See related commentary on pg 2569

ORIGINAL ARTICLE

Possible Pathogenic Role of Th17 Cells for Atopic Dermatitis

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The critical role of IL-17 has recently been reported in a variety of conditions. Since IL-17 deeply participates in the pathogenesis of psoriasis and keratinocyte production of certain cytokines, the involvement of T helper cell 17 (Th17) in atopic dermatitis (AD) is an issue to be elucidated. To evaluate the participation of Th17 cells in AD, we successfully detected circulating lymphocytes intracellularly positive for IL-17 by flow cytometry, and the IL-17$^+$ cell population was found exclusively in CD3$^+$CD4$^+$ T cells. The percentage of Th17 cells was increased in peripheral blood of AD patients and associated with severity of AD. There was a significant correlation between the percentages of IL-17$^+$ and IFN-γ^+ cells, although percentage of Th17 cells was not closely related to Th1/Th2 balance. Immunohistochemically, IL-17$^+$ cells infiltrated in the papillary dermis of atopic eczema more markedly in the acute than chronic lesions. Finally, IL-17 stimulated keratinocytes to produce GM-CSF, TNF-α, IL-8, CXCL10, and VEGF. A marked synergistic effect between IL-17 and IL-22 was observed on IL-8 production. The number of Th17 cells is increased in the peripheral blood and acute lesional skin of AD. Th17 cells may exaggerate atopic eczema.

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INTRODUCTION

IL-17-producing CD4$^+$ T helper cells (Th17 cells) have crucial functions in host defense, and dysregulated Th17 cell responses mediate a variety of autoimmune and inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, and experimental autoimmune encephalomyelitis (McKenzie *et al.*, 2006). IL-6 and transforming growth factor-β are both required for induction of Th17 cells, whereas IL-23 is necessary for establishment of the Th17 lineage (Bettelli *et al.*, 2006; Mangan *et al.*, 2006). Th17 cells coexpress IL-22 (Liang *et al.*, 2006; Zheng *et al.*, 2007), which belongs to the IL-10 family of cytokines and its receptor is expressed on a variety of epithelial tissues (Wolk *et al.*, 2004). IL-17 and IL-22 cooperatively enhance some immunological responses (Liang *et al.*, 2006).

An important function of IL-17 is to coordinate local tissue inflammation through upregulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines, including IL-6, GM-CSF, tumor-necrosis factor-α (TNF-α), IL-1β, KC/CXCL1, MCP-1/CCL2, MIP-2/CXCL2, MCP-3/CCL7, and MIP-3α/CCL20, as well as matrix metalloproteases, to enable activated T cells to migrate through the extracellular matrix (Nakae *et al.*, 2002; Kolls, 2006). A close relationship between IL-17 and the cutaneous milieu has been suggested by a number of observations. IL-17 induces production of certain cytokines, chemokines, and antimicrobial peptides by keratinocytes (Albanesi *et al.*, 1999, 2000; Liang *et al.*, 2006). Its cooperation with IL-22 has been documented in the antimicrobial peptide elaboration (Liang *et al.*, 2006).

Recent findings have suggested that Th17 cells profoundly participate in the pathogenesis of certain skin disorders, in particular, psoriasis (Albanesi *et al.*, 2000; Zheng *et al.*, 2007). In this Th1-mediated (Lew *et al.*, 2004), chronic inflammatory disease with epidermal hyperplasia, the role of IL-22 for dermal inflammation and acanthosis is stressed (Boniface *et al.*, 2005; Zheng *et al.*, 2007). On the other hand, involvement of IL-17 has also been shown in allergen-specific immune responses (Nakae *et al.*, 2002; Kolls, 2006). IL-17 mRNA has been detected in skin affected by allergic contact dermatitis (Teunissen *et al.*, 1998) as well as psoriasis (Zheng *et al.*, 2007). Considering the proinflammatory property of IL-17, it is an interesting issue whether and how Th17 cells are involved in the pathogenesis of atopic dermatitis (AD), a representative skin disease with a chronic clinical course (Homey *et al.*, 2006; Maintz and Novak, 2007). AD merits Th17 investigation because it is known as a Th2 cell-mediated disease, and one can evaluate the biased relationship between Th17 cells and Th1/Th2 balance in relation to psoriasis. Perhaps more importantly, the effects of IL-17 and resultant outcomes on keratinocytes and dermal vasculature may provide some insights to AD pathophysiology.

