The target search for innate allergic immune modulatory proteins in TSLP-activated NKT cells using proteomics

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The target search for innate allergic immune modulatory proteins in TSLP-activated NKT cells using proteomics

Directed by Professor Kwang Hoon Lee

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Chang Ook Park

June 2015

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June 2015

ACKNOWLEDGEMENTS

First of all, I would like to express my great appreciation to my supervisor, Professor Kwang Hoon Lee, for his guidance and encouragement to complete this thesis.

I appreciate Professors Jin-Wou Kim, Min-Geol Lee, Jae Myun Lee, and Myung Hyun Sohn who gave me great suggestions, supports and encouragements. I also specially thank to Professor Jin Young Kim for her guidance and technical assistance.

I appreciate Dr. Jung U Shin, Hyeran Kim, Dr. Shan Jin, Dr. Ji Yeon Noh, Dr. Jung Soo Lee, Dr. Hemin Lee, and SeoHyeong Kim for their kind support.

Finally, I am truly grateful to my family members, especially my parents, my wife, and my two sons, who have been my side with love during the years of my study. I give my love and admiration to them.

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ABSTRACT

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Although adaptive allergic inflammation, including IgE sensitization, has long been regarded as a therapeutic target, especially in allergic patients with atopic dermatitis (AD), the treatments of nonallergic AD or recalcitrant severe AD patients are often challenging, thus new immunologic approaches such as innate immunity are required to control symptoms in these patients. The innate allergic immune response including an epidermis-derived pro-Th2 cytokine, thymic stromal lymphopoietin (TSLP)-activated invariant natural killer T (iNKT) cells is considered a principal innate immune axis in AD, and we previously demonstrated that the number of iNKT cells increased in lesional skin of AD patients. Therefore, the aim of this study is to identify the innate allergic immune modulatory proteins in TSLP-activated iNKT cells using proteomics, and validate the candidates of innate allergic immune modulatory factors in peripheral blood and skin from patients with AD.

Using a new quantitative proteome analysis using a TMT-labeling method, I identified, quantitated, and compared the proteins in non-treated, TSLP-6 h treated,

and TSLP-24 h treated iNKT cells to identify sequentially significantly changed proteins after TSLP treatment. Among initially identified 1404 proteins, I could identify significantly differentially expressed 28 proteins (3 up-regulated and 25 down-regulated proteins). Finally, I chose CXCR4 for further investigation because CXCR4 was the most significantly up-regulated protein in TSLP 24 h-treated iNKT cells and human iNKT cells express CXCR4.

I demonstrated that $CXCR4^{+}$ iNKT cells were significantly infiltrated into AD skin compared to normal skin while circulating iNKT cells from AD patients expressed less CXCR4 rather than normal circulating iNKT cells. Next, I also demonstrated that its cognate ligand, SDF1α was significantly increased in AD skin while iNKT cells did not express SDF1α, but resided next to surrounding SDF1α-producing cells. Human dermal fibroblasts and T cells (both CD4 and CD8) in AD skin produced SDF1α significantly compared to those in normal human skin. TSLP significantly induced SDF1α expression in normal human T cells (both CD4 and CD8) and dermal fibroblasts 24 hours after TSLP treatment. SDF1α levels were also increased in the peripheral blood of AD patients.

In summary, circulating TSLP-activated iNKT cells induce CXCR4 expression, and migrated into the AD skin, which is enriched with its cognate ligand, SDF1α produced by TSLP-activated dermal fibroblasts and T cells. Increased circulating SDF1α of AD patients also may potentiate CXCR4-mediated iNKT cell infiltration into the AD skin.

Key words: atopic dermatitis, thymic stromal lymphopoietin, invariant natural killer T cell, CXCR4, SDF1α

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I. INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic, chronic relapsing inflammatory skin disease, characterized by skin barrier dysfunction resulting in dry skin and allergic sensitization to food and environmental antigens.[1,2] The most common form of AD, accounting for 70-80% of cases, is allergic AD with elevated concentrations of total and allergen-specific IgE in serum and skin.[3] The remaining 20-30% patients have nonallergic AD, with normal total IgE levels and negative serum allergen-specific IgE, reflecting multiple different pathogenic mechanisms and indicating that AD represents a collection of heterogeneous groups.[4]

Although adaptive allergic inflammation, including IgE sensitization, has long been regarded as a therapeutic target, especially in allergic AD patients, the clinical efficacy of anti-IgE monoclonal antibody in these AD patients is still controversial.[5] The treatments of nonallergic AD or recalcitrant severe AD patients are often challenging, thus new immunologic approaches such as innate immunity are required to control symptoms in these patients. The innate allergic immune response, including thymic stromal lymphopoietin-activated invariant natural killer T cells or

mast cells, is considered a principal innate immune axis in AD.[6,7] Natural killer T (NKT) cells represent a subpopulation of thymus-derived T cells that co-express a T-cell receptor (TCR) along with typical surface receptors for natural killer cells.[8] More than 80% of NKT cells express invariant TCRα chain Vα24-Jα18 paired with Vβ11 in humans (or Vα14-Jα18 paired with Vβ8, Vβ7, or Vβ2 in mice) and are referred to as invariant NKT (iNKT) cells.[9] iNKT cells uniquely recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like molecule CD1d. A glycolipid isolated from a marine sponge, α-galactosylceramide (α-GalCer), binds CD1d and activates mouse and human iNKT cells,[10] but the natural ligands presented in vivo by CD1d to iNKT cells remain unclear except for a lysosomal glycosphingolipid, isoglobotrihexosylceramide.[11] iNKT cells rapidly produce large quantities of cytokines, including IL-4 and IFN-γ, as a manifestation of innate-like immunity. This rapid production of cytokines regulates adaptive immune responses by activation of dendritic cells (DCs), natural killer cells, B cells, and conventional T cells, thus linking innate and adaptive immunity.[12]

Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine produced by human keratinocytes in AD.[13] TSLP exerts its biological activities by binding to a heterodimeric receptor consisting of the IL-7 receptor α-chain (IL-7Rα) and the TSLP receptor chain (TSLPR).[14] TSLP not only triggers adaptive allergic immune responses by stimulating myeloid DCs to induce T_H2 cell responses, but also rapidly induces an innate type of allergic immune response by activating mast cells and NKT cells.[15] In our previous study, we demonstrated that the number of iNKT cells increased in lesional skin of atopic dermatitis patients. Then, we suggested that iNKT cells, unlike conventional T cells, may contribute to the innate allergic immune response in the pathogenesis of atopic dermatitis.[6]

Thus, the aim of my study is to identify the innate allergic immune modulatory proteins in TSLP-activated iNKT cells using proteomics. Also, I would like to validate the candidates of innate allergic immune modulatory factors in peripheral blood and skin from patients with AD.

II. MATERIALS AND METHODS

1. Subjects

Blood samples were obtained from non-atopic healthy controls (HCs) and patients with AD as defined by the criteria of Hanifin and Rajka.[16] Skin biopsy specimens were taken from the patients with AD and some of the HCs. The patients did not receive any systemic or topical treatment with immunosuppressive drugs for at least four weeks before collection of blood samples and skin biopsies. For sorting of iNKT cells using proteomics, isolated peripheral blood mononuclear cells (PBMCs) from normal blood donor buffy coat preparations were washed and incubated with FITC-anti-human 6B11 mAb against the complementarity-determining–region 3 of the Vα24-Jα18 TCR of iNKT cells (BD Biosciences, San Jose, CA, USA). Cells were sorted with an EPIC Altra cell sorter (Beckman Coulter, Fullerton, CA, USA). Sorted iNKT cells were incubated with or without 50 ng/ml TSLP for 6 or 24 hours. All subjects provided written informed consent to participate in the study and the institutional review board approved this study.

2. Proteome sample preparation

The protein mixtures (100 μg) from each cell population were reduced with 500 mM tris (2-carboxyethyl) phosphine (TCEP) at room temperature for 60 min and then alkylated with 500 mM IAA at room temperature in the dark for 60 min. The samples were desalted using membrane filter of 10 KMW and dissolved in 200 mM triethylammonium bicarbonate (TEAB) buffer to a final concentration of 1 μg/μL. Protein concentration was measured by a bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA), following the manufacturer's protocol. Sequencing grade trypsin (Promega, Madison, WI, USA) was added at 1:20 (wt/wt) into proteins in TEAB buffer and incubated overnight at 37°C. Samples were individually labeled using TMT-126, 129 (control group: untreated iNKT cells); TMT-127, 130 (TSLP 6-

h-treated iNKT cell group) and TMT-128, 131 (TSLP 24-h-treated iNKT cell group) following the manufacturer's protocol (Thermo Scientific). Aqueous hydroxylamine solution (5% w/v) was added to quench the reaction. The six samples were then combined, speed-vacuum dried, and then dissolved in 50 μL of water containing 0.1% formic acid for 2D-LC-MS/MS analysis. The general experimental procedure is shown in Fig. 1.

