
Mean ± SD	37.7 ± 13.4			5.0 ± 5.9	100 ± 0	
12	33	F	Focal	0.33	2	Cryotherapy
13	33	F	Focal	0.08	1	None
14	61	F	Focal	0.33	1	Topical steroid
15	37	F	Focal	0	1	Phototherapy
16	34	M	Focal	0.5	40	Oral and topical steroid
17	34	M	Focal	0.33	70	Topical steroid/cryotherapy
18	39	M	Focal	0.91	70	Topical steroid
19	40	M	Focal	0.91	40	Topical steroid/cryotherapy
Mean ± SD	38.9 ± 9.3			0.4 ± 0.3	28.1 ± 30.9	
20	36	F	Ophiasis	2	5	Oral steroid
21	30	F	Ophiasis	4	40	Topical steroid
22	31	M	Ophiasis	1	30	Topical steroid
23	31	F	Ophiasis	0.16	30	Topical steroid
24	29	F	Ophiasis	4	40	Cryotherapy
25	65	F	Ophiasis	1	10	None
26	26	F	Ophiasis	1	80	Cryotherapy
Mean ± SD	35.4 ± 13.4			1.9 ± 1.5	33.6 ± 24.6	
1	37	M	Control			
2	31	M	Control			
3	23	F	Control			
4	49	F	Control			
5	44	F	Control			
6	52	F	Control			
7	33	F	Control			
8	32	M	Control			
Mean ± SD	37.6 ± 9.9					

PBMCs were separated as $CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells using a $CD4^+CD25^+$ regulatory T cell Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ T cells were incubated in 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Life Technologies, Paisley, UK) at 37 °C for 10 min and activated with anti-CD3 and anti-CD28-bound beads (Miltenyi Biotec) for 4 days with and without $CD4^+CD25^+$ T cells ($CD4^+CD25^-$ T cells: $CD4^+CD25^+$ T cells = 2×10^5:1×10^3) in serum-free X-VIVO15 medium (Lonza, Basel, Switzerland). Treg cell suppressive ability was determined based on the Treg functional ratio by comparing the proliferative rate of $CD4^+CD25^-$ T cells cultured with and without $CD4^+CD25^+$ T cells, as previously reported [7].

Although the Treg cell count tended to be increased in all the AA patients compared with controls, the difference was not statistically significant ($4.69 \pm 1.24\%$ *vs.* $3.81 \pm 1.12\%$, $p = 0.074$; Fig. 1a). There was no significant difference between the three types of AA and controls (Fig. 1c). The Treg functional ratio, however, was significantly lower in all the AA patients than in controls ($69.87 \pm 23.76\%$ *vs.* $89.30 \pm 6.98\%$, $p = 0.038$; Fig. 1b). All the AA patients exhibited impaired Treg cell function compared with controls. Treg cell function differed significantly between ophiasis patients and controls, and between with focal AA patients and ophiasis patients (Fig. 1d).

$CD4^+CD25^+$ Treg cell number and function in AA patients ($n = 43$) were first examined by Zoller et al. [8] in 2004, using an *in vitro* proliferation assay with ^{3}H-thymidine incorporation. In contrast to the mouse model of AA, the $CD4^+CD25^+$ Treg cell number was significantly increased in AA patients. In our study, using Foxp3 as a specific Treg cell marker, we found no difference in Treg cell number between total AA patients and controls. The slight increase in the Treg cell number might be a compensatory mechanism for impaired Treg cell function. Zoller et al. [8] also evaluated $CD4^+CD25^+$ Treg cell function based on the proliferative activity of PBMC, $CD8^+$, $CD4^+CD25^-$ T cells and found that inhibition of those cells proliferation in patients with progressive AA was significantly reduced compared with healthy controls; patients with stable or regressive AA, however, did not differ from controls. In this proliferation assay, suppressive capacity might be underestimated based on proliferation of the Treg cells themselves, and ^{3}H-thymidine might not have been added at the time of maximal proliferation throughout the culture period [4,7]. Our study using CFSE revealed that $CD4^+CD25^+$ Treg cell inhibitory activity was further reduced in ophiasis patients than in focal AA patients and controls, suggesting that $CD4^+CD25^+$ Treg cell dysfunction plays a particularly important role in ophiasis, and the level of dysfunction corresponds clinically with an active or progressive state. However, AT/AU, another poor prognostic factor, was not related to Treg cell dysfunction and might be corresponded to a stable disease state. To our knowledge, this is the first *in vitro* suppression assay based on the dilution of fluorescent labeled CFSE in human AA. Together these findings suggest that the decreased inhibitory function of Treg cells is related to the disease activity state, and that the ophiasis form is more strongly related to the disease activity state. We also conducted a comixing experiment using allogeneic $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells in two patients to test whether the decreased inhibitory function was due to increased resistance of the effector $CD4^+CD25^-$ T cells [4]. The findings revealed no increased resistance of these effector T cells to $CD4^+CD25^+$ Treg cells in AA patients (data not shown). Because recent evidence suggests $CD25^+CD127^{low}$ cells manifested suppressive activity [9], $CD4^+CD25^+$ cells isolated using beads from three patients and controls were stained with CD127 antibody. There was no difference between AA patients and controls (Supplementary Fig. 1).

