The Role of CCCTC-Binding Factor for Skin-Resident Memory T Cells in Atopic Dermatitis

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Directed by Professor Kwang Hoon Lee

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“Be joyful always,
pray continually,
give thanks in all circumstances,
for this is God's will for you in Christ Jesus.”

1 Thessalonians 5:16-18

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Seo Hyeong Kim

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ABSTRACT

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Atopic dermatitis (AD) is a highly pruritic, chronic relapsing inflammatory skin disease characterized by significant T-cell infiltration and frequently recurs in the same areas of the skin, and even resolved skin keeps the disease's memory. While the role of tissue-resident memory T cells (T_{RM} cells) has been well studied in the chronic inflammatory skin diseases such as psoriasis which relapses in the same sites, that of T_{RM} cells in AD has not been investigated in detail. CCCTC-binding factor (CTCF) is a ubiquitously expressed regulator of fundamental genetic processes including transcription, intra- and interchromosomal interactions, and chromatin structure. Because of its critical role in genome function, CTCF binding patterns have long been assumed to be largely invariant across different cellular environments. Therefore, the

functional analysis of CTCF in the different types of cells and tissues would further expand our knowledge in the roles of CTCF. In this dissertation, I focus on the role of CTCF in T_{RM} cells in AD.

To explore the function of T_{RM} cells in AD. I examined how many T_{RM} cells expressing the canonical maker CD69 and CD103 existed and which cytokines including IL-4, IL-13, IL-17, IL-22 and IFN-γ these cells produced in human AD and age-matched normal skin tissues and in AD-like mouse models generated with the treatment of allergens (1-Fluoro-2,4-dinitrobenzene (DNFB), oxazolone, ovalbumin) or mixture thereof. I also evaluated which AD triggering factors affected the development of T_{RM} cells and the production of cytokines using purified cutaneous lymphocyte-associated antigen (CLA) expressing T cells from normal peripheral blood mononuclear cells (PBMCs). Finally, using transcriptomics, I evaluated the cytokine signatures and novel genes associated with skin T_{RM} cells compared to migratory memory T cells (T_{MM}) in human normal and AD skin.

I observed that $CD69^+$ and $CD103^+$ T_{RM} cells significantly infiltrated into chronic AD skin compared to normal skin. However, normal skin mostly contained $CD103^-$ and $CD69^-$ T cells. Significant numbers of $CD69^+$ AD T_{RM} cells produced Th2 cytokines (IL-4, IL-13), Th17 cytokines (IL-17, IL-22), and Th1 cytokines (IFN-γ). In AD-like mouse model induced by allergen mixture, $CD69^+$ skin T_{RM} cells produced various cytokines (IL-4, IL-17, IL-22 and IFN-γ) compared to a single allergen induced AD-

like mouse models. During the incubation of CLA^+ T cells for 3 weeks, $CD69^+$ expression of $CD4^+$ and $CD8^+$ T cells was continuously increased in a time-dependent manner while $CD103^+$ expression was not significantly changed. Repeated exposure to thymic stromal lymphopoietin (TSLP), a well-known AD triggering factor, induced more $CD69^+$ T_{RM} cells during the development of AD T_{RM}. Various cytokines (IL4, IL-17, IL-22 and IFN-γ) were considerably produced in $CD69^+$ T_{RM} cells compared to $CD69^-$ T cells in AD mimicking mice after 3 weeks. I further confirmed that AD $CD69^+$ T_{RM} cells expressed higher levels of Th1, Th2, Th17 and Th22 cytokines and their transcription factors (T-bet, GATA3, RORγt and Ahr) showing unique transcriptional profiles distinct from those of AD $CD69^-$ T_{MM} cells or normal skin $CD69^+$ T_{RM} cells. I further identified various genes associated with tissue egress and residency specific to AD T_{RM} cells. Among those, I chose CCCTC-binding factor (CTCF) because of its role in immune diseases. I confirmed the decrease of CTCF in $CD69^+$ T_{RM} cells from AD patient skin. In conclusion, CTCF may play an important role in regulating the expression of multiple cytokines from $CD69^+$ T_{RM} cells in AD skin. Through this mechanism, repeated exposure to AD-triggering factors might induce AD T_{RM} cells to produce multiple cytokines and sustain the recurrence and chronicity of AD patients.

