Defective Epidermal Innate Immunity and Resultant Superficial Dermatophytosis in Adult T-cell Leukemia/Lymphoma

Yu Sawada1, Motonobu Nakamura1, Rieko Kabashima-Kubo1, Takatoshi Shimauchi2, Miwa Kobayashi1, and Yoshiki Tokura2

Abstract

Purpose: Superficial dermatophytosis is quite commonly seen in patients with adult T-cell leukemia/lymphoma (ATLL), as approximately 50% of the patients develop cutaneous mycotic infections. Because superficially infected fungi in the stratum corneum of the epidermis cannot directly contact with T cells infiltrating in the upper dermis, some perturbation of epidermal innate immunity has been postulated. Interleukin (IL)-17–producing helper T cells (Th17) can induce the keratinocyte production of antimicrobial peptides such as human β defensin (HBD)-2 and LL-37, which play an essential role in cutaneous innate immunity.

Experimental Design: We investigated the frequency of circulating Th17 cells, serum levels of cytokines, and epidermal expression of HBD-1, 2, 3, and LL-37 in ATLL patients with or without superficial dermatophytosis.

Results: The frequency of peripheral Th17 cells and the serum level of IL-17 was significantly decreased in ATLL patients, whereas the serum IL-10 and TGF-β1 levels were increased as compared with healthy controls. Furthermore, ATLL patients with dermatophytosis had higher IL-10 and TGF-β1 levels and lower IL-17 levels than did those without dermatophytosis. Immunohistochemical study revealed that the epidermal expression of both HBD-2 and LL-37 were significantly lower in ATLL patients with dermatophytosis than in non-ATLL patients with dermatophytosis.

Conclusions: Taken together, these results suggest that the keratinocyte production of antimicrobial peptides promoted by Th17 cells is reduced in ATLL patients, leading to the perturbed innate immunity and the frequent occurrence of superficial dermatophytosis.

Introduction

Adult T-cell leukemia/lymphoma (ATLL) is a malignancy of mature CD4+ T cells caused by human T-cell lymphotropic virus type 1 (HTLV-1; refs. 1–3). HTLV-1 infection is prevalent in southern Japan, especially in Kyushu area (4, 5), Caribbean country, and Africa (6, 7). On the basis of the number of abnormal lymphocytes, organ involvement, and severity, ATLL is divided into 4 clinical categories: acute type, lymphoma type, chronic type, and smoldering type (Shimoyama’s classification; ref. 8). Superficial dermatophytosis, such as tinea and candidiasis, is quite common in ATLL patients, as approximately 50% of the patients develop cutaneous mycotic infections (9, 10), and the incidence of dermatophytosis of indolent type of ATLL patients was higher than that of aggressive type (10). Dermatophytosis of ATLL patients tended to be intractable even with antifungal treatments, and inflammatory reactions to fungi are less prominent in ATLL patients than non-ATLL dermatophytosis patients (10). These findings have suggested the defective immunity against dermatophytes in ATLL patients. Because superficially infected fungi in the stratum corneum of the epidermis cannot directly contact with T cells infiltrating in the upper dermis, some perturbation of epidermal innate immunity has been postulated. Interleukin (IL)-17–producing helper T (Th17) cells represent a lineage of effector T cells critical in host defense, and dysregulated Th17 cell responses mediate a wide variety of autoimmune and inflammatory conditions such as rheumatoid arthritis (11), inflammatory bowel disease (12), and atopic dermatitis (13). IL-17 stimulates keratinocytes to produce various cytokines, such as granulocyte macrophage colony–stimulating factor, tumor necrosis factor α, CXC
chemokine ligand 10, and vascular endothelial growth factor (13). IL-17 can also enhance the expression of antimicrobial peptides such as human β defensin (HBD)-2 and LL-37 in keratinocytes (14, 15), which play an essential role in cutaneous innate immunity against fungi. These reports suggested that Th17 cells and IL-17 might play an important role in host defense against superficial dermatophytosis.

ATLL cells produce various chemokines and cytokines, such as IL-10 and transforming growth factor (TGF)-β1 (16, 17), which cause immunosuppression in ATLL patients. In particular, IL-10 exerts an inhibitory effect on macrophages and suppresses the cytokine production by Th17 cells (18). In this study, we investigated the frequency of circulating Th17 cells, serum levels of cytokines, and epidermal expression of antimicrobial peptides such as HBDs and LL-37 in ATLL patients.