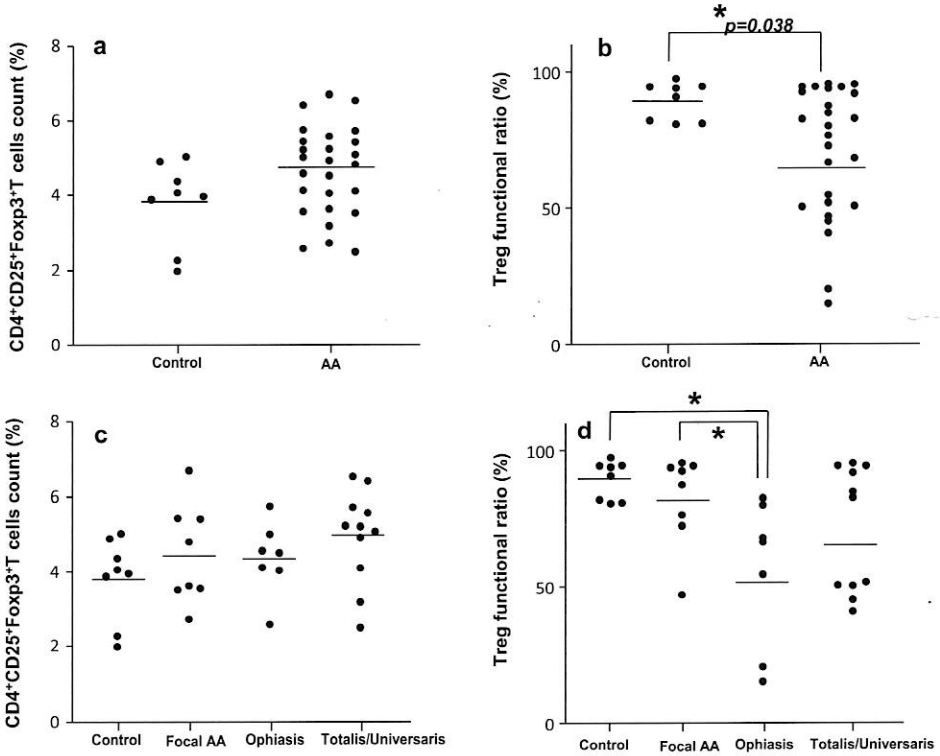

Fig. 1. $CD4^+CD25^+Foxp3^+$ T cell counts (a) and Treg functional ratio (b) in total AA patients and controls (Mann–Whitney *U*-test. $^*P < 0.05$). $CD4^+CD25^+Foxp3^+$ T cell counts (c) and Treg functional ratio (d) in each of the three types of AA and controls (Dunnett's test). Treg functional ratio = (1 − proliferative response of $CD4^+CD25^-$ T cells with $CD4^+CD25^+$ T cells/proliferative response of $CD4^+CD25^-$ T cells without $CD4^+CD25^+$ T cells) × 100 (%).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.01.006

In conclusion, we suggest that $CD4^+CD25^+$ Treg cells function rather than number has a key role in determining susceptibility to AA, especially the ophiasis type. Treatment modalities for the management of AA, such as phototherapy and vitamin D [10]) therapy that may restore the regulatory activity of Treg cells, warrant further exploration.

Acknowledgment

This work was supported in part by Grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

[1] Christoph T, Muller-Rover S, Audring H, Tobin DJ, Hermes B, Cotsarelis G, et al. The human hair follicle immune system: cellular composition and immune privilege. Br J Dermatol 2000;142:862–73.
[2] Gilhar A, Paus R, Kalish RS. Lymphocytes, neuropeptides, and genes involved in alopecia areata. J Clin Invest 2007;117:2019–27.
[3] Ito T, Ito N, Bettermann A, Tokura Y, Takigawa M, Paus R. Collapse and restoration of MHC class-I-dependent immune privilege: exploiting the human hair follicle as a model. Am J Pathol 2004;164:623–34.
[4] Buckner JH. Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol 2010;10:849–59.
[5] Zoller M, McElwee KJ, Engel P, Hoffmann R. Transient CD44 variant isoform expression and reduction in CD4(+)/CD25(+) regulatory T cells in C3H/HeJ mice with alopecia areata. J Invest Dermatol 2002;118:983–92.
[6] Zoller M, McElwee KJ, Vitacolonna M, Hoffmann R. The progressive state, in contrast to the stable or regressive state of alopecia areata, is reflected in peripheral blood mononuclear cells. Exp Dermatol 2004;13:435–44.
[7] McMurchy AN, Levings MK. Suppression assays with human T regulatory cells: a technical guide. Eur J Immun 2012;42:27–34.
[8] Zoller M, McElwee KJ, Vitacolonna M, Hoffmann R. Apoptosis resistance in peripheral blood lymphocytes of alopecia areata patients. J Autoimmun 2004;23:241–56.
[9] Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006;203:1693–700.
[10] Chambers ES, Hawrylowicz CM. The impact of vitamin D on regulatory T cells. Curr Allergy Asthma Rep 2011;11:29–36.