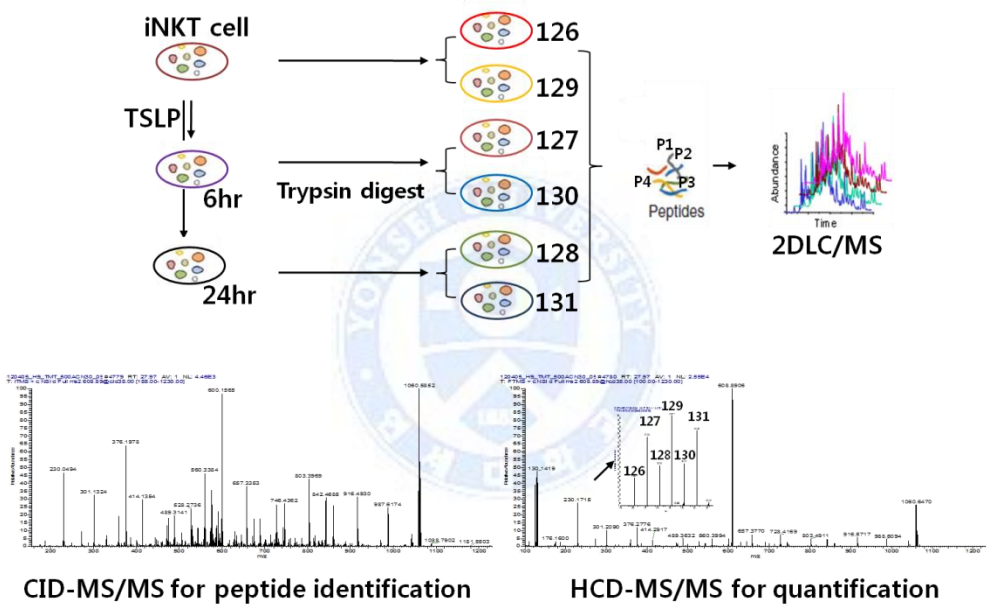


Figure 1. Protein quantification using in-vitro labeling, Tandem Mass Tag (TMT). I used a quantitative mass spectrometry-based proteomics experiments with stable-isotope-containing isobaric tags (TMT). TSLP was treated on purified iNKT cells from normal donors. 6 and 24 hours after TSLP treatment, I extracted protein products and performed TMT analysis after dividing individual samples into two and labeling in vitro.

3. 2D-LC-MS/MS

The TMT-labeled samples were analyzed using a 2D-LC-MS/MS system consisting of a nanoACQUITY UltraPerformance LC System (Waters, Milford, MA, USA) and a LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nano-electrospray source. Briefly, a strong cation exchange (5 μm, 3 cm) column was placed just before the C18 trap column (id 180 μm, length 20 mm, and particle size 5 μm; Waters). Peptide solutions were loaded in 5 μL aliquots for each run. Peptides were displaced from the strong cation exchange phase to the C18 phase by a salt gradient that was introduced through an autosampler loop and then desalted for 10 min at a flow rate of 4 μL/min. Then, the trapped peptides were separated on a 200 mm homemade microcapillary column consisting of C18 Aqua (particle size 3 μm) (Phenomenex, Torrance, CA, USA), packed into 100 μm silica tubing with an orifice id of 5 μm.

An eleven-step salt gradient was performed using 3 μL of 0, 25, 50, 100, 250, and 500 mM ammonium acetate (0.1% formic acid in 5% ACN) and 4, 5, 9 and additional 9 μL and 500 mM ACN, (0.1% formic acid in 30% ACN). The mobile phases, A and B, were composed of 0 and 100% acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 1 min and was ramped to 20% B over 5 min, to 55% B over 90 min, to 95% B over 1 min, and remained at 95% B over 13 min and 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. The voltage applied to produce an electrospray was 2.0 kV. During the chromatographic separation, the LTQ Orbitrap Elite was operated in a data-dependent mode. The MS data were acquired using the following parameters: five data-dependent collision induced dissociation-high energy collision dissociation (CID-HCD) dual MS/MS scans per full scan; CID scans were acquired in linear trap quadrupole (LTQ) with two-microscan averaging; full scans and HCD scans were acquired in Orbitrap at resolution 60,000 and 15,000 respectively, with two-microscan averaging; 35% normalized collision

energy (NCE) in CID and 45% NCE in HCD; ±1 Da isolation window. Previously fragmented ions were excluded for 60 sec. In CID-HCD dual scan, each selected parent ion was first fragmented by CID and then by HCD.

4. Protein identification and quantification

MS/MS spectra were analyzed using the following software analysis protocols with the IPI mouse database (IPI.MOUSE. 7.26.2012). The reversed sequences of all proteins were appended into the database for calculation of false discovery rate (FDR). ProLucid[17] was used to identify the peptides, a precursor mass error of 25 ppm, and a fragment ion mass error of 600 ppm. Trypsin was selected as the enzyme, with three potential missed cleavage. TMT modification (+ 229.1629) at the N-terminus and lysine residue by the labeling reagent and carbamidomethylation at cystein were chosen as static modifications. Oxidation at methionine was chosen as variable modification. The CID and HCD tandem MS spectra from the same precursor ion are often combined by software to allow better peptide identification and quantification.[18] I used homemade software where reporter ions from HCD spectrum were inserted into CID spectrum with the same precursor ion at the previous scan. Reporter ions were extracted from small windows (±20 ppm) around their expected m/z in the HCD spectrum. The output data files were filtered and sorted to compose the protein list using the DTASelect[19] (The Scripps Research Institute, La Jolla, CA, USA) with two and more peptides assignments for protein identification and a false positive rate less than 0.01.

A quantitative analysis was conducted using Census in IP2 pipeline (Integrated Proteomics, San Diego, CA, USA). The intensity at a reporter ion channel for a protein was calculated as the average of this reporter ion's intensities from all constituent peptides in the identified protein.[20] The measured intensity ratios of proteins were transformed to Gaussian fit ratio scale. Ratios were averaged and proteins with $P < 0.05$ were defined as

significantly regulated. Heat map for differential expression analysis was undertaken using the limma software package for R.[21]

5. Immunohistochemical staining for CXCR4 and Vα24-Jα18

Acetone-fixed 6 μm cryostat sections were stained with anti-CXCR4 mAb (Abcam, Cambridge, UK), anti-Vα24-Jα18 (6B11) mAb (BD Biosciences). Double staining was performed by using anti-Vα24-Jα18 and anti-CXCR4. Development of the staining was performed with a G|2 Doublestain System kit (Dako, Carpinteria, CA, USA) according to the manufacturer's recommendations. Two independent investigators evaluated staining intensity in a blind manner.

6. Transwell migration assay

Human iNKT and T cells were sorted from normal PBMCs using iNKT and CD3 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, according to manufacturer's protocol. The purity was greater than 95% in all cases. The migration assay was performed using 6.5 mm Transwell filter inserts with 5.0 μm pore (Corning Costar, Tewksbury, MA, USA) in 24 well culture plates. The migration assay was started by the addition of 100 μl serum-free buffer (RPMI 1640 medium, 0.5% BSA) containing 1×10^6 cells to the upper chamber and the addition of 600 μl serum-free RPMI1640 medium with 300 ng/ml of stromal cell-derived factor-1α (SDF1α) (R&D systems, Minneapolis, MN, USA) to the lower chamber. After 3 h of incubation, the cells in the bottom chamber were counted using the Beckman Coulter counter (Beckman Coulter). The migration index was expressed as a percentage of the cell population migrating to the lower chamber.

7. Immunofluorescence staining

A. Cells

For surface staining, cells were stained with PE-anti CD8 mAb (BD Biosciences), PE-anti CD4 mAb (Abcam), PE-anti CD3 mAb (Novus Biologicals, Littleton, CO, USA). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience, San Diego, CA, USA), then stained with FITC-anti-human SDF1α mAb (Abcam). Cells were treated with 50 ng/ml TSLP for 24 h.

B. Tissues

Acetone-fixed 6 μm cryostat sections were washed with phosphate buffered saline (PBS), blocked with 10% goat non-immune serum (Invitrogen, Camarillo, CA, USA) at room temperature for 1 h and then incubated with anti-human SDF1α mAb (Abcam), anti-CXCR4 mAb (Abcam) at 4°C overnight. After washing with PBS, the sections were incubated with FITC-conjugated anti-Rabbit IgG (Abcam) at room temperature for 1 h. After washing with PBS, the sections were incubated with PE-anti-Vα24-Jα18 TCR mAb (eBioscience), PE-anti CD8 mAb (BD Biosciences), PE-anti-CD4 mAb (Abcam), PE-anti-CD3 mAb (Novus Biologicals) at room temperature for 2 h. For vimentin staining, the sections were incubated with anti-vimentin mAb (R&D) at room temperature for 2 h, then, after washing with PBS, incubated with PE-conjugated anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. After washing with PBS, the sections were incubated with 4′, 6-Diamidino-2-phenylindole dihydrochloride (Sigma, St. Louis, MO, USA) for 5 min, and then mounted with the mounting medium. Sections were observed and imaged with LSM 700 (Carl Zeiss, Jena, Germany). All incubations were performed at room temperature in the dark using the antibody concentrations according to manufacturer's recommendation.

8. Western blot

Western blotting was carried out by using 20 μg protein from whole-cell lysates of healthy human T cells, fibroblasts, and human serum. And the resulting supernatant was used as the cytosol fraction. Protein extracts were prepared by lysing the cells in pro-prep lysis buffer (Intron, Seoul, Korea) and the concentration of cellular protein was determined using Copper(II) sulfate solution in Bicinchoninic Acid solution (Sigma). Equal concentrations of cellular protein mixed with a 5× sample buffer were heated at 100°C for 5 min and separated on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto ECL nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) with Tris buffer (0.025 M Tris-HCI, 0.192 M glycine, and 20% MeOH). The membrane was blocked for 1 h at room temperature with 5% skim milk in TBS-Tween 20, incubated overnight at 4°C with specific antibodies, and then incubated with horseradish peroxidase-conjugated anti-mouse or rabbit secondary antibodies (Santa Cruz) for 1 h at room temperature. Finally, membrane was developed with the enhanced chemiluminescence Western blotting detection reagent (Santa Cruz) according to the manufacturer's protocol. Goat anti-human TSLPR (R&D) and Rabbit anti-human SDF1α (Abcam) antibodies were used at a dilution of 1:1000. Rabbit anti-GAPDH antibody (Santa Cruz) was used as a loading control at 1:1000.

9. RT-PCR analysis

Total RNA was extracted with a commercially available kit (RNeasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription reactions were performed using 1 μg of total RNA. The following primers were used: CXCR4, 5'-CAC TTC AGA TAA CTA CAC CG-3' (sense) and 5'-ATC CAG ACG CCA ACA TAG AC-3' (antisense); TSLPR, 5'-CTG ACC TGT CCT ACG GGG AT-3' (sense) and 5'-CCT GGA AGT TCC CTT GGT GT-3' (antisense); and GAPDH, 5'-CAT TGC CCT CAA TGA CCA CT-3' (sense) and 5'-TCC TTG GAG GCC ATG TAG AC-3'

(antisense). Amplification was performed on a GeneAmp polymerase chain reaction (PCR) system 2700 (Applied Biosystems, Foster city, CA, USA). PCR was conducted under the following conditions: for GAPDH, denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 7 min; for TSLPR and CXCR4, 94°C for 30 s, 53.7°C (TSLPR) and 58°C (CXCR4) for 1 min, and 72°C for 1 min for 40 cycles, followed by a final extension at 72°C for 7 min. Specific PCR fragments were separated on a 2% agarose gel and visualized using ethidium bromide staining.