Materials and Methods

Patient background and clinical evaluation

Eight ATLL patients with dermatophytosis, 7 ATLL patients without dermatophytosis, 6 non-ATLL patients with dermatophytosis, and 8 healthy controls were enrolled in this study. The diagnosis of ATLL was based on the clinical features, histopathologically, cytologically proven mature T-cell malignancy, presence of anti–HTLV-1 antibody, and monoclonal integration of HTLV-1 proviral DNA into blood and/or skin tumor cells as described previously (2, 8, 19, 20). Dermatophytosis was diagnosed with microscopic examinations using KOH preparations. Five of 8 ATLL patients with dermatophytosis had tinea unguium in all nails, and 6 patients also had tinea corporis. None of the patients took systemic steroids or immunosuppressants. The study design was approved by the review board of University of Occupational and Environmental Health. Skin biopsies and blood examinations were carried out after informed consent had been obtained from the patients. All experiments were conducted in accordance with the Declaration of Helsinki Principles.

Table 1. Statistical analysis of characteristics

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<td>2.63 ± 2.00</td>
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NOTE: Intragroup comparisons were carried out using the χ² test and one-way ANOVA. Each clinical factor showed no statistical differences between each group except the percentage of atypical cells and CD4/CD8 ratio.
Patient characteristics
The patients were categorized into 2 groups of age: 70 years or older and younger than 70 years. Lymphocytosis was defined as total lymphocyte count more than 2.0 × 10^9/L. We also examined the percentage of atypical cells, CD4/CD8 ratio, and percentages of CD4^+CD25^+ cells and CD4^+CCR4^+ cells.

Flow cytometric analysis of CD4^+ T cells, CD8^+ T cells, CD4^+CD25^+ T cells, and CD4^+CCR4^+ T cells
Peripheral blood was obtained by vein puncture from the subjects with heparin as anticoagulant. Peripheral blood mononuclear cells (PBMC) were purified by a standard method using Ficoll-Paque (GE healthcare Bio-sciences AB; ref. 13) and washed twice with PBS containing 2% fetal calf serum (FCS; Thermo Scientific). HBSS containing 0.1% NaN_3 and 1% FCS was used as the staining buffer. After incubation for 30 minutes with monoclonal antibodies (mAb), 10,000 labeled cells were analyzed on a FACSCalibur (Becton Dickinson) in each sample. Fluorescein isothiocyanate (FITC) conjugated mAb to CD4 (SK3) and phycoerythrin (PE) labeled mAb to CD8 (SK1), CD25 (2A3), and CCR4 (1G1) were purchased from BD Immunocytometry Systems.

Intracellular cytokine staining of PBMCs
PBMCs were isolated from patients and control subjects by standard Ficoll-Paque method (Phamacia). Intracellular cytokines were stained according to the protocol of Cytofix/C Perm/Wash solution as manufacturer’s protocol (BD Biosciences). They were stained with PE-labeled anti-IL17 mAb (BD Biosciences) and washed twice with PBS containing 2% fetal calf serum (FCS; Invitrogen), 5 x 10^{-5} mol/L 2-mercaptoethanol (Gibco), 2 mmol/L l-glutamine (MP Biomedicals), 25 mmol/L HEPES (Cellgro), 1 mmol/L non-essential amino acids (Gibco), 1 mmol/L sodium pyruvate (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) in a 24-well plate with 10 ng/mL of phorbol-12-myristate 13-acetate (Sigma Chemical Co.) containing 10% FCS (Invitrogen). 5 x 10^{-5} mol/L 2-mercaptoethanol (Gibco), 2 mmol/L l-glutamine (MP Biomedicals), 25 mmol/L HEPES (Cellgro), 1 mmol/L non-essential amino acids (Gibco), 1 mmol/L sodium pyruvate (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) in a 24-well plate with 10 ng/mL of phorbol-12-myristate 13-acetate (Sigma Chemical Co.), 10^{-6} mol/L of ionomycin (Wako), and 0.7 μL of Goldistop (BD Biosciences) for 8 hours. Then, cells were washed and directly stained with PE-labeled anti-IL17 mAb (BD Biosciences) for 20 minutes at 4°C. Fluorescence profiles were analyzed by flow cytometry in FACSCanto (BD Biosciences).