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Abbreviations: AD, atopic dermatitis; HPF, high-power field; NHEK, normal human epidermal keratinocyte; PBMC, peripheral blood mononuclear cell; Th, T helper; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor

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In this study, we investigated Th17 cells in the peripheral blood and skin lesions of AD, with a study of the effect of IL-17 on the production of cytokines/chemokines and vascular endothelial growth factor (VEGF) by keratinocytes. In particular, to enumerate Th17 cell, we successfully analyzed the cells by intracellular cytokine staining. Results suggest that Th17 cells are elevated in the blood and can function as an upmodulator in skin lesions of AD.

RESULTS

Increased percentage of IL-17$^+$CD4$^+$ T cells in PBMCs from severe AD patients

The atopic patients enrolled in this study were divided into three different severity groups (mild, moderate, and severe) according to the criteria of Rajka and Langeland (1989). The grouping was validated with a blood severity marker for AD, lactate dehydrogenase (Mukai *et al.*, 1990; Jacyk and Ungerer, 1991; Figure 1). As reported previously (Mukai *et al.*, 1990), severity of AD correlates well with lactate dehydrogenase level (Figure 1).

Circulating T cells bearing IL-17 in the cytoplasm were examined in AD patients. Since CD4 expression on T cells is downregulated during culture with the stimulants, CD3$^+$ and CD8$^+$ T cells positive for intracytoplasmic IL-17 were analyzed by flow cytometry. Figure 2 shows representative flow cytometry data from an AD patient. There was a discernible population of IL-17$^+$CD3$^+$ and IL-17$^+$CD8$^-$ T cells in the peripheral blood mononuclear cells (PBMCs), whereas the number of IL-17$^+$CD8$^+$ T cells was not substantial, indicating that CD4$^+$ T cells are the major source of IL-17.

Figure 3a summarizes the percentages of IL-17$^+$CD4$^+$ cells in the patients tested. The values differed significantly among the three groups of AD patients, with different severity. IL-17$^+$CD4$^+$ T-cell number was higher in the severe group ($0.71 + 0.09$) than the healthy control ($0.42 + 0.07$). The mean percentage of IL-17$^+$CD4$^+$ cells in psoriasis vulgaris ($1.3 + 0.2$) was slightly higher than that of severe AD but there was no statistical significance. As for expression of other cytokines, there was a tendency of both

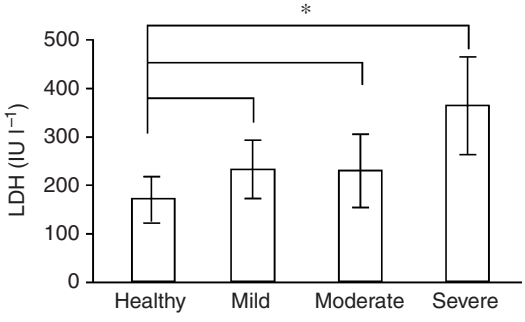

Figure 1. Lactate dehydrogenase levels in different severity groups of AD patients. The AD patients were classified as acute ($n=23$), mild ($n=7$), moderate ($n=5$), and severe ($n=11$) by clinical findings, and serum levels of lactate dehydrogenase from AD patients were compared with that in healthy donors. Student's *t*-test was performed between the indicated groups and an asterisk indicates $P< 0.05$.