Bong Seok Shin[a,b,*], Takuya Furuhashi[a], Motoki Nakamura[a], Kan Torii[a], Akimichi Morita[a]
[a]*Department of Geriatric and Environmental Dermatology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*;
[b]*Department of Dermatology, Chosun University Medical School, 588 Seosuk-dong, Dong-gu, Gwangju, Republic of Korea*

*Corresponding author. Tel.: +81 52 853 8261; fax: +81 52 852 5449
E-mail address: derm75@chosun.ac.kr (B.S. Shin)

8 May 2012

http://dx.doi.org/10.1016/j.jdermsci.2013.01.006

Letter to the Editor

Three-dimensional imaging of epidermal keratinocytes and dermal vasculatures using two-photon microscopy

The skin structures have conventionally been observed by histology; however, the morphology of the skin components may be distorted as a result of the multiple fixation and staining steps. In addition, stationary sections only provide two-dimensional information, thus the development of a method to observe bona-fide skin structures in unfixed specimen should widen our insight on skin biology. Two-photon microscopy (TPM) is a powerful tool for deep-tissue observation in living animals [1]. Recently, TPM has been applied to *ex vivo* observation of human skin specimens. Using TPM, the collagen and elastin fibers are detectable by second harmonic generation (SHG) or auto-excited fluorescent signals, respectively [2]. However, other components in the skin are hardly identified in unprocessed samples. In mice, various kinds of intravital labeling strategies have been developed; for example, blood flows were visualized with intravenous injection of fluorescein-conjugated dextran [3] and transgenic animals and adoptive transfer of *in vitro* labeled cells enables a tracking of specified cells [4]. However, these methods are difficult to apply to human and alternative methodology must therefore be developed.

In the present study, we succeeded in visualizing epidermal keratinocytes in human skin biopsy samples using alexa594-conjugated isolectin B4 (IB4; Invitrogen, Carlsbad, CA). To label epidermal keratinocytes, 2 μg IB4 in 40 μL phosphate buffered saline (PBS) was intradermally injected into the remnants of skin biopsy samples with informed patient consent. The samples were then placed at room temperature for 30 min. Next, the skin was observed from the surface side using TPM (IX-81; Olympus, Tokyo, Japan). A vertical slice image showed that IB4 penetrated the epidermis up to 60 μm from the basement membrane (Fig. 1a), which is consistent with the tight junction in the granular layer [5]. Twenty-five micrometers from the skin surface, large and flattened matured keratinocytes were observed (longitudinal diameter = 44.7 ± 4.4 μm) (Fig. 1b). The cellular size was decreased with distance from the surface (17.5 ± 3.5 μm at 40 μm from the surface and 11.1 ± 1.6 μm at 55 μm from the surface of the skin) (Fig. 1c–e).

In addition, we found that the intradermal injection of a lipophilic dye, BODIPY (493/503; Invitrogen, 10 mM in 40 μL PBS), enabled the labeling of epidermal keratinocyte cytoplasm (Fig. 1f). When keratinocyte cytoplasm was simultaneously stained with BODIPY, the identification of individual keratinocytes became much easier because the nuclei of keratinocytes escaped BODIPY staining. In this manner, we sometimes observed keratinocytes that contained two nuclei within an IB4-circumscribed area that might be under division when skin was biopsied (Fig. 1f, arrows).

We then found that a keratinocyte labeling strategy with IB4 was also applicable to murine and we succeeded in the live-visualization of the cell divisions of keratinocytes *in vivo*. Two micrograms of IB4 in 40 μL PBS were subcutaneously injected into the ear of C57BL/6N (purchased from SLC, Shizuoka, Japan). Thirty minutes later, image stacks were acquired every five minutes. A large, round IB4-circumscribed keratinocyte was divided into two in a sequential movie (Fig. 1g and Supplementary movie 1).

Supplementary data related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.01.005.