Measurement of serum cytokine levels
Peripheral blood samples were transferred to serum separating tubes and centrifuged at 1,000 × g at 20°C for 20 minutes after clot formation. The supernatants were carefully harvested, and aliquots were frozen at −80°C until analysis. The serum IL-17, IL-10, and TGF-β1 levels were measured by ELISA using the Human IL-10 or TGF-β1 Immunoassay Kit (R&D Systems) and the Human IL-17A Immunoassay Kit (eBioscience), respectively, according to the manufacturer’s instructions.

Skin biopsy and immunohistochemical staining
Skin biopsy specimens were taken from dermatophytosis lesions of ATLL patients (n = 8) and non-ATLL patients (n = 6) and from dermatophytosis-unaffected sites of ATLL patients (n = 7) and non-ATLL patients (n = 8). Formalin fixed, paraffin-embedded tissue sections were first deparaffinized and dehydrated, then washed in TBS (Quartett GmbH) containing 0.1% bovine serum albumin 3 times. Endogenous peroxidase activity was quenched by incubating the slides in a solution of 700 μL H_2 O_2 (30% WAKO) in 70 mL ethanol (WAKO). To carry out antigen retrieval, the sections were pretreated with pepsin (0.4%) for 30 minutes at 37°C. After blocked with normal rabbit or goat serum for 20 minutes, the sections were incubated with 1:50 diluted primary antibody against HBD-1, 2, 3, and LL-37 (Santa Cruz Biotechnology) at room temperature for 60 minutes. The slides were washed in TBS buffer 3 times and incubated with the horseradish peroxidase-conjugated rabbit anti-human IgG or goat anti-human IgG antibody (DAKO) at room temperature for 30 minutes.
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minutes. Afterwards, the slides were washed with TBS 3 times and incubated with diamino benzidine tetrahydrochloride as substrate and counterstained with hematoxylin (Merk Eurolab). Negative controls without primary antibody were examined simultaneously in each experiment to verify antibody specificity.

To quantify the staining intensity, digitalized specimens were exported to JPG files by NDP view software (Hamamatsu Photonics). The following processes were carried out with Adobe Photoshop CS (J) (Adobe System, Inc.). Three different areas of the cytoplasm of keratinocytes were selected and expressed as Red channel histograms. In the bar graph, the horizontal and vertical axes represent tone and quantity, respectively. The histogram shows 255 different shades from pitch black (0) to pure white (255), and the number represents the level of brightness of each color. We analyzed the mean intensity of the histogram in the cytoplasm and averaged the value of 3 different areas. To obtain density, we calculated: 255 − “mean” of each color. These values were named as “red density” (RD) and used for further investigation (21).

**Statistical analysis**

The frequency of Th17 cells and CD4⁺CD25⁺ T cells and the serum concentrations of each cytokine were compared between the ATLL with dermatophytosis patients, ATLL without dermatophytosis patients, dermatophytosis patients without ATLL, and healthy controls. All statistical analyses were carried out using GraphPad Prism 4.0. The Student t test was used to calculate statistical differences. All

**Figure 2.** CD4⁺CD25⁺ T-cell frequencies and IL-10 and TGF-β1 levels in peripheral blood. A, the frequencies of CD4⁺CD25⁺ T cells were measured by flow cytometry. B, the serum IL-10 and TGF-β1 levels were measured by ELISA. C, the correlations between the frequency of CD4⁺CD25⁺ T cells and the serum IL-10 (left) and TGF-β1 (right) were analyzed.


Results

**Patients’ background**

The clinical data of all patients are summarized in Table 1. According to the Shimoyama’s classification, all ATLL patients belonged to the smoldering type. All dermatophytosis patients with or without ATLL had *tinea pedis* et *unguium* and/or *tinea corporis*. No dermatophytosis patients regardless of the presence of ATLL had taken any immuno-suppressants, oral steroids, or chemotherapy. The χ² test and one-way ANOVA revealed that in the individual clinical factors including male/female ratio, age, and counts of lymphocytes, CD4⁺CD25⁺ cells, and CD4⁺CCR4⁺ cells, there were no statistical differences between the groups, except for the percentage of atypical cells and CD4/CD8 ratio.