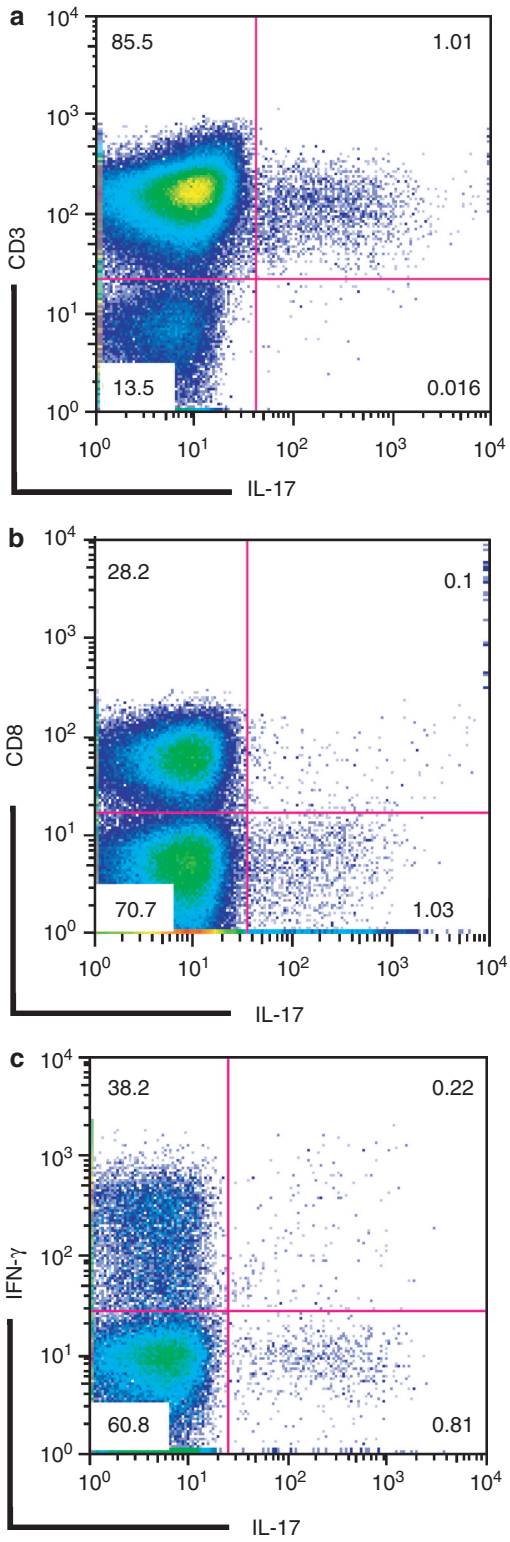

Figure 2. Flow cytometric analysis of IL-17$^+$ cells in peripheral blood lymphocytes. PBMCs isolated from a 30-year-old man with AD were stimulated with PMA and ionomycin for 8 hours and Golgistop was added. Then, production of IL-17 by PBMCs was determined at the single-cell level by intracellular cytokine staining and flow cytometric analysis. The numbers in the upper and lower right quadrants represent the percentage of IL-17$^+$ cells with or without expression of CD3$^+$ or CD8$^+$ cells in the total lymphocyte populations gated.

