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ORIGINAL ARTICLE

Comparative Analysis of Human Epidermal and Peripheral Blood γδ T Cell Cytokine Profiles

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Background: Human epidermal γδ T cells are known to play crucial roles in the defense and homeostasis of the skin. However, their precise mechanism of action in skin inflammation remains less clear. **Objective:** In this study, we analyzed the cytokine expression profile of human epidermal γδ T cells and compared it to that of peripheral blood γδ T cells to investigate the specific activity of epidermal γδ T cells in modulating skin inflammation. **Methods:** We isolated γδ T cells from epidermal tissue or peripheral blood obtained from healthy volunteers. Isolated γδ T cells were stimulated using immobilized anti-CD3 antibody and interleukin-2 plus phytohaemagglutinin, and were then analyzed using a cytokine array kit. **Results:** Both epidermal and peripheral blood γδ T cells produced comparable levels of granulocyte-macrophage colony-stimulating factor, I-309, interferon-γ, macrophage migration inhibitory factor, macrophage inflammatory protein-1α, and chemokine (C-C) ligand 5. The epidermal γδ T cells produced significantly higher levels of interleukin-4, -8, -13, and macrophage inflammatory protein-1β than the peripheral blood γδ T cells did. Notably, the epidermal γδ T cells produced several hundred-fold higher levels of interleukin-13 than interleukin-4. **Conclusion:** These results suggest that the epidermal γδ T cells have a stronger potential to participate in the Th2-type response than the peripheral blood γδ T cells do. Furthermore, epidermal γδ T cells might play an important role in the pathogenesis of Th2-dominant skin diseases because of their active production of interleukin-13. **(Ann Dermatol 26(3) 308～313, 2014)**

-Keywords-
Epidermis, Interleukin-4, Interleukin-13, γδ T cells

INTRODUCTION

The skin, composed of epidermis, dermis, and subcutaneous tissue, is one of the most active organs in the human body. The epidermis, the outermost barrier tissue, is composed of keratinocytes, melanocytes, and immune cells such as Langerhans cells and γδ T cells[1]. Epidermal cells actively communicate with each other through a variety of soluble signaling factors (growth factors, cytokines, chemokines, and inflammatory mediators) and through direct contact mediated by cell surface molecules. γδ T cells are the first T cells that emigrate from the thymus, and many of these γδ T cells take residence in epithelial tissues, including the skin, intestine, lung, and reproductive tract[2]. Epidermis-resident γδ T cells are derived from fetal thymic precursor cells and move to skin before birth[3]. Evidence from multiple laboratories indicates that epidermal γδ T cells play specialized roles in the maintenance of epithelial homeostasis, wound healing, tumor surveillance, infection, and inflammation[1-8]. These γδ T

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cells have been shown to express interleukin (IL)-2, IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN γ), tumor necrosis factor-α, chemokine (C-C) ligand (CCL)-3 (macrophage inflammatory protein [MIP]-1α), CCL-4 (MIP-1β), CCL-5 (RANTES), and chemokine (C motif) ligand 1[2,5,6]. Similar to chemokines, these cytokines are detected at low levels under resting conditions, but are rapidly upregulated upon stimulation. The ability of epidermal γδ T cells to produce numerous cytokines and chemokines suggests that these molecules are key players in the immune responses stimulated by the epidermal γδ T cells.

Epidermal γδ T cells are significantly different from those of the peripheral blood in terms of ontogeny, tissue tropism, and antigen receptor diversity[6,9]. In humans, most of the peripheral blood γδ T cells express the *Vδ2* gene segment[10]. Peripheral γδ T cells recognize, expand, and release cytokines in response to non-peptide antigens, which are mostly of microbial origin. In contrast, most γδ T cells in epithelial tissues express the *Vδ1* gene segment and respond to poorly defined self-antigens expressed by stressed cells[3,11-14]. In addition, epidermal γδ T cells primarily possess tissue-specific T cell receptors (TCRs) with limited or no diversity compared to the diverse TCRs expressed by γδ T cells found in the peripheral lymphoid organs and blood[13]. However, it is unclear whether human epidermal γδ T cells exert any functions different from those of peripheral γδ T cells resulting from their differential cytokine production profiles.