10. Statistical analysis

All statistical analysis was conducted by using SPSS version 12.0 software (SPSS, Inc, Chicago, IL, USA). Data were analyzed with the Mann-Whitney *U* test. Correlations were determined by calculating the Pearson correlation coefficient. $P < 0.05$ was considered statistically significant.

III. RESULTS

1. Quantitative proteome analysis using a TMT-labeling method

Proteins extracted from iNKT cells of un-treated, TSLP 6 h-treated, and TSLP 24 h-treated group were labeled with TMT isobaric tag individually for quantitative proteomic analysis. Samples were then combined, speed-vacuum dried, and then dissolved in 50 μL of water containing 0.1% formic acid for 2D-LC-MS/MS analysis. Initially, I identified 1404 proteins in TSLP-treated and un-treated iNKT cells and then I performed Gene Ontology (GO) analysis (Fig. 2).

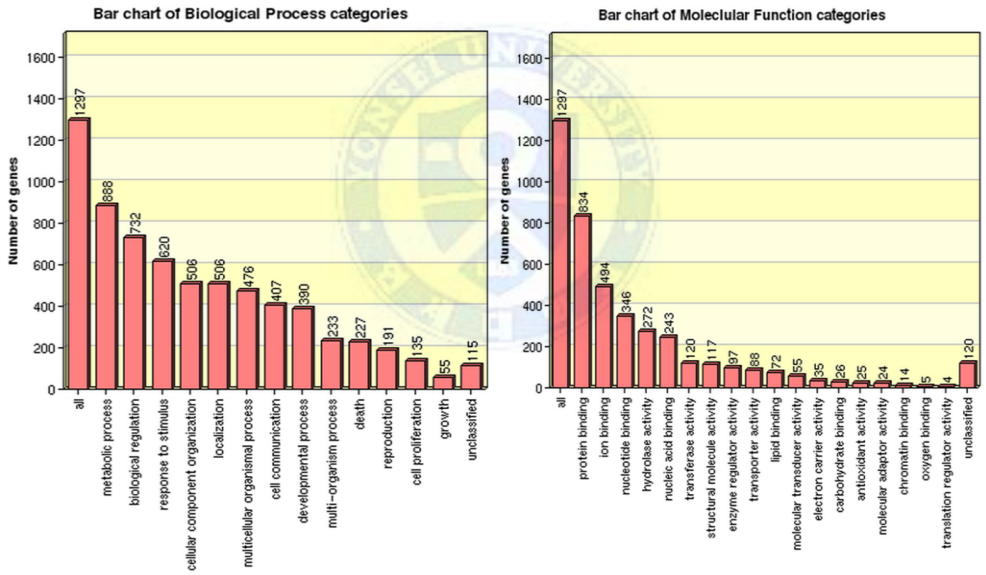


Figure 2. Gene Ontology (GO) analysis. I identified 1,404 proteins after TMT analysis and then I performed GO analysis (http://bioinfo.vanderbilt.edu/webgestalt/) using 1312 genes extracted from these proteins. 15 genes among these genes are excluded (KIAA1949, TUBB2C, HSPE1, C20orf3, HBD, MOBKL2A, CCDC72, LOC727848, THOC4, COPG, PKM2, SNRPEL1, MT-CO2, C9orf167, C2orf85).

Afterwards, I attempted to identify proteins that either increased or decreased depending on time after stimulation with TSLP. Considering distributions of Gaussian fit ratios, proteins with $P < 0.05$ (ratio $\leq$ -0.37 or $\geq$ 0.37) were considered as differentially expressed ones. Finally, I identified 28 proteins as candidates for further investigation. Among these proteins, 3 proteins were up-regulated (Table 1) and 25 proteins were down-regulated (Table 2). I also found that these proteins were differentially expressed in un-treated, TSLP 6 h-treated, and TSLP 24 h-treated group in a heat-map analysis of the corresponding 24 genes (Fig. 3). Among these proteins, CXCR4 increased most significantly in TSLP 24 h-treated group compared to other 8 proteins clustering in the same gene group (Fig. 4). Only CXCR4 was gradually up-regulated depending on the time. CXCR4 was confirmed two times in TMT analysis, and I also found that up-regulated CXCR4 is linked with up- and down-regulated proteins using protein-protein interaction (PPI) analysis (Fig. 5). Thus, I chose CXCR4 for further validation and functional studies using peripheral blood and skin from AD patients compared to HCs.

Table 1. Up-regulated proteins identified using TMT labeling method

NO.	IPI NO.	DESCRIPTION	Fit ratio (TSLP6/Cont)	Fit ratio (TSLP24/Cont)
1	IPI00005154.1	SSRP1 FACT complex subunit SSRP1	0.728	0.262
2	IPI00216130.8	OGFR Isoform 2 of Opioid growth factor receptor	0.462	0.445
3	IPI00216445.1	CXCR4 Isoform 2 of C-X-C chemokine receptor type 4	0.452	0.585

Table 2. Down-regulated proteins identified using TMT labeling method

NO.	IPI NO.	DESCRIPTION	Fit ratio (TSLP6/Cont)	Fit ratio (TSLP24/Cont)
1	IPI00553211.1	ERMN Isoform 2 of Ermin	0.096	-0.839
2	IPI00021304.1	KRT2 Keratin, type II cytoskeletal 2 epidermal	-0.517	-0.735
3	IPI00853068.2	HBA2;HBA1 Hemoglobin alpha-2	0.383	-0.698
4	IPI00410714.5	HBA2;HBA1 Hemoglobin subunit alpha	0.380	-0.694
5	IPI00930351.1	HBD Hbbm fused globin protein (Fragment)	0.418	-0.657
6	IPI00018769.4	THBS2 Thrombospondin-2	-0.403	-0.654
7	IPI00654755.3	HBB Hemoglobin subunit beta	0.397	-0.630
8	IPI00554676.2	HBG2 Hemoglobin subunit gamma-2	0.445	-0.625
9	IPI00815947.1	HBB Truncated beta-globin (Fragment)	0.437	-0.615
10	IPI00942405.1	LSM7 R30783_1	0.415	-0.611
11	IPI00473011.3	HBD Hemoglobin subunit delta	0.403	-0.608
12	IPI00791534.2	SLC4A1 Solute carrier family 4, anion exchanger, member 1	0.233	-0.604
13	IPI00829896.1	HBD Hemoglobin Lepore-Baltimore (Fragment)	0.390	-0.564
14	IPI00215983.3	CA1 Carbonic anhydrase 1	0.277	-0.532
15	IPI00707101.0	AHSG Alpha-2-HS-glycoprotein	0.593	-0.514
16	IPI00477420.2	SLC17A6 Vesicular glutamate transporter 2	0.436	-0.506
17	IPI00216187.2	ZNF268 zinc finger protein 268 isoform c	0.280	-0.460
18	IPI00218494.3	HSD3B2 Isoform 1 of 3 beta-hydroxysteroid dehydrogenase/Delta 5-- 4-isomerase type 2	-0.346	-0.443
19	IPI00412713.4	SAMM50 Sorting and assembly machinery component 50 homolog	0.142	-0.443
20	IPI00480077.3	PMFBP1 Isoform 3 of Polyamine-modulated factor 1-binding protein 1	-0.426	-0.433
21	IPI00056357.3	C19orf10 UPF0556 protein C19orf10	-0.080	-0.431
22	IPI00885104.1	MPHOSPH8 Isoform 2 of M-phase phosphoprotein 8	0.145	-0.430
23	IPI00029132.3	BTK Tyrosine-protein kinase BTK	-0.200	-0.420
24	IPI00010257.1	AHSP Alpha-hemoglobin-stabilizing protein	0.179	-0.394
25	IPI00785113.1	LRRFIP1 Isoform 1 of Leucine-rich repeat flightless-interacting protein 1	-0.402	-0.377