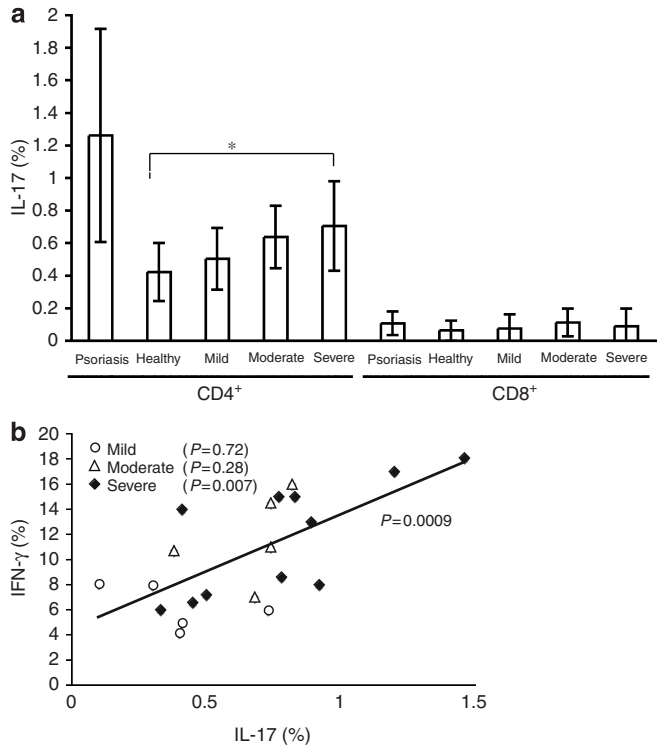

Figure 3. Percentage of circulating IL-17$^+$ T cells in relation to AD severity and IFN-γ$^+$ T cells. AD patients were divided into three different severity groups (mild, $n=7$; moderate, $n=5$; and severe, $n=11$). (**a**) Healthy subjects ($n=9$), three groups of AD, psoriatic patients ($n=16$) were compared for percentage of IL-17$^+$ and CD4$^+$ or CD8$^+$ T cells, and a significant correlation between healthy donors and severe AD patients was found ($P=0.03$). (**b**) IFN-γ$^+$ T cells of all AD patients were also analyzed. A significant correlation between percentage of IL-17$^+$ CD4$^+$ T cells and IFN-γ$^+$ T cells was found ($P=0.0009$). In addition, the correlation (P-value) between the percentage of IL-17$^+$ CD4$^+$ T cells and IFN-γ$^+$ T cells among acute, moderate, and severe AD patients was depicted.

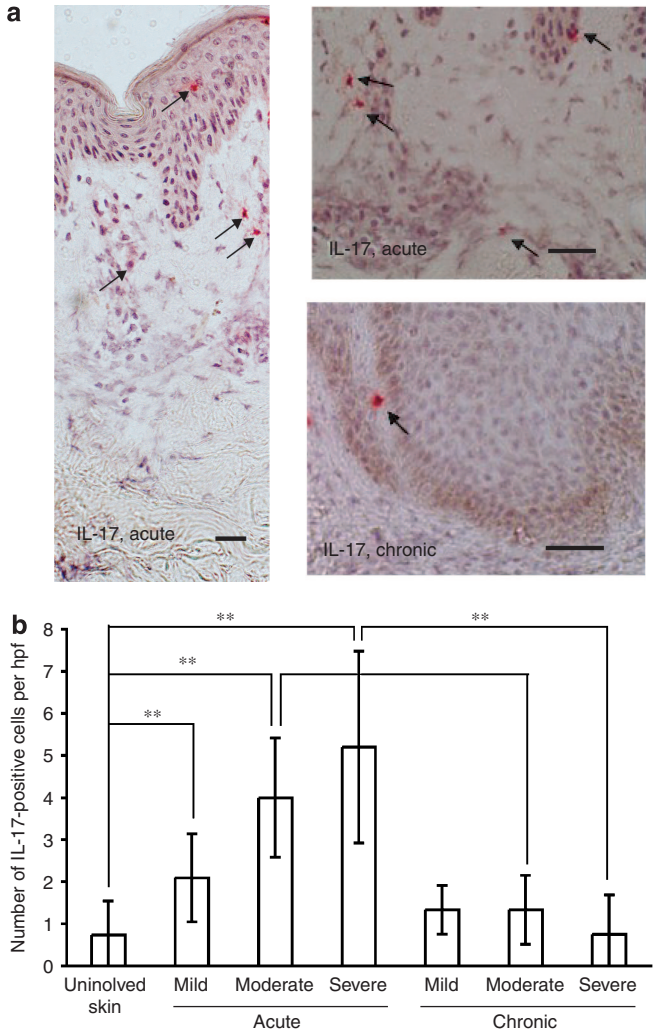

Figure 4. Immunohistochemistry of IL-17$^+$ cells in skin lesions of AD. (**a**) A skin specimen from an AD patient was immunohistochemically stained for IL-17. Left, low-magnification image of acute lesion; top right, high-magnification image of acute lesion; and bottom right, high-magnification image of chronic lesion. Bar = 30 μm. (**b**) The number of IL-17$^+$ cells was enumerated in acute and chronic lesions from mild, moderate, and severe cases of AD. Columns show mean ± SD. Student's t-test was performed between the indicated groups and an the asterisk indicates $P<0.05$.