**Absence of dermatophytosis-associated elevations in circulating Th17 cells and serum IL-17 in ATLL patients**

Intracellular cytokine staining of PBMCs revealed that the frequency of circulating Th17 cells of the ATLL patients was significantly lower than that of the healthy controls (P < 0.001; Fig. 1A; Supplementary Fig. S1). Whereas the presence of dermatophytosis was associated with elevation of Th17 cell frequency in the non-ATLL patients, the frequency was significantly lower in ATLL patients with dermatophytosis than in those without dermatophytosis (P = 0.006). Moreover, the IL-17⁺ cell frequencies in the skin biopsies of ATLL with dermatophytosis were lower than those of dermatophytosis (Supplementary Fig. S2A and B). Thus, the results suggested that ATLL patients have a low frequency of circulating Th17 cells, which is further lowered when the patients suffer from dermatophytosis.

In the non-ATLL patients with dermatophytosis, the serum IL-17 level was significantly increased compared with the healthy controls (Fig. 1B). In ATLL patients either with or without dermatophytosis, the IL-17 levels were significantly lower than those in the healthy controls. There was no significant difference in the IL-17 level between ATLL patients with dermatophytosis and those without dermatophytosis. Again, it is suggested that ATLL patients have low levels of IL-17, which cannot be enhanced by the presence of dermatophytosis.

**Increased frequency of circulating CD4⁺CD25⁺ T cells and elevated serum IL-10 and TGF-β1 levels in ATLL patients**

The number of CD4⁺CD25⁺ T cells reflects both malignant ATLL cells and normal regulatory T cells (Treg) with the former being the majority. The frequencies of circulating CD4⁺CD25⁺ T cells in the ATLL patients, irrespective of having dermatophytosis, were significantly higher than those of the healthy controls and the non-ATLL dermatophytosis patients (Fig. 2A). However, there is no significant difference in the number of CD25⁺ cells infiltrating in the skin biopsies among the groups (Supplementary Fig. S2C).

In both ATLL patient groups with and without dermatophytosis, the levels of serum IL-10 (Fig. 2B, left) and TGF-β1 (Fig. 2B, right) were significantly increased compared with healthy controls. Moreover, the elevations of the serum IL-10 and TGF-β1 levels were significantly more prominent in the ATLL group with dermatophytosis than in that without dermatophytosis. In the non-ATLL dermatophytosis patients, the serum TGF-β1 level was significantly higher than that of the healthy controls. When the correlations between the frequency of CD4⁺CD25⁺ T cells and the serum IL-10 or TGF-β1 levels were analyzed, the frequency of CD4⁺CD25⁺ T cells correlated with the serum level of IL-10 (Fig. 2C, left) but not TGF-β1 level (Fig. 2C, right). These data suggested, in association with the increase of CD4⁺CD25⁺ T cell number, regulatory cytokine IL-10 and TGF-β1 are elevated in ATLL patients. It is thought that dermatophytosis is associated more prominently with the increased levels of IL-10 and TGF-β1 in ATLL patients and non-ATLL individuals, respectively.

The correlation between these regulatory cytokines and Th17/IL-17 was also analyzed. The percentage of circulating Th17 cells (Fig. 3, top left) and the serum IL-17 level (Fig. 3, top right) correlated inversely with the serum IL-10 level. On the other hand, there was no significant correlation between TGF-β1 and Th17 cells (Fig. 3, bottom left) or IL-17 (Fig. 3, bottom right), suggesting that IL-10 exerts a more opposite effect on Th17 cells.

**No induction of antimicrobial peptides in ATLL patients with dermatophytosis in association with IL-17 reduction**

We carried out immunohistochemical staining for HBDS and IL-37 in the dermatophyte-infected and noninfected skin samples of ATLL and non-ATLL individuals. Skin
biopsy specimens were taken from dermatophytosis lesions of ATLL patients ($n=8$) and non-ATLL patients ($n=6$) and from dermatophyte-unaffected sites of ATLL patients ($n=7$) and non-ATLL patients ($n=8$). A representative photograph is shown in Fig. 4A, and the mean expression levels in all specimens are in Fig. 4B. In the non-ATLL dermatophytosis lesions, HBD-2 and LL-37, which are IL-17-inducible antimicrobial peptides, were highly expressed in the epidermis. However, in the ATLL patients, these antimicrobial peptides were not induced even in the dermatophytosis lesions. On the other hand, HBD-3 was induced by dermatophyte infection in both non-ATLL and ATLL patients. The HBD-1 expression was unchanged in all groups.