In the present study, we compared the cytokine profiles of human epidermal and peripheral blood γδ T cells to investigate the differential activities of epidermal γδ T cells, which affect their neighboring epidermal cells.

MATERIALS AND METHODS

Human skin specimens and blood samples

Skin and peripheral blood samples were collected from human volunteers after informed consent had been obtained according to the approval of the Institutional Review Board at Seoul National University Hospital (IRB No. H-1012-054-344).

Preparation of human epidermal γδ T cells

Whole skin specimens larger than 3×5 cm^2 were obtained from three healthy volunteers undergoing elective breast surgery. Skin specimens were incubated in RPMI media containing 2.4 U/ml dispase II (Roche Applied Science, Indianapolis, IN, USA) overnight at 4°C. Epidermal sheets were separated from the dermis by using forceps, and then cultured in complete media (RPMI 1640 media supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10% heat-inactivated fetal calf serum, 100 mM nonessential amino acids, 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, and 50 mM 2-b-mercaptoethanol in the presence of 50 U/ml IL-2 at 37°C in a 5% CO_2 incubator for 3 to 4 days. Epidermal sheets were then gently agitated to isolate the epidermal cells. Harvested epidermal cell suspensions were enriched for T cells by using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation at $400\times g$ for 30 minutes at room temperature. Epidermal γδ T cells were selected from epidermal mononuclear cell suspensions by using the TCR γδ$^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and a magnetic cell separator, according to the manufacturer's instructions.

Preparation of human peripheral blood γδ T cells

Human peripheral blood mononuclear cells were isolated from peripheral blood, collected from the antecubital veins of three healthy volunteers, by performing Histopaque 1077 gradient centrifugation at $400\times g$ for 30 minutes at room temperature. Peripheral γδ T cells were selected using the TCR γδ$^+$ T Cell Isolation Kit and a magnetic cell separator, according to the manufacturer's instructions.

Flow cytometry

The purity of γδ T cells was analyzed by flow cytometry using fluorescein isothiocyanate-conjugated anti-human CD3 monoclonal antibodies (mAbs; BD Biosciences, Bergisch Gladbach, Germany), APC-conjugated anti-human TCR γδ complex mAbs (BD Biosciences, San Jose, CA, USA), or the corresponding fluorescently conjugated isotype-matched control Abs. Two-color flow cytometry was performed using the BD FACSCalibur Flow Cytometer, and the results were analyzed using the Cell Quest software (BD Biosciences, USA).

Cytokine array

Epidermal and peripheral blood γδ T cells were stimulated using immobilized 10 mg/ml anti-human CD3 mAb (BD Biosciences, USA) and 100 U/ml IL-2 plus 500 ng/ml phytohaemagglutinin for 36 hours. The supernatants of the cultures were analyzed using the Proteome Profiler Human Cytokine Array Kit, Panel A (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The cytokine blot intensities on the array membranes were measured using the ChemiDoc gel documentation system (Bio-Rad, Hercules, CA, USA). The relative intensities of the blots were calculated using the following formula: $100\times$(intensity of sample − intensity of negative control)/