IFN-γ$^+$ T cells (11.4 + 1.4) and IL-4$^+$CD4$^+$ cells (0.49 + 0.07) to be elevated in AD patients compared with in healthy subjects (IFN-γ, 9.2 + 1.5; IL-4, 0.35 + 0.07). IFN-γ$^+$ T cells were also elevated markedly in psoriasis (41.7 + 10.6). In AD patients, a significant correlation ($R=0.67$, $P=0.0037$) between the percentage of IL-17$^+$CD4$^+$ cells and IFN-γ$^+$ T cells was found (Figure 3b), but not between IL-17$^+$CD4$^+$ cells and IL-4$^+$CD4$^+$ cells (data not shown). We also examined whether IL-17$^+$CD4$^+$ T cells are related to Th1/Th2 balance. No significant correlation was found between the number of IL-17$^+$CD4$^+$ T cells and the ratio of IFN-γ$^+$ T cells/IL-4$^+$CD4$^+$ cells. These data suggest that AD patients have a high percentage of Th17 cells depending on the severity of atopic eczema, and the level of Th17 cells is not closely associated with Th balance.

Infiltration of IL-17$^+$ T cells in skin lesions of AD

Skin biopsy specimens were taken from acute and chronic skin lesions of patients with mild, moderate, and severe AD. The chronic form was defined by the presence of lichenified or pruriginous eruptions, whereas scaly erythematous lesions were categorized as the acute form. They were subjected to immunohistochemical staining for IL-17, IFN-γ, and IL-4. IL-17-positive lymphocytes were found in the papillary areas in the upper dermis, and some epidermotropic lymphocytes also bore IL-17 (Figure 4a). The percentage of IL-17-bearing lymphocytes was higher in the acute than in chronic lesions. In acute lesions, the number of IL-17$^+$ lymphocytes per high-power field (HPF) depended on the severity of AD (Figure 4b). There was no significant difference in the number of IFN-γ$^+$ cells/HPF between the acute and chronic lesions, and IFN-γ$^+$ cells/HPF tended to infiltrate at a higher percentage in the severe form (1.18 + 0.20) than in the mild form (0.70 + 0.01). As to IL-4$^+$ cells/HPF, no significant difference existed between acute and chronic lesions, but again, severity

dependency was observed in acute lesions (severe, 0.99 + 0.20; mild, 0.68 + 0.16).

Augmentation of keratinocyte production of GM-CSF, TNF-α, IL-8, and VEGF by IL-17