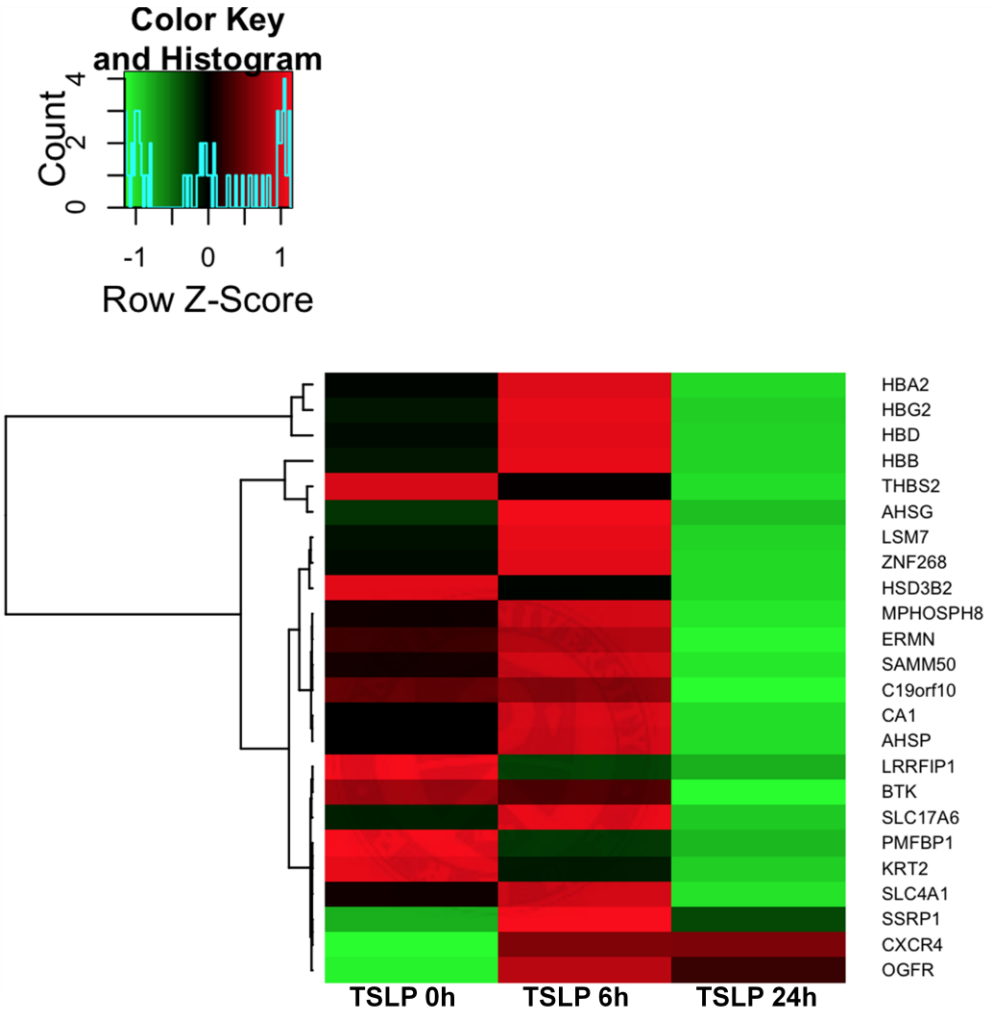


Figure 3. Heat map of expression profiles of un-treated, TSLP 6 h-treated, and TSLP 24 h-treated group. Heat map shows the corresponding 24 genes with the most significant difference between these 3 groups.

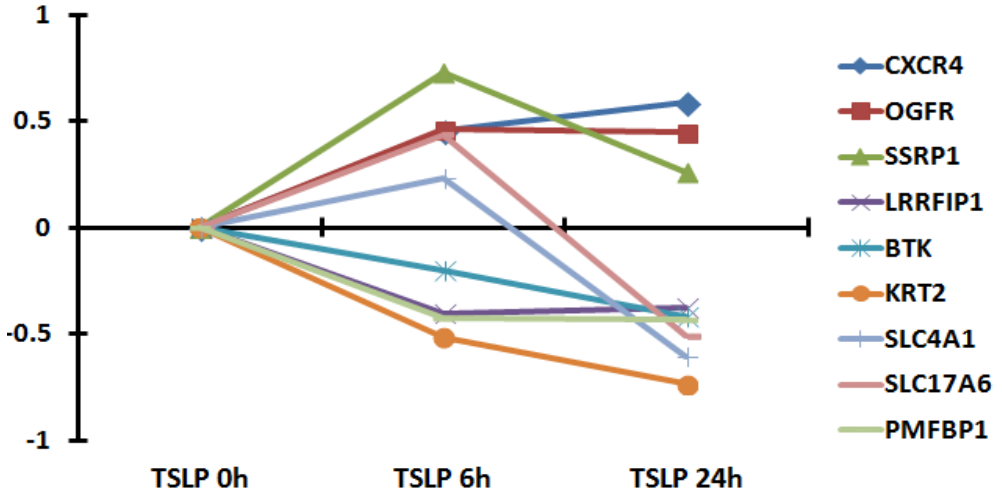


Figure 4. Gaussian fit ratios of 9 proteins clustering in the same gene group that have significant different expression in un-treated, TSLP 6 h-treated, and TSLP 24 h-treated group. CXCR4 increased most significantly in TSLP 24 h-treated group compared to other 8 proteins clustering in the same gene group while OGFR and SSRP1 increased in TSLP 6 h-treated group, but not TSLP 24 h-treated group. Only CXCR4 was gradually up-regulated depending on the time.

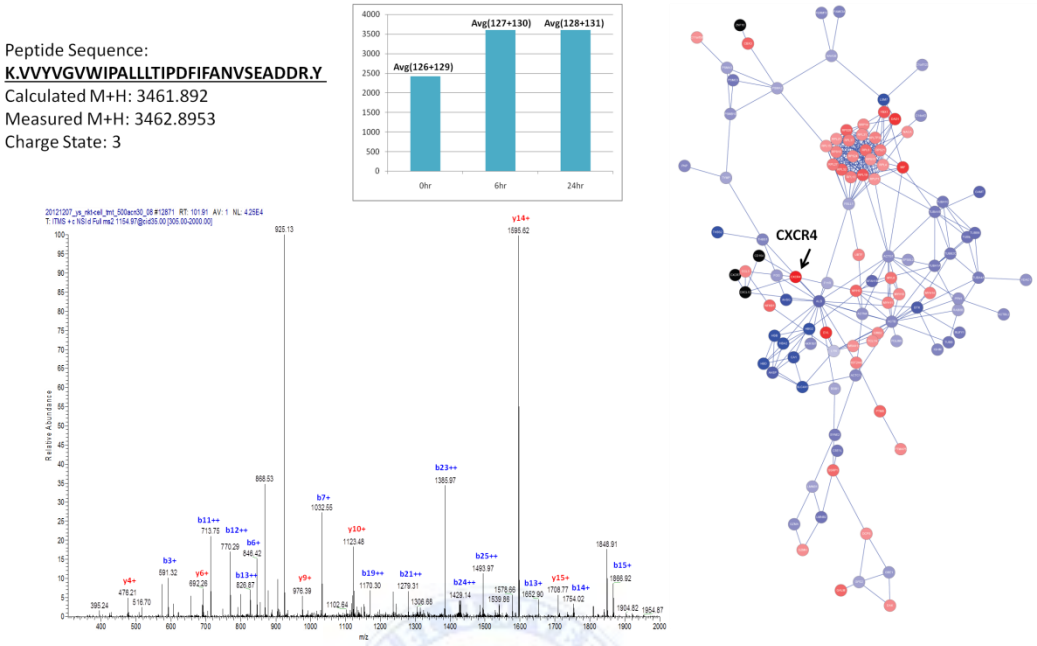


Figure 5. Confirmation of CXCR4 and protein-protein interaction (PPI) analysis. CXCR4 was confirmed two times in TMT analysis. Although both are peptides sharing same sequence, one was found from 3+ charge ion and another from 4+ charge ion. MS/MS data were gathered more efficiently from 3+ charge ion. Reporter ions which represent quantitative changes were calculated from sums of averaged of each values from charge ions. Up-regulated CXCR4 is linked with up- and down-regulated proteins using PPI analysis.

2. CXCR4 expression in iNKT cells from lesional skin and peripheral blood of AD patients

To validate CXCR4 expression in AD patients, I first evaluated whether Vα24^{+} iNKT cells in the AD skin express CXCR4 using immunohistochemical staining. I could confirm that infiltrated Vα24^{+} iNKT cells in the lesional skin of AD patients expressed CXCR4 significantly compared to normal skin (Fig. 6).

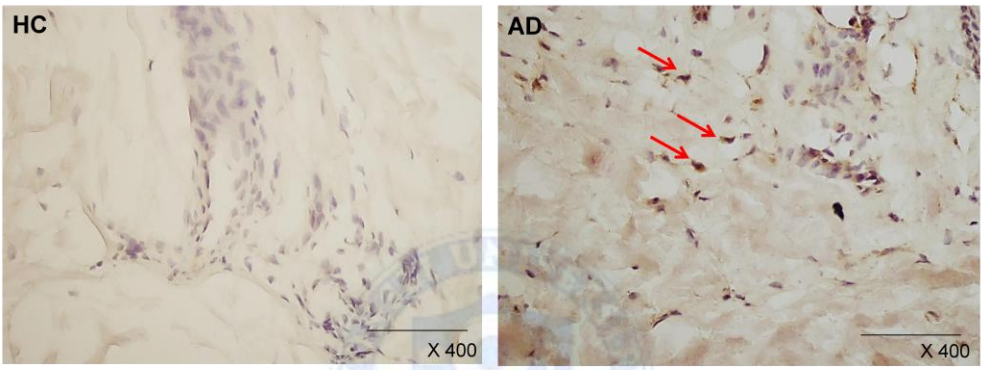


Figure 6. Vα24 and CXCR4 expression in the skin of HCs and AD patients. CXCR4 was co-stained with infiltrated Vα24^{+} iNKT cells in AD skin (CXCR4: brown, Vα24: red). Original magnification x 400. Arrows indicate Vα24/CXCR4 double positive cells. Both Vα24 and CXCR4 expression were up-regulated in the skin of AD patients in contrast to HCs.

To further confirm expression levels of Vα24 and CXCR4 in the lesional skin of AD patients, I performed immunofluorescence staining. I found that Vα24 expression was significantly increased in AD skin compared to HCs while CXCR4 expression of AD skin was comparable to normal skin. I also found that Vα24/CXCR4 co-stained iNKT cells from lesional skin from AD patients significantly increased compared to HCs (Fig. 7).