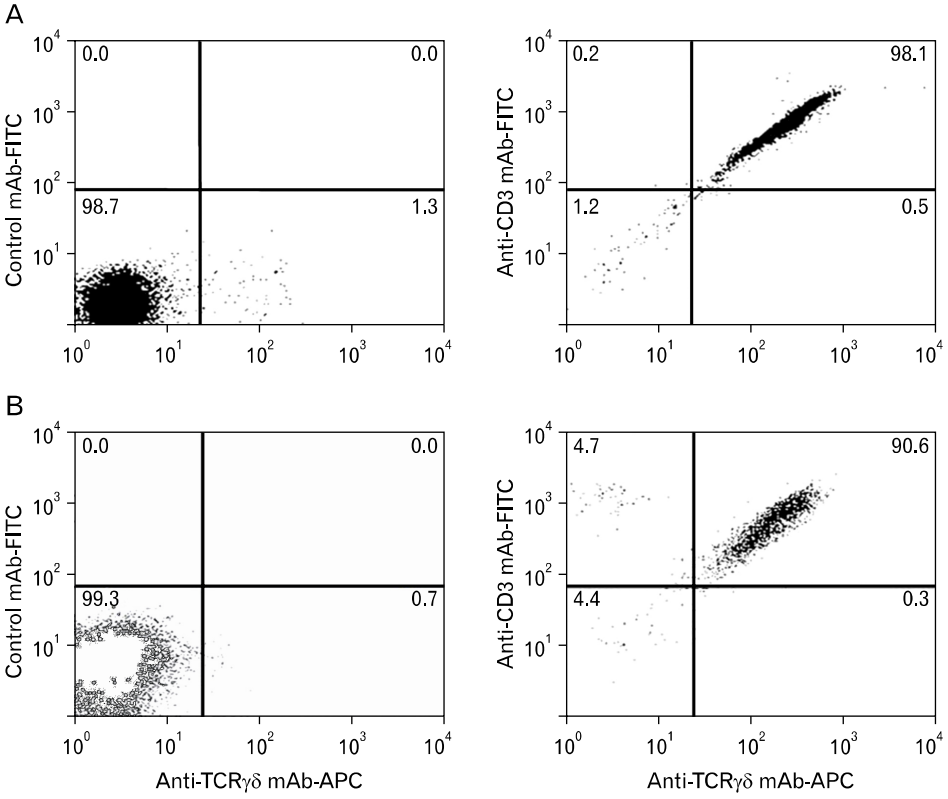

Fig. 1. Flow cytometric analysis of the purified epidermal and peripheral blood $\gamma\delta$ T cells. Purified $\gamma\delta$ T cells obtained from human epidermal tissue (A) and from the peripheral blood (B) of healthy volunteers were stained using a fluorescein-conjugated anti-CD3 monoclonal antibody (mAb) and APC-conjugated anti-$\gamma\delta$ T cell receptor mAb or the corresponding fluorescently conjugated isotype-matched control Abs. Three independent experiments were performed for epidermal cells and peripheral blood cells respectively, and the representative results are presented. FITC: fluorescein isothiocyanate.

(intensity of positive control−intensity of negative control). Data are presented as means±standard deviation.

Enzyme-linked immunosorbent assays

IL-4 and IL-13 levels were measured in the culture supernatants by using the OptEIA™ human IL-4 enzyme-linked immunosorbent assay (ELISA) set and human IL-13 ELISA set (BD Biosciences, USA), respectively, according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using the Student's t-test to determine the significant differences between the cytokine levels in epidermal $\gamma\delta$ T cells versus those in peripheral $\gamma\delta$ T cells. SPSS ver. 15.0.1. software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. A p-value of <0.05 was considered statistically significant.

RESULTS

Flow cytometric analysis of the purified human epidermal and peripheral blood $\gamma\delta$ T cells

The purity of $CD3^+$ TCR $\gamma\delta^+$ cells isolated from the epidermis was 98.1% (Fig. 1A) and 90.6% from the peripheral blood (Fig. 1B). The results confirmed that the purities of $\gamma\delta$ T cells were sufficient for them to be used in the cytokine array experiments.

Comparison of the cytokine expression profiles of epidermal and peripheral blood $\gamma\delta$ T cells

The blot intensities on the cytokine array membranes from activated epidermal (Fig. 2A∼C) and activated peripheral blood $\gamma\delta$ T cells (Fig. 2D∼F) were measured as described in the Materials and Methods. A summary of these data is presented in Fig. 2G (epidermal $\gamma\delta$ T cells) and Fig. 2H (peripheral blood $\gamma\delta$ T cells). These data showed that both epidermal and peripheral blood $\gamma\delta$ T cells produced comparable levels of GM-CSF, I-309 (CCL-1), IFN γ, macrophage migration inhibitory factor, MIP-1 α (CCL-3), and RANTES (CCL-5). In addition, we found that epidermal $\gamma\delta$ T cells produced significantly higher levels of IL-4, IL-8, IL-13, and MIP-1 β than peripheral blood $\gamma\delta$ T cells did ($p<0.05$).