The effect of IL-17 on keratinocyte production of GM-CSF, TNF-α, IL-8, CCL5, CXCL10, CCL22, and VEGF was examined in normal human epidermal keratinocytes (NHEKs). GM-CSF and TNF-α are proinflammatory cytokines and induce maturation of Langerhans cell and dermal dendritic cells (Bechetoille *et al.*, 2006). IL-8/CXCL8 attracts neutrophils (Keller *et al.*, 2005). VEGF stimulates vascular endothelial cells to proliferate (Breier *et al.*, 1992). Since IL-22 cooperates with IL-17 for Th17 to function as an immunomodulator in certain conditions (Boniface *et al.*, 2007; Zheng *et al.*, 2007), the synergistic effects of these two cytokines were also tested. IL-17 and/or IL-22 was added at the starting of experimental NHEK culture. Three-day culture supernatants were measured for the above-mentioned cytokines, chemokines, and VEGF. As compared with the non-addition control, IL-17 markedly augmented production of proinflammatory cytokine GM-CSF (Figure 5) and TNF-α (data not shown), whereas IL-22 enhanced TNF-α but not GM-CSF. As for chemokines, IL-17 highly increased IL-8 production (Figure 5), but decreased CCL5 that of (data not shown), and IL-22 virtually shared these effects with IL-17, but to a lesser extent. CXCL10 production was augmented by both IL-17 and IL-22, with the latter being stronger, whereas CCL22 level was not changed by either IL-17 or IL-22 (data not shown). The production of VEGF was upmodulated by IL-17 but not IL-22. The results suggest that IL-17 is a powerful stimulator for keratinocytes to produce skin inflammation-associated molecules. Synergistic effects of IL-17 and IL-22 were remarkably found on IL-8 production (Figure 5), whereas they did not exhibit additive or synergistic effects on the other products.

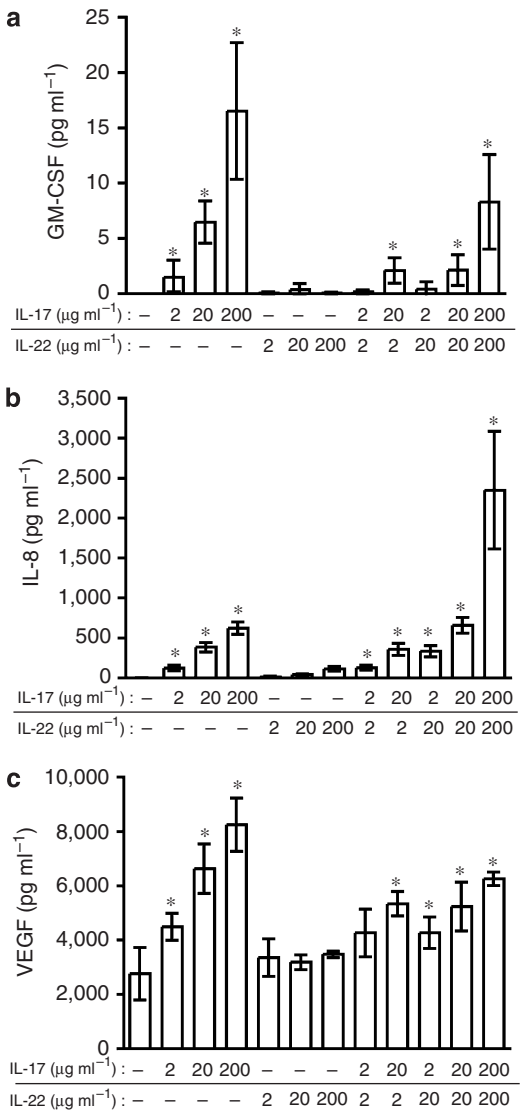

Figure 5. Effects of IL-17 and/or IL-22 on keratinocyte production of cytokines/chemokines and VEGF. NHEKs were cultured with or without IL-17 and/or IL-22 at the indicated doses. Three-day culture supernatants were subjected to analysis with the cytometric beads array system or ELISA. Columns show mean ± SD of data using triplicated wells. The asterisk indicates statistically significant differences compared with the untreated group ($P<0.05$, unpaired two-tailed *t*-test). Data are representative of those from three independent experiments.

DISCUSSION

Intracellular detection of IL-17 is necessary for enumeration of Th17 cells in PBMCs. In this study, we successfully counted their percentage by flow cytometry and investigated whether this particular T-cell population was changed in number in AD patients. Our study demonstrated that Th17 cells were increased in percentage in PBMCs of AD patients and infiltrated in the eczematous lesions of AD, and IL-17 stimulated keratinocytes to produce cytokines/chemokines and VEGF. It is noteworthy that percentage of IL-17$^+$CD4$^+$ cells in AD patients was slightly lower than that of psoriatic patients, a well-known disease whose pathogenesis involves Th17 (Zheng *et al.*, 2007).