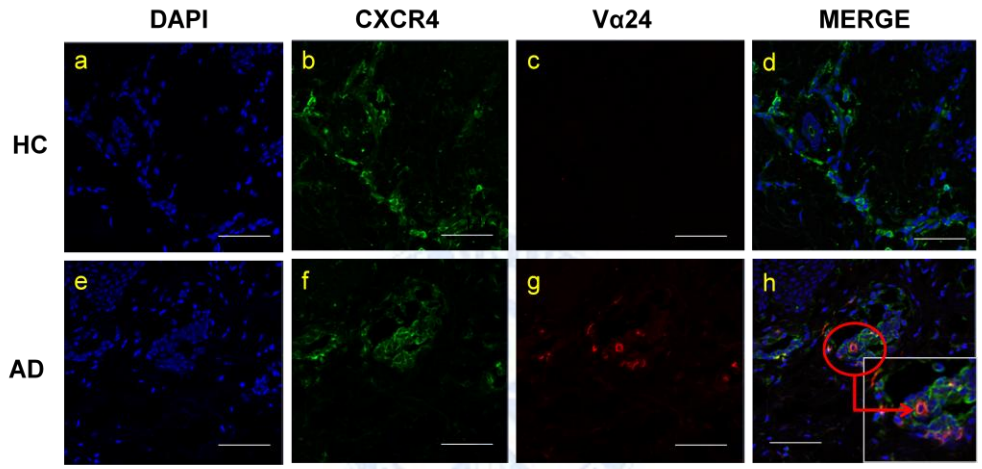


Figure 7. Vα24/CXCR4 expression in the skin of HCs and AD patients. Vα24/CXCR4 was strongly expressed in the AD tissues (e, f, g, h) in contrast to HCs (a, b, c, d). Co-localization of CXCR4 (green) and Vα24 (red) was noted in the lesional skin of AD patients indicated by the red arrow. The figures (d, h) are merged images in which the expression of CXCR4 is green (b, f), and the expression of Vα24 is red (c, g). Nuclei were stained with DAPI (blue). Co-staining of Vα24 and CXCR4 was significantly increased in the skin of AD patients in contrast to HCs. Original magnification x 200.

Next, to evaluate CXCR4 expression in Vα24^{+} iNKT cells in the peripheral blood of AD patients, I examined CXCR4 mRNA levels using RT-PCR. In contrast to AD skin, I found that sorted Vα24^{+} iNKT cells expressed less CXCR4 in the peripheral blood of AD patients than HCs (Fig. 8).

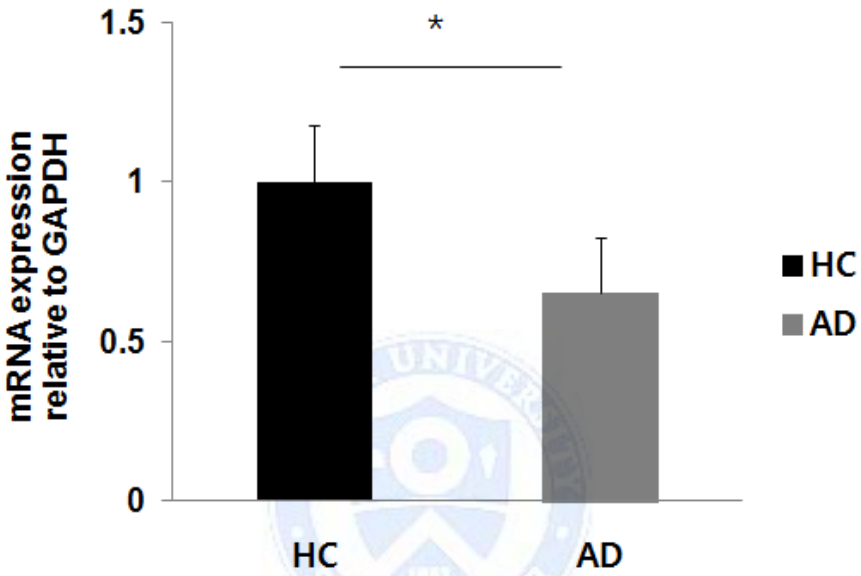
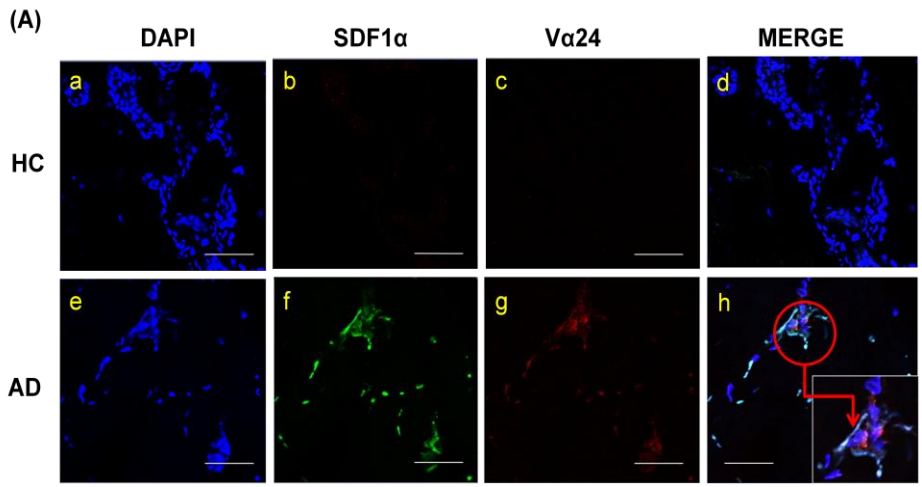
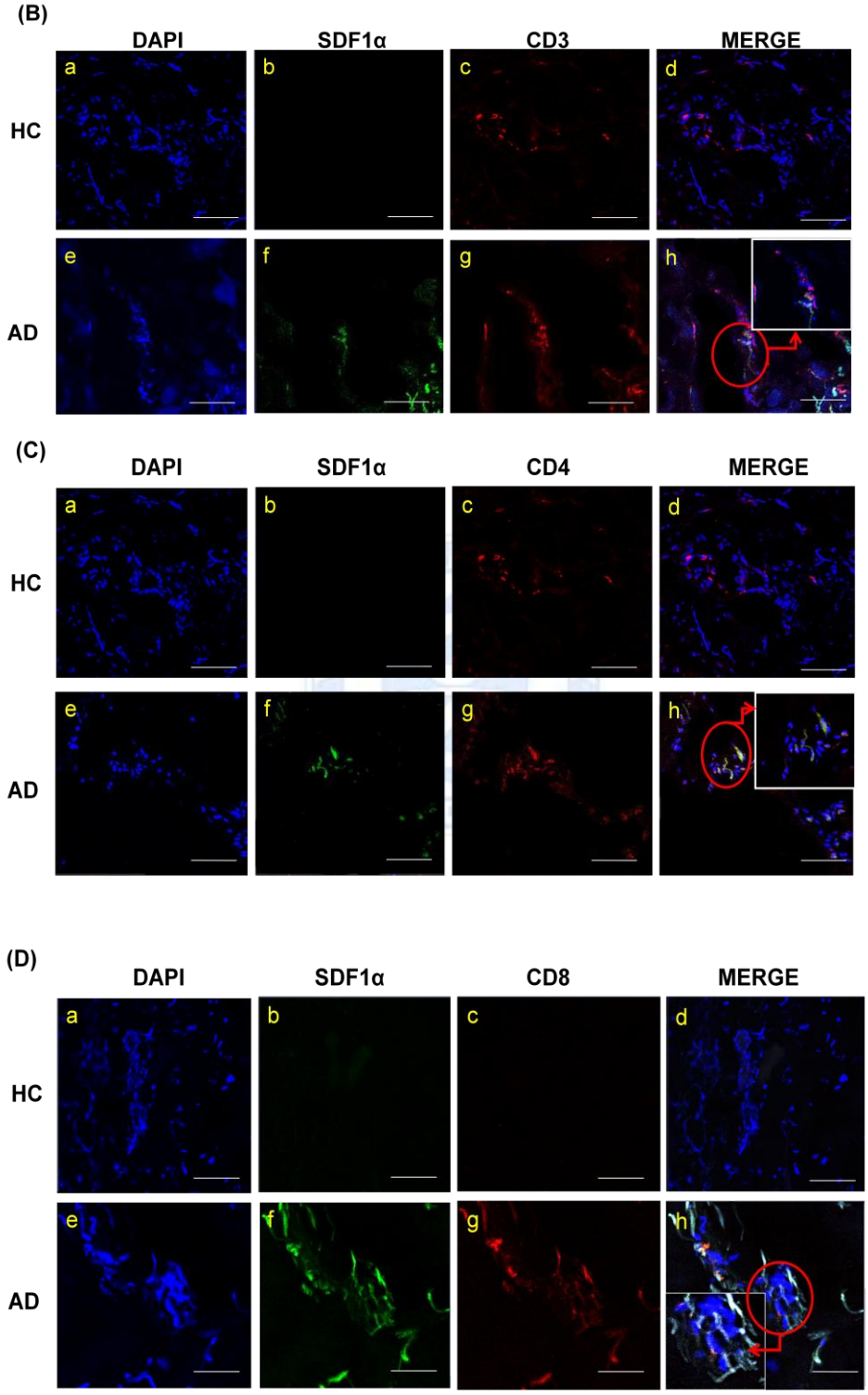


Figure 8. CXCR4 mRNA expressin in sorted iNKT cells in the peripheral blood of AD patients and HCs. I observed CXCR4 mRNA levels in sorted Vα24^{+} iNKT cells from PBMCs in both AD patients and HCs (n = 5), but the CXCR4 mRNA levels in sorted iNKT cells of AD were decreased rather than HCs in contrast to the results found in skin tissues (*$P < 0.05$).

3. Increased SDF1α expression in the lesional skin of AD patients

As SDF1α is the cognate ligand for chemokine receptor, CXCR4,[22] I evaluated whether AD skin expresses SDF1α using immunofluorescence staining. First, I examined how Vα24^{+} iNKT cells interact with SDF1α, a ligand for CXCR4 because skin iNKT cells express high levels of CXCR4. SDF1α was significantly expressed in the dermis of AD skin (f) in contrast to normal skin (b). Interestingly, Vα24^{+} iNKT cells did not express SDF1α (h), but skin iNKT cells were located in juxtaposition to SDF1α-producing cells indicating that skin CXCR4^{+}Vα24^{+} iNKT cells may be directly affected by surrounding SDF1α-producing cells (Fig. 9A). SDF1α-producing cells in AD skin had both longitudinal and round shapes. Thus, I stained these cells with CD3, CD4, and CD8 Abs for round-shaped cells, and vimentin Ab for spindle-shaped cells because there are many T cells with round-shape and longitudinal dermal fibroblasts in AD skin (Fig. 9B-E). CD3 T cells expressed SDF1α (Fig. 9B), and both CD4 and CD8 T cells expressed SDF1α (Fig. 9C and D). Spindle-shaped SDF1α-positive cells were strongly stained with vimentin Ab (Fig. 9E). Thus, I demonstrated that major source of SDF1α is dermal fibroblasts, and CD4 and CD8 T cells also produce SDF1α in AD skin implying that SDF1α-mediated migration of CXCR4^{+}Vα24^{+} iNKT cells may be regulated by dermal fibroblasts and T cells.