Quantitative analyses of interleukin-4 and interleukin-13 production in epidermal and peripheral blood $\gamma\delta$ T cells

The ELISA results presented in Fig. 3 confirmed that epidermal $\gamma\delta$ T cells produced significantly higher levels of IL-4 and IL-13 than peripheral blood $\gamma\delta$ T cells did. At the same time, epidermal $\gamma\delta$ T cells produced IL-13 at levels several hundred-fold higher than the levels of IL-4

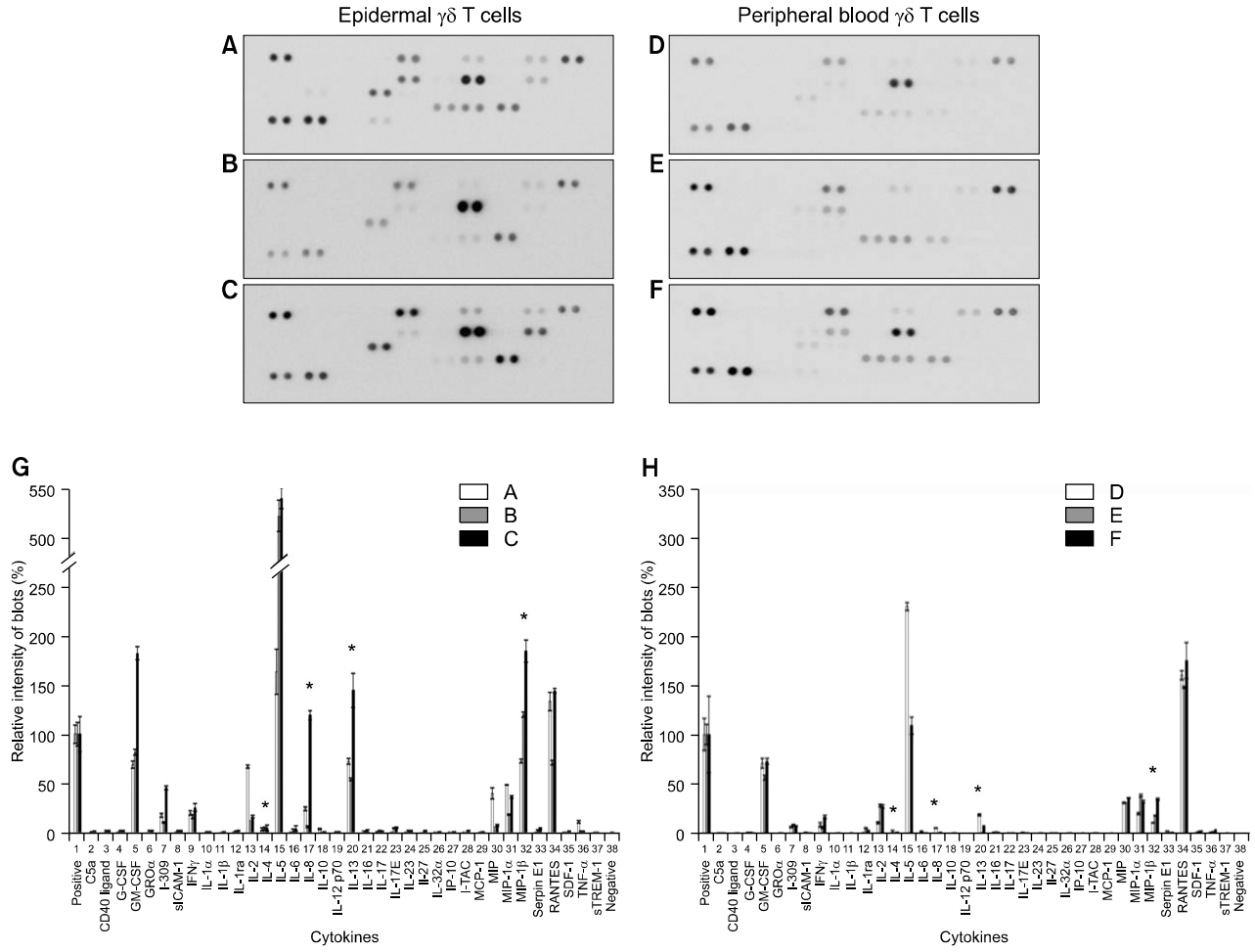

Fig. 2. Comparison of cytokine expression profiles of epidermal and peripheral blood γδ T cells. Epidermal γδ T cells were isolated from the epidermes of three healthy volunteers (A∼C) by using the method described in the Materials and Methods section. Peripheral blood γδ T cells were isolated from the peripheral blood of three healthy volunteers (D∼F) by using conventional Histopaque 1077 gradient centrifugation, followed by magnetic-activated cell sorting. Epidermal and peripheral blood γδ T cells were stimulated using immobilized anti-CD3 antibody and IL-2 plus phytohaemagglutinin. The culture supernatants were analyzed after 36 hours by using the Human Cytokine Array Panel A. (A∼F) cytokine array membranes. (G, H) The blot intensities on the array membranes were measured using a gel documentation system. Data are presented as the mean±standard deviation. Statistical analyses were performed using the Student's t-test. C5a: complement component 5a, G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte-macrophage