The important role of Th17 cells for AD is suggested by the finding that percentage of Th17 was associated with severity of AD. The percentage of Th17 was significantly correlated with the percentage of IFN-γ-producing Th1 cells, but not with that of IL-4-producing Th2 cells. Moreover, there was no strong association of Th17 cells with Th1/Th2 balance. These results suggest that Th17 cells participate in the development of AD as an enhancer, but not an immune-polarizer, of AD. While IFN-γ-producing T cells are involved in the chronic lesions of AD (Grewe *et al.*, 1994), Th17 cells possibly play a role for prolonged exaggeration of AD lesions.

An immunohistochemical study of atopic eczema revealed infiltration of IL-17-secreting cells in the lesional skin. In accordance with the previous study (Toda *et al.*, 2003), Th17 infiltrated more markedly in acute eczematous lesions than in chronic ones in a severity-dependent manner. Thus, Th17 is considered to serve as an initial cytokine source for development of the skin lesions. T-cell-driven keratinocyte activation plays a relevant role in the pathogenesis of prolonged inflammatory skin disorders, including AD. Our

evaluation of the effects of IL-17 on keratinocyte production of cytokines, chemokines, and VEGF demonstrated that IL-17 stimulates NHEKs to produce GM-CSF, TNF-α, IL-8, CXCL10, and VEGF. IL-17 increases production of GM-CSF, IL-6 (Albanesi *et al.*, 2000), and IL-8 (Albanesi *et al.*, 1999) directly and in synergism with IFN-γ, IL-4, and/or TNF-α, whereas CCL5 production is decreased (Albanesi *et al.*, 1999). Their observations are virtually the same as ours, except for CXCL10, which we found to be upregulated by IL-17 and more remarkable by IL-22. IL-17 also stimulates keratinocytes to express CD54 in the presence of IFN-γ (Albanesi *et al.*, 1999). Thus, keratinocyte production of proinflammatory cytokines is augmented in Th17-infiltrating atopic skin lesions. Th17 also may promote proliferation of dermal vessels by VEGF, a sequential event for exacerbation of AD (Wakita *et al.*, 1994).

In previous studies, the synergistic effect of IL-17 and IL-22 has not been studied fully. Since IL-17 and IL-22 are coexpressed by Th17 cells and expression of both cytokines is initiated by transforming growth factor-β (Liang *et al.*, 2006; Zheng *et al.*, 2007), their synergistic effects on keratinocytes are an issue to be clarified, but has been reported in only one study, which demonstrated synergism in keratinocyte expression of antimicrobial peptides (Zheng *et al.*, 2007). We found significant synergism in IL-8 production, but not for any other products.

Psoriasis and AD are two representative inflammatory skin diseases. Although mediated by Th1 (Gudjonsson *et al.*, 2004) and Th2 cells (Leung and Soter, 2001), respectively, there are exacerbating factors common to these disorders, as exemplified by keratinocyte-derived proinflammatory cytokines and VEGF (Wakita *et al.*, 1994; Bhushan *et al.*, 1999). It is suggested that IL-17 is involved in this common process of skin inflammation as a proinflammatory cytokine.

MATERIALS AND METHODS

All experiments were conducted in accordance with the Declaration of Helsinki Principles.

Subjects

A total of 23 patients with AD (aged 9–51 years; 13 men and 10 women), and nine healthy non-AD volunteers with low serum IgE levels (aged 24–38 years; 5 men and 4 women) were enrolled in this study. AD was diagnosed according to the criteria of Hanifin and Rajka (1980). Severity of AD was evaluated by the criteria of Rajka and Langeland (1989) and AD was classified into mild, moderate, or severe. Serum IgE levels of the patients ranged from 43 to 160,000 IU ml^{-1} and averaged 14,149 IU ml^{-1}. None of the patients had oral steroids or immunosuppressants. Sixteen patients with psoriasis vulgaris (aged 26–77 years all men; average PASI score, $11.3 + 2.9$) were also tested as a disease control. The study design was approved by the review board of University of Occupational and Environmental Health. Measurements in this study were performed after informed consent had been obtained.