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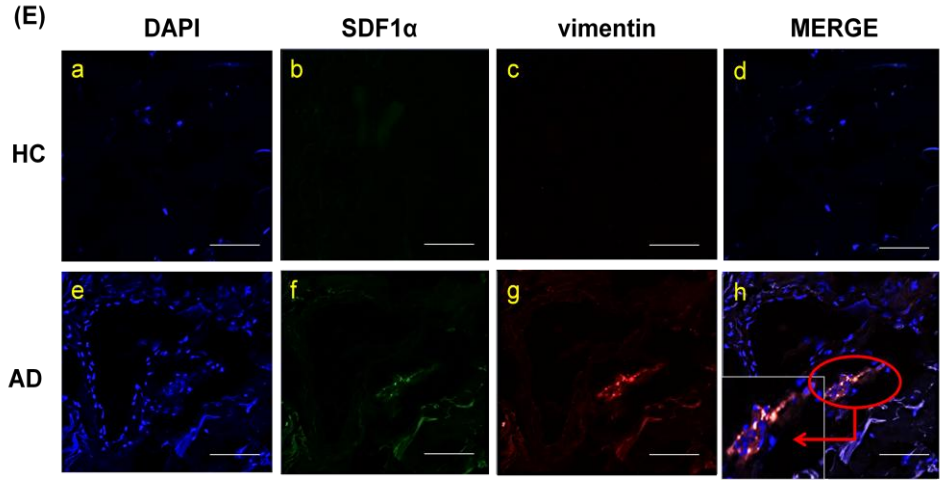


Figure 9. Expression of Vα24, CD3, CD4, CD8, vimentin, and SDF1α in the skin of HCs and AD patients. SDF1α was strongly expressed in the dermis of lesional AD skin (e, f, g, h) in contrast to HCs (a, b, c, d). To investigate which cells express SDF1α in AD skin, SDF1α Ab (green), and Vα24, CD3, CD4, CD8, vimentin Abs (red) were stained in the skin of HCs and AD patients. Nuclei were stained with DAPI (blue). Original magnification x 200. (A) Expression of Vα24 and SDF1α in the skin of HCs and AD patients. SDF1α was not expressed in $V\alpha24^+$ iNKT cells. (B) Expression of CD3 and SDF1α in the skin of HCs and AD patients. SDF1α was co-stained in CD3 T cells. (C) Expression of CD4 and SDF1α in the skin of HCs and AD patients. SDF1α was co-expressed in CD4 T cells. (D) Expression of CD8 and SDF1α in the skin of HCs and AD patients. SDF1α was focally expressed in CD8 T cells. (E) Expression of vimentin and SDF1α in the skin of HCs and AD patients. SDF1α was strongly co-stained in $vimentin^+$ spindle-shaped cells indicating human dermal fibroblasts.

4. SDF1α induces migration of human iNKT cells

Next, I evaluated how SDF1α affects migration properties of iNKT cells using transwell migration assay. I found that SDF1α significantly enhanced migration of sorted Vα24^{+} iNKT cells from normal human PBMCs compared to purified T cells that did not migrate significantly after 3 h incubation (Fig. 10). These results indicate that circulating Vα24^{+} iNKT cells expressing CXCR4 respond to SDF1α and migrate into AD skin, which is enriched with SDF1α produced by human dermal fibroblasts and T cells.

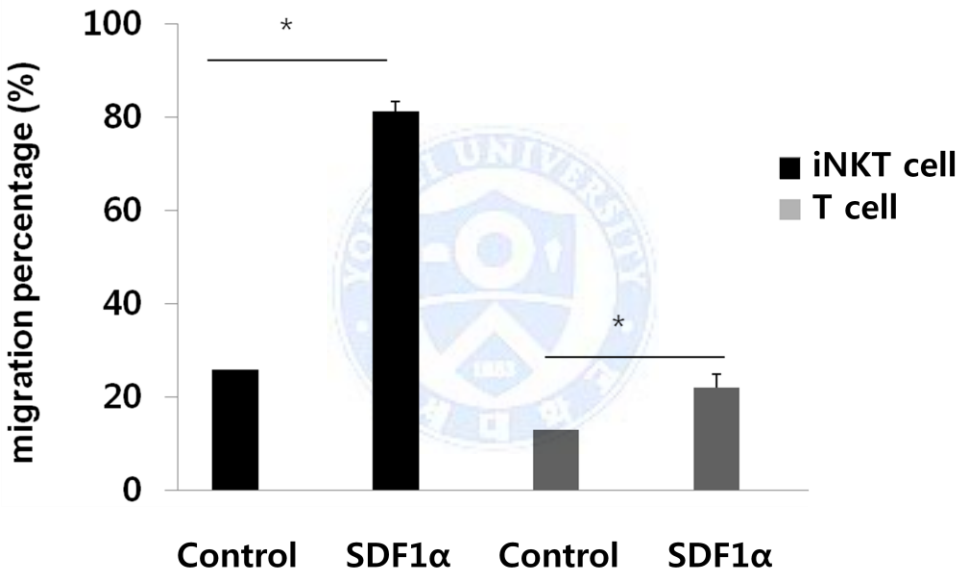
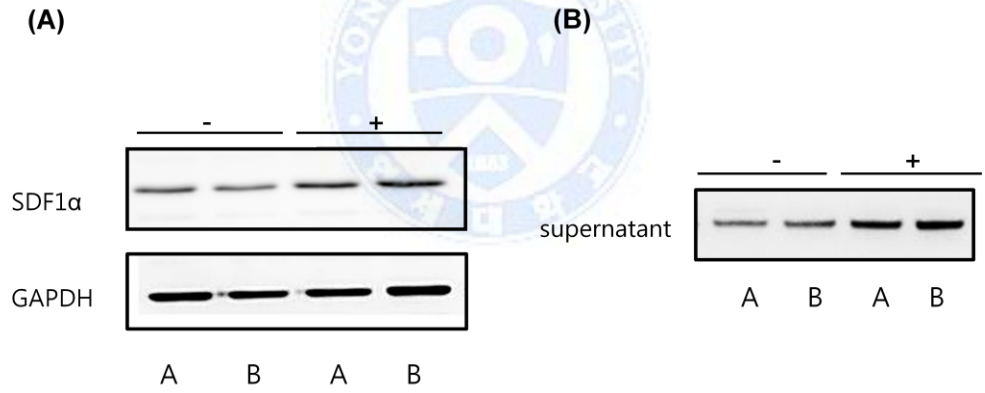


Figure 10. SDF1α promotes the migration of iNKT cells. The effect of SDF-1α on migration of normal human iNKT cells and T cells was assessed by transwell migration assay. The percentage of cells that migrated through the 8-μm pore-size membrane of Transwell insert is shown (*$P < 0.05$). After 3 h of incubation, 300 ng/ml SDF-1α significantly increased the migration of Vα24^{+} iNKT cells while T-cell migration was not significantly affected by SDF-1α.

5. TSLP induces SDF1α expression in T cells

I demonstrated that skin T cells produce SDF-1α in atopic dermatitis. However, which mechanism involves the production of SDF-1α by T cells in AD is not known yet. As T cells are already proven to have TSLP receptor and directly activated,[15] I examined whether TSLP stimulates T cells to produce SDF-1α. I demonstrated that TSLP-activated T cells produced more SDF-1α than non-activated T cells (Fig. 11A), and extracellular (secreted) SDF-1α is also significantly increased in TSLP 24h-treated group compared to non-treated group (Fig. 11B) using Western blotting. I also confirmed that TSLP-treated CD3 T cells significantly expressed SDF-1α compared to non-activated CD3 T cells using immunofluorescence staining (Fig. 11C). Both CD4 and CD8 T cells also produced SDF-1α 24 hours after TSLP treatment (Fig. 11D and E).