colony stimulating factor, GROα: growth-related oncogene α, sICAM-1: soluble intercellular adhesion molecule-1, IFNγ: interferon-γ, IL: interleukin, IP-10: interferon gamma-induced protein 10, I-TAC: interferon-inducible T cell α chemoattractant, MCP-1: monocyte chemotactic protein 1, MIP: macrophage inflammatory protein, RANTES: regulated on activation, normal T cell expressed and secreted, SDF-1: stromal cell-derived factor 1, TNF-α: tumor necrosis factor α, sTREM-1: soluble triggering receptor expressed on myeloid cells 1. *$p<0.05$, epidermal vs. peripheral blood γδ T cells.

produced.

DISCUSSION

In this study, we analyzed and compared the cytokine expression profiles of human epidermal and peripheral blood γδ T cells to determine the specific activity of epidermal γδ T cells in modulating skin immune responses. Although activated epidermal γδ T cells are a rich source of several cytokines and chemokines, the transcripts of IL-1α, IL-1β, IL-6, IL-7, IL-10, and IL-13 are in low abundance, and intracellular cytokine staining fails to detect IL-4, IL-5, and IL-10[15,16]. Thus, it was unexpected that we found that activated human epidermal γδ T cells produced significant levels of both Th1-type cytokines (IFNγ) and Th2-type cytokines (IL-4, IL-5, and IL-13) at the protein level (Fig. 2A). Next, we checked the purity of the cells to confirm that IL-4 and IL-13 were produced by γδ T cells and not by other cells contaminating the cultures. Our flow cytometry results showed that 98.1% and 90.6%