Intracellular cytokine staining of PBMCs

PBMCs were isolated from patients and control subjects by standard Ficoll–Paque method (Phamacia, Uppsala, Sweden). Intracellular cytokines were stained according to the protocol of Cytostain (Immunotech, Marseille, France), with a few modifications. Briefly, cells (2×10^6 cells ml^{-1}) were incubated in complete RPMI (RPMI-1640 (Sigma Chemical Co., St Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (Cellgro, Herndon, VA), 1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin) in a 24-well plate with 10 ng ml^{-1} of phorbol-12-myristate 13-acetate (Sigma Chemical Co.), 10^{-6} M of ionomycin (Wako, Osaka, Japan), and 0.7 μl of Golgistop (BD Biosciences, San Diego, CA) for 8 hours. Then, cells were washed and directly stained with PerCP-conjugated anti-CD8 mAb (BD Biosciences) and subsequently with APC-conjugated anti-CD3 mAb (BD Biosciences) for 20 minutes at 4 °C. After washing, 100 μl of Cytofix/Cytoperm buffer (BD Biosciences) was added to each well and incubated for 20 minutes at room temperature, and washed with Perm/Wash solution as per manufacture's protocol (BD Biosciences). They were stained with phycoerythrin-labeled anti-IL-17, IL-4, or IL-5, and FITC-labeled anti-IFN-γ mAb, for 20 minutes at 4 °C. Fluorescence profiles were analyzed by flow cytometry in FACSCanto (BD Biosciences).

Immunohistochemical staining

We obtained 27 biopsy specimens from 13 atopic patients. The specimens were obtained from acute and chronic lesions of AD and frozen in Tissue-Tek OCT compound. Chronic lesions were defined as lichenified or pruriginous eruptions, and scaly erythematous lesions were categorized as acute lesions. Cryostat sections (7 μm) were fixed in acetone and stained as described previously (Kabashima *et al.*, 2005) with rat anti-human IFN-γ, IL-4, and IgG1 (BD Biosciences), and mouse anti-human IL-17 and IgG1 (eBiosciences, San Diego, CA). Rat and mouse antibodies were detected using biotinylated anti-rat and mouse IgG (Dako Cytomation, Kyoto, Japan), followed by treatment with horseradish peroxidase-conjugated streptoavidin (KPL, Gaithersburg, MD). Peroxidase enzyme reactions were developed with conventional substrates, diaminobenzidine (Sigma Chemical Co.). The sections were lightly counterstained with hematoxylin. The number of immunoreactive lymphocytes was enumerated from five HPFs in the dermis of each immunostained section. Data were expressed as the number of IL-17 cells/HPF.

Keratinocyte culture and measurements of cytokines and VEGF

NHEKs were purchased from Cascade Biologics (Portland, OR). They were grown in the serum-free keratinocyte Epilife growth medium (Cascade Biologics) and used at the third passage in all experiments (Kobayashi *et al.*, 2007). Growth supplement was omitted 48 hours before experiments. IL-17 and/or IL-22 (R&D Systems, Minneapolis, MN) were added at the beginning of experimental culture using 24-well plates (Corning Glass Works, Corning, NY). Three-day culture supernatants were examined for GM-CSF, TNF-α, IL-8, RANTES/CCL5, IP-10/CXCL10, MDC/CCL22, and VEGF levels. The concentration of these substances, except for that of CCL22, was measured using cytometric beads array system (BD Bioscience) according to the manufacturer's protocol. The concentration of CCL22 was measured using ELISA kits (BD Bioscience) according to the manufacturer's directions.

Statistical analysis

Student's *t*-test (impaired) was employed to determine statistical differences between means. Correlations were studied by Pearson product-moment correlation coefficient.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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