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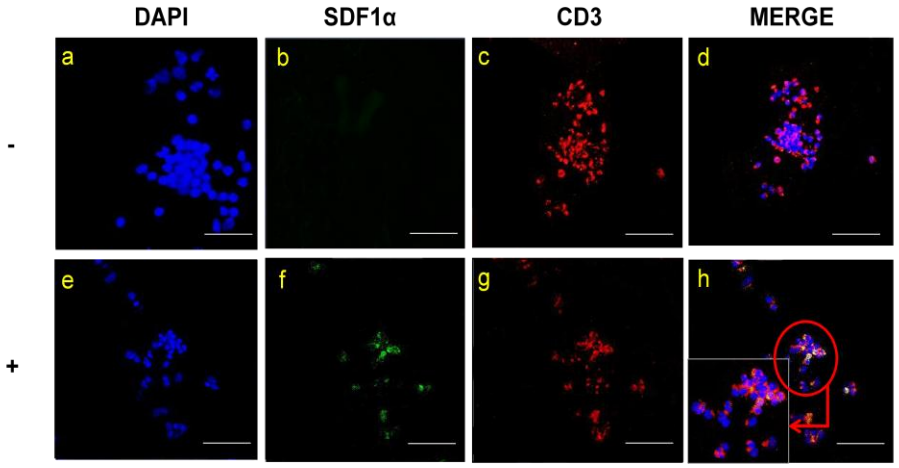
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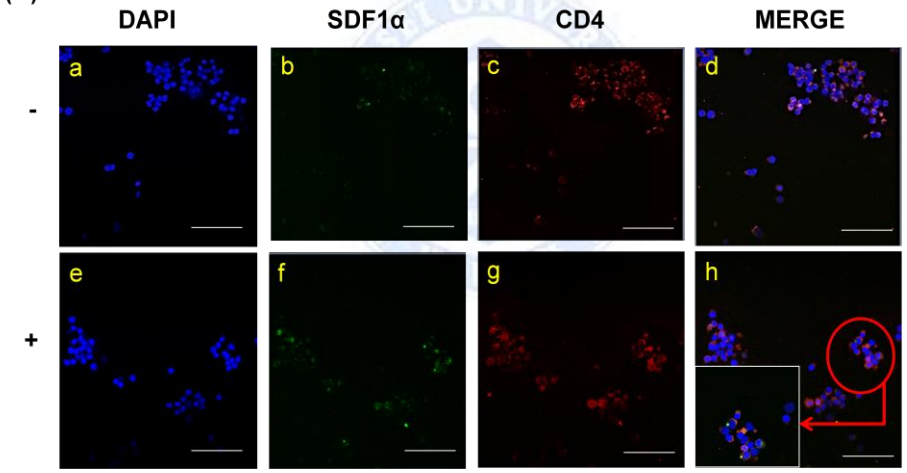
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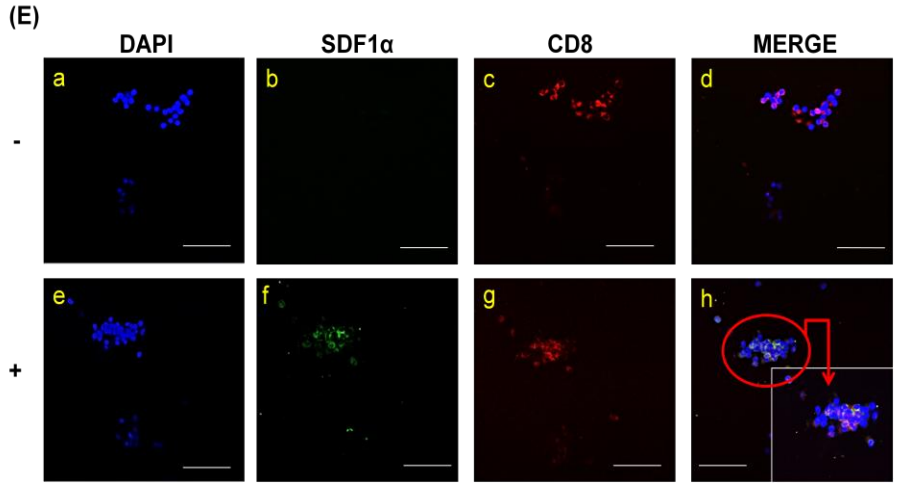
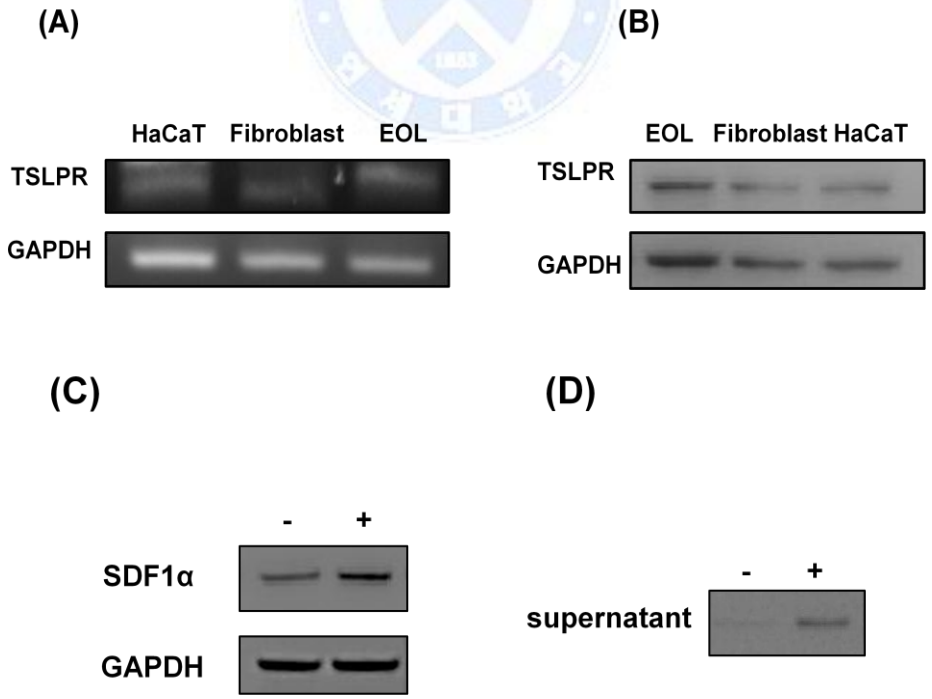


Figure 11. Up-regulated expression of SDF1α in T cells after TSLP treatment.

(A) Western blot analysis of SDF1α in sorted T cells after treatment with 50 ng/㎖ of TSLP for 24 h. Intracellular SDF1α was increased after TSLP treatment. (B) Secreted extracellular SDF1α was increased after TSLP treatment. (C), (D), (E) Immunofluorescence staining analysis of CD3, CD4, CD8, and SDF1α in T cells after TSLP treatment. Immunofluorescence staining for CD3 (C), CD4 (D), CD8 (E) (all Red), and SDF1α (green) was evaluated by confocal laser microscopy. CD8, CD4, CD3 staining (all c, g), and SDF1α staining (all b, f) in T cells after non-treatment (a, b, c, d) and treatment (e, f, g, h) with 50 ng/㎖ of TSLP for 24 h. SDF1α was increased in CD3, CD4, and CD8 T cells after TSLP treatment. Nuclei were stained with DAPI (blue) (all a, e). Original magnification x 200.

6. TSLP induces SDF1α expression in dermal fibroblasts

I also demonstrated that human dermal fibroblasts produce SDF-1α in atopic dermatitis. To evaluate direct action of TSLP on dermal fibroblasts, I first examined if human dermal fibroblasts express TSLP receptor. I found that dermal fibroblasts expressed TSLP receptor comparable to eosinophils or keratinocytes using RT-PCR (Fig. 12A) and Western blot analysis (Fig. 12B). Next, I examined whether TSLP stimulates human dermal fibroblasts to produce SDF-1α. I demonstrated that TSLP-activated fibroblasts produced more SDF-1α than non-activated fibroblasts (Fig. 12C), and extracellular (secreted) SDF-1α is also increased in TSLP 24h-treated fibroblasts (Fig. 12D) using Western blotting. As TSLP-activated T cells and dermal fibroblasts produce and secrete SDF-1α extracellularly, I also evaluated serum levels of SDF-1α in AD patients and HCs. I found that SDF-1α expression was higher in the serum of AD patients than HCs (Fig. 12E).



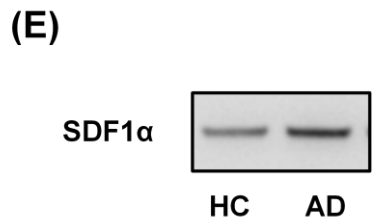


Figure 12. Up-regulated expression of SDF1α in TSLP-activated fibroblasts and serum of AD patients. (A & B) Expression of TSLP receptor (TSLPR) in eosinophil cell line (EOL), human dermal fibroblast (HDF), and immortalized human keratinocyte (HaCaT) cells. EOL, HDF, and HaCaT cells expressed TSLPR in semi-quantitative RT-PCR (A) and Western blot analysis (B). (C) Up-regulated expression of intracellular SDF1α in fibroblasts after TSLP treatment. (D) Secreted extracellular SDF1α was increased after TSLP treatment. (E) SDF1α expression in the serum of HCs and AD patients. SDF1α was highly expressed in the AD serum compared to normal human serum.

IV. DISCUSSION

The epidermis of AD skin is highly expressed with TSLP,[13] and iNKT cells are also increased in the lesional skin of AD patients.[6] We previously demonstrated that TSLP-activated iNKT cells produce Th2 cytokine (IL-4 and IL-13). However, how iNKT cells are recruited into the lesional skin of AD and how TSLP interacts with iNKT cells to migrate into the AD skin, and which factors significantly affect this mechanism are still unknown. Thus, to identify new immunomodulatory proteins in TSLP-mediated iNKT cell immune response, I used new quantitative proteome analysis using a TMT-labeling method that has enhanced accuracy and reproducibility as a tool of high-throughput proteomics.[18,23] I compared non-treated, TSLP-6 h treated, and TSLP-24 h treated iNKT cells to identify sequentially significantly changed proteins after TSLP treatment. Among initially identified 1404 proteins, I could identify significantly differentially expressed 28 proteins (3 up-regulated and 25 down regulated proteins). Finally, I chose CXCR4 for further investigation because CXCR4 was most significantly up-regulated in TSLP 24 h-treated iNKT cells and human iNKT cells express CXCR4.[24,25]

CXCR4 is one of chemokine receptor having a 7 transmembrane domain, G-protein-coupled cell surface receptor which was initially cloned in 1994,[26] and initially identified as a co-receptor for entry of T-tropic (X4) HIV viruses into CD4 T cells.[27] CXCR4 is now known to essential for organ developmental processes,[28,29] cancer metastasis,[30] and trafficking of immune cells.[31-33] As CXCR4-mediated chemotaxis on NK cells is already proven,[33] my proteomics data that TSLP induces CXCR4 expression in iNKT cells suggest that CXCR4-mediated iNKT cells trafficking to AD skin is plausible. I demonstrated that $CXCR4^+$ iNKT cells were significantly infiltrated into AD skin compared to normal skin also supporting the importance of CXCR4-mediated chemotatic signal for trafficking of iNKT cells into AD skin. Interestingly, circulating iNKT cells from AD patients expressed less CXCR4 compared to normal circulating iNKT cells. One possibility is that $CXCR4^+$ iNKT cells in AD patients are already sequestered to the AD skin and only iNKT

cells expressing less CXCR4 may circulate within the blood.