The staining intensity of the antimicropeptides was analyzed as described (21). The relationships between the keratinocyte expression of antimicrobial peptides and the serum IL-17 level were examined. The levels of HBD-2 (Fig. 5, top right) and LL-37 (Fig. 5, bottom right) correlated significantly with the serum IL-17 level. On the other hand, neither HBD-1 (Fig. 5, top left) nor HBD-3 (Fig. 5, bottom left) showed a correlation with the IL-17 level. These results are in agreement with the known notion that the expression...
of HBD-1 is constitutional, and those of HBD-2 and LL-37, but not HBD-3, are promoted by IL-17 (14, 15). It is thus suggested that HBD-2 and LL-37 can be induced by IL-17 only in healthy subjects, and IL-17 might play an important role in the epidermal innate immunity against dermatophytes.

Discussion

Patients with ATLL often suffer from various infections, such as pneumocystis carinii, pathogenic fungi, viruses, and parasites, and these infections occasionally result in death (22). They also often exhibit intractable superficial dermatophytosis, and its pathomechanism has been an issue to be clarified. Th17 cell-derived cytokines stimulate keratinocytes to produce antimicrobial peptides (14, 15), and ATLL malignant T cells are assumed to reduce the number and/or function of Th17 cells. Therefore, we addressed the relationship between the abnormality of Th17 cells and the susceptibility to dermatophytosis in ATLL patients. As the circulating CD4+CD25+ T-cell frequency and the serum IL-10 level were increased, Th17 cells and IL-17 were decreased in ATLL patients, especially when they had dermatophytosis. Therefore, ATLL cells are considered to play a suppressive role for Th17 cells. Both in vivo and in vitro studies have shown that cellular immune responses are markedly impaired in ATLL patients (23), and ATLL cells secrete various immunosuppressive cytokines such as IL-10 and TGF-β1. In particular, IL-10 produced by ATLL cells suppresses the secretion of Th17-mediated cytokines (24). Although the source of IL-10 is not definitive yet, ATLL cells as well as other Treg or Th2 cells residing in the blood might be activated to produce IL-10 in the patients. Thus, it seems that ATLL cells lead to defective epidermal innate immunity and resultant dermatophytosis by suppressing Th17 cells.

Consistently, we found that the production of antimicrobial peptides by epidermal keratinocytes was depressed in ATLL patients, which presumably attenuated the innate immunity of skin surface environment. IL-17 enhances various antimicrobial peptides such as HBD-2 and LL-37 in keratinocytes (14, 15), but neither hBD-1 nor hBD-3 (25). HBD-2 is active against fungi and effective for *tinea corporis* (26), and LL-37, one of the peptide forms of human cathelicidin, has an activity against fungi (27). Defective IL-17 production has been observed in several fungal infectious disorders such as recurrent vulvovaginal candidiasis, onychomycosis, and chronic mucocutaneous candidiasis (28, 29). Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy had severely reduced IL-17 responses to *Candida albicans* antigens, and it was strongly associated with neutralizing autoantibodies to IL-17 (29). These findings imply that Th17-mediated antimicrobial peptides play an essential role in innate immunity of the skin.

Our study on the ATLL immune condition showed that Th17 cells are deeply involved in the mechanism underlying the keratinocyte production of dermatophyte-eliminating antimicrobial peptides. Other infectious conditions might also be associated with the reduced number of Th17 cells in ATLL patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Sawada, M. Nakamura, R. Kabashima-Kubo, M. Kobayashi, Y. Tokura

Development of methodology: Y. Sawada, M. Nakamura, R. Kabashima-Kubo, Y. Tokura

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sawada, M. Nakamura, R. Kabashima-Kubo

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sawada, M. Nakamura, R. Kabashima-Kubo, Y. Tokura

Writing, review, and/or revision of the manuscript: Y. Sawada, M. Nakamura, Y. Tokura

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Nakamura, Y. Tokura

Study supervision: M. Nakamura, T. Shimauchi, Y. Tokura

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