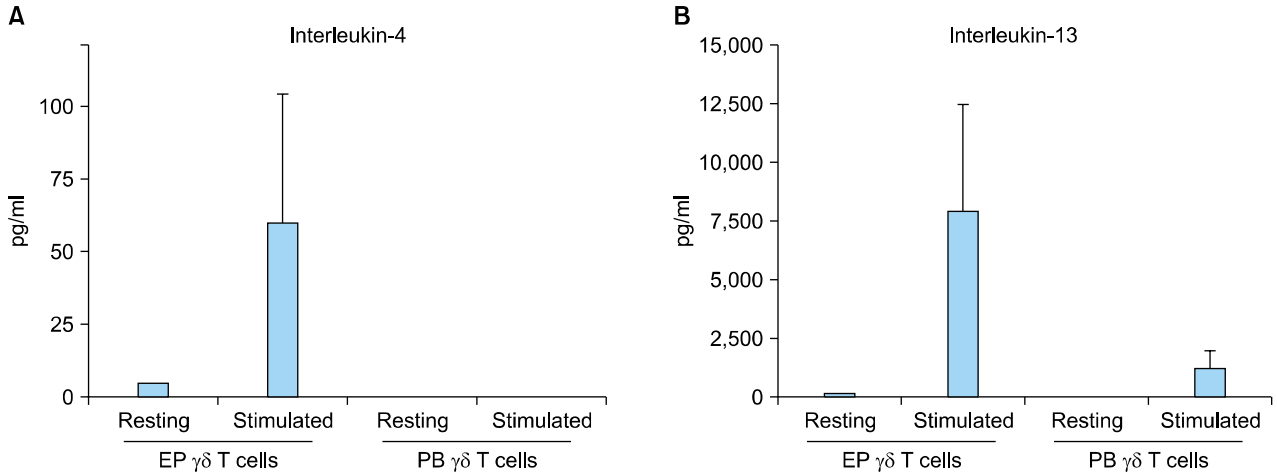

Fig. 3. Quantitative analyses of interleukin-4 and interleukin-13 production in epidermal $\gamma\delta$ T cells and peripheral blood $\gamma\delta$ T cells. Epidermal and peripheral blood $\gamma\delta$ T cells were each isolated from three healthy volunteers. Cells were stimulated using immobilized anti-CD3 antibody and interleukin-2 plusphytohaemagglutinin, or cultured without any stimulation. The culture supernatants were analyzed after 36 hours by using ELISAs. Data are presented as the mean±standard deviation of three independent experiments. Statistical analyses were performed using the Student's t-test. EP: epidermal, PB: peripheral blood. *$p<0.05$, epidermal vs. peripheral $\gamma\delta$ T cells.

of epidermal and peripheral blood cells, respectively, were $CD3^+TCR\gamma\delta^+$ (Fig. 1).

Interestingly, our results showed that activated epidermal $\gamma\delta$ T cells produced significantly higher levels of IL-4, IL-8, IL-13, and MIP-1β than peripheral blood $\gamma\delta$ T cells (Fig. 2). IL-8 and MIP-1β are well-known chemotactic factors that are produced from epithelial cells as well as from lymphocytes. In this study, we focused on the predominant cytokines produced by T cells, IL-4 and IL-13, which are typical Th2-type cytokines[17]. These cytokines affect a variety of cell types including T cells, B cells, natural killer cells, mast cells, monocytes/macrophages, endothelial cells, epithelial cells, dendritic cells, fibroblasts and keratinocytes[18,19]. In the skin, IL-4 and IL-13 are known to play multiple roles in diseases such as atopic dermatitis (AD) and vitiligo[20,21]. The concentration of IL-13 is reported to be abnormally high in the serum of AD and vitiligo patients[22-25], and the acute skin lesions of AD patients contain increased numbers of cells expressing IL-4, IL-5, and IL-13 mRNA[26-28]. IL-4 might be important during the initial phase of allergic responses and in the priming and development of Th2 cells, whereas IL-13 plays a more critical role in immunoglobulin E induction and chronic AD pathogenesis[29]. Our quantitative ELISA data confirmed that epidermal $\gamma\delta$ T cells produced markedly higher levels of IL-4 and IL-13 than peripheral blood $\gamma\delta$ T cells, and the amount of IL-13 produced by the cells was several hundred-fold higher than the levels of IL-4 produced (Fig. 3). In summary, our results suggest that epidermis-resident $\gamma\delta$ T cells have stronger potential to participate in Th2-type responses than $\gamma\delta$ T cells existing in the periphery. These data suggest that epidermal $\gamma\delta$ T cells play an important role in the pathogenesis of Th2-dominant skin diseases through the active production of IL-13.

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