Stromal cell-derived factor-1α (SDF1α), which is now designated as CXCL12,[34] is a cognate ligand for CXCR4.[35] SDF1α is a highly conserved chemokine with 99% homology between mouse and man, and have its essential functions in developmental processes across species barrier.[36,37] In the adult, bone marrow stromal cells,[38] cancer-associated fibroblasts,[30] and human dermal fibroblasts[39] produce SDF1α. I demonstrated that vimentin^{+} dermal fibroblasts in AD skin expressed SDF1α significantly compared to normal human skin. Interestingly, T cells (both CD4 and CD8) also produced SDF1α significantly compared to T cells in normal skin. Thus, I assumed that AD microenvironments like high TSLP micromillieu could affect the production of SDF1α in AD skin. TSLP significantly induced SDF1α expression in normal human T cells (both CD4 and CD8) and dermal fibroblasts 24 hours after TSLP treatment.

Migration of pathogenic T cells to the affected skin in inflammatory skin diseases[40] is a key event in developing cutaneous manifestations in atopic dermatitis. T cell trafficking to the inflamed skin has well been studied.[41] Cutaneous lymphocyte antigen (CLA) and CCR4 is essential for skin homing T cells.[42,43] In contrast to conventional T cells, however, migration properties of innate T cells like iNKT cells into the skin have been not well evaluated. Human iNKT cells have different migration properties depending on organ and disease status. Liver iNKT cells express CXCR6 highly and respond to CXCL16, then accumulate in liver, providing immune surveillance, anti-tumor activity, or promoting inflammation and liver fibrosis.[44-46] Kidney NKT cells expressing CXCR6 also attenuates severe nephritis in a murine model.[47] In systemic lupus erythematosus (SLE) patients, circulating iNKT cells express high levels of CCR6 and CCR4, and iNKT cells from cutaneous lesion of SLE patients also express CCR4 and IFN-γ showing the activity of Th1 type iNKT cells.[48] But I observed that iNKT cells in the lesional skin from patients with AD, a Th2-dominant disease, expressed CXCR4 interacting with surrounding SDF1α-producing cells suggesting that Th2 type iNKT cells from AD (or TSLP-activated iNKT cells) may have different migratory properties compared

to Th1 type iNKT cells. Th17 type iNKT cells also contribute to developing atopic dermatitis. Future studies about migratory properties and in situ function of skin IL-17 producing iNKT cells will be an interesting topic.

In AD skin, an epidermis-derived pro-Th2 cytokine, TSLP is triggered by several stimuli such as allergens, microbial agents, and mechanical injury like scratching.[7,49,50] Increased TSLP as AD skin also circulates in the peripheral blood of AD patients.[51,52] TSLP-activated iNKT cells induce CXCR4 expression, and migrate into the AD skin which is enriched with its cognate ligand, SDF1α produced by TSLP-activated dermal fibroblasts and T cells. SDF1α levels are also increased in the peripheral blood of AD patients, which may potentiate CXCR4-mediated iNKT cell infiltration into the AD skin. A diagram of SDF1α/CXCR4-mediated iNKT cell infiltration in AD skin is shown in Fig. 13. Future targeting therapy against SDF1α/CXCR4/iNKT cell axis in atopic dermatitis[53,54] is to be determined to develop new therapeutic alternatives for severe, or recalcitrant AD patients.

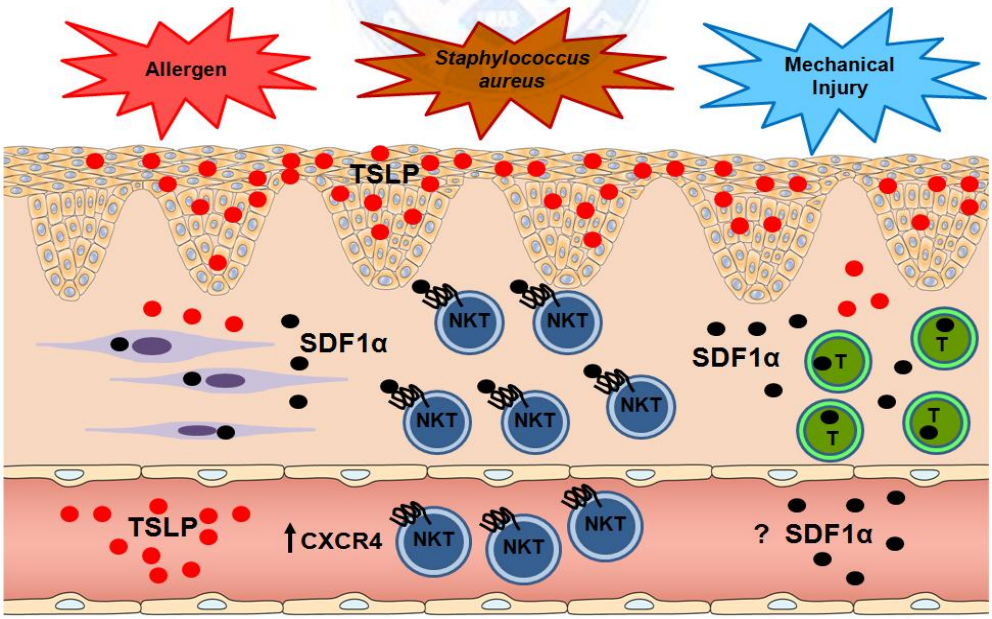


Figure 13. SDF1α/CXCR4-mediated iNKT cell infiltration in AD skin. TSLP is induced by several stimuli such as allergens, microbial agents, and mechanical injury like scratching in the epidermis of AD skin. Increased TSLP also circulates in the peripheral blood of AD patients. TSLP-activated iNKT cells express CXCR4, and are recruited into the AD skin enriched with its cognate ligand, SDF1α produced by TSLP-activated dermal fibroblasts and T cells. Increased circulating SDF1α of AD patients may potentiate CXCR4-mediated iNKT cell infiltration into the AD skin.

V. CONCLUSION

Using an advanced quantitative proteome analysis with a TMT-labeling method, I identified a new innate immunomodulatory protein, CXCR4 in iNKT cells in atopic dermatitis. I further validated the functional role of CXCR4 and its ligand, SDF1α to recruit iNKT cells into the AD skin using immunohistochemical, immunofluorescence staining and migration assay. New therapeutic approaches targeting against SDF1α-CXCR4 axis could give me alternative opportunity to develop an innate immune modulatory drug in atopic dermatitis.

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ABSTRACT (IN KOREAN)

프로테오믹스 기법을 이용한 TSLP 처리 iNKT 세포에서의 선천성 알레르기 면역 조절 단백 발굴

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박 창 욱

아토피피부염은 유소아에서 발생하여 흔히 성인까지 지속되는 심한 소양증과 특징적인 피부 소견을 보이는 만성 염증성 질환이다. 아토피피부염의 병인으로는 유전적 배경과 면역학적 기전, 환경적 요인 등의 다양한 인자가 복합되어 있는 것으로 알려져 있지만 주된 발병 기전이 무엇인지는 아직 명확히 밝혀져 있지 않다. 항 IgE 항체가 아토피피부염 환자에서 효과가 없는 경우가 많고 IgE와 연관이 없는 내인성 아토피피부염의 경우도 있어 IgE와 관련된 후천성 면역 기전뿐만 아니라 선천성 알레르기 면역 기능에 대한 중요성이 최근 보고되고 있다. 이전 연구에서 TSLP에 의해 활성화된 iNKT 세포가 아토피피부염 환자에서 증가되어 있어 iNKT 세포가 선천성 알레르기 면역 반응에 중요한 역할을 한다고 보고하였으나 아직 그 정확한 기전은 밝혀져 있지 않다. 따라서 이번 연구에서는 프로테오믹스 기법을 이용하여 TSLP 처리 iNKT 세포에서 발현하는 선천성 알레르기 면역 조절 단백을 발굴하여 아토피피부염에서 그 역할을 확인하고 검증하여 새로운 선천성 알레르기 면역 조절 기전을 밝히고자 하였다.

정량적 프로테오믹스 분석을 위해 TSLP 미처리, 6시간 및 24시간 TSLP 처리 iNKT 세포에서 단백질을 추출한 후 가수분해하고, Tandem Mass Tag (TMT) 방법을 이용하여 표지하였으며, 2D-LC-MS/MS 분석을 실시하여 각각의 그룹간에 발현이 차이 나는 단백질을 발굴하였다. 전체 1404 개의 단백질이 동정되었고, 3개 단백질의 발현이 TSLP 처리 군에서 증가 되었으며, 25개 단백질의 발현이 TSLP 처리 군에서 감소되었다. 케모카인 수용체의 일종인 CXCR4가 24시간 TSLP 처리 iNKT 세포에서 가장 현저히 증가하고 문헌 고찰상 human iNKT 세포와의 연관성이 관찰되어 CXCR4와 이의 리간드인 SDF1α를 이용하여 추가적으로 기능 검증을 실시하였다. 아토피피부염 환자의 피부 병변에서 CXCR4 양성 iNKT 세포가 현저히 증가하였으며 아토피피부염 환자 혈액에 있는 iNKT 세포의 경우 CXCR4 발현이 정상인의 혈액에서보다 조금 감소하였다. 리간드인 SDF1α의 경우 아토피피부염 피부 병변에서 증가하였으며 dermal fibroblast 및 T 세포 (CD4 및 CD8)가 SDF1α를 발현하였고 iNKT 세포의 경우 SDF1α 발현 세포의 주위에서 관찰되었다. 또한 TSLP를 24시간 처리한 후 정상 dermal fibroblast 및 T 세포에서 SDF1α 발현이 유도되는 것을 관찰하였다. 아토피피부염 환자의 혈청에서 또한 SDF1α 발현이 증가되어 있었다. 따라서 아토피피부염의 혈액에 존재하는 iNKT 세포가 TSLP에 활성화되어 CXCR4의 발현을 유도하면 아토피피부염의 피부 병변에 존재하는 TSLP에 의해 활성화된 dermal fibroblast와 T 세포가 분비하는 SDF1α에 반응하여 CXCR4 양성 iNKT 세포가 아토피피부염 피부 병변으로 유주하게 되고 혈중에 증가되어 있는 SDF1α 또한 이 과정을 증폭시켜 아토피피부염의 피부 병변 유발 및 악화 기전에 관여하리라 생각된다.

핵심되는 말: 아토피피부염, thymic stromal lymphopoietin, invariant natural killer T cell, CXCR4, SDF1α