Key words : atopic dermatitis, tissue-resident memory T cells, cytokines, CCCTC-binding factor

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I. INTRODUCTION

1. Atopic dermatitis

Atopic dermatitis (AD) is a chronic, relapsing skin disease with pruritus and inflamed, eczematous skin lesions. It usually presents during early infancy and childhood, but it can persist into or start in adulthood.[1-4] Two types of AD have been identified. The nonallergic or “intrinsic” form, which affects around 20% of adult patients with AD, is accompanied by low IgE serum levels and the absence of any detectable allergen sensitization[5,6] The allergic form or “extrinsic” form, which affects about 70% of the adult patients with AD, occurs due to sensitization toward environmental allergens and increased levels of serum IgE. In extrinsic AD, memory T cells expressing a skin

homing receptor, cutaneous lymphocyte-associated antigen (CLA), produce increased levels of Th2 cytokines, IL-4 and IL-13, which are known to induce isotype switching to IgE synthesis.[7] These CLA^+ T cells also produce low levels of IFN-γ, which is known to inhibit Th2 cell function. Intrinsic AD is associated with less production of IL-4 and IL-13 than extrinsic AD. The histology of dermatitis is highly similar to that of allergic contact dermatitis.[7] "Acute" skin lesions are characterized by marked intercellular edema of the epidermis. In the dermis, there is marked T cell infiltration. The lymphocytic infiltration also consists predominantly of activated memory T cells bearing CD3, CD4, HLA-DR, CD25, and CD45RO. Basophils and neutrophils are rarely present, but eosinophils are seen in acute lesions, and mast cells are also present in various stages of degranulation. "Chronic" lichenified lesions are characterized by a hyperplastic epidermis with elongation of the rete ridges, prominent hyperkeratosis, and minimal edema. There is an increase in IgE-bearing dendrite cells (DC) in the epidermis, and though the number of mast cells is increased, they are generally fully granulated. The pathogenesis of AD appears to result from interactions among genetic, environmental, and immunological factors.[8-10] As for the pathogenesis of AD, whether skin barrier breakdown precedes allergic inflammation (the "outside-in" model) or inflammatory response caused by immune abnormality precedes barrier changes (the "inside-out" model) remains unresolved. Recently, AD pathogenesis has been attributed to the "outside-inside-outside" model, which considers the complex interplay of both

hypotheses.[11] Thus, AD appears to be induced by complex interactions between dysfunction of epidermal barrier and abnormal immune response. Damage to the skin barrier could lead to the enhanced penetration of antigens and reduce the moisture of the skin. On the other hand, the innate immune system quickly responds, causing adaptive immune responses to take place in the skin. Therefore, both skin barrier impairment and immune abnormality contribute to the pathogenesis of AD.

Pathogenesis associated with skin barrier dysfunction includes filaggrin gene mutations, ceramide reduction of the stratum corneum, antimicrobial peptides, and serine proteases inhibitors.[12-14] Among patients with AD, mutations in the filaggrin gene result in reduced production of filaggrin. These patients tend to have more severe symptoms and allergic reactions. The abnormal immune responses in AD include elevated serum IgE and allergen sensitization, elevated Th2 cytokines in acute lesions, increased T cells expressing CLA, increased Langerhans cells and inflammatory dendritic cells, and up-regulated FcεRI expression in inflammatory dendritic epidermal cells.[15] In order to prevent the penetration by external antigens and bacteria, the skin barrier can be damaged by keratinocytes and antigen-presenting cells, and the innate immune response can occur. In the case of AD, the responses of innate immune system and acquired immune system are abnormal, and allergic inflammatory reactions occur. Increased serum IgE levels are related to the worsening of AD and also detected in asthma and allergic rhinitis, responsible to stimulate basophils after antigen exposure.[16]

When toll-like receptors (TLRs) are stimulated by external bacteria or damage to the skin barrier, antimicrobial peptides, cytokines, and chemokines are secreted and induced to tighten tight junctions in order to prevent further infiltration of external bacteria. In AD patients the function of these TLRs is decreased, which is a typical congenital immune system abnormality found in AD patients such as a missense mutation of the TLR2 gene.[17]

Adverse reactions in the acquired immune system occur when foreign antigens penetrate the damaged skin barrier and the dendritic cells (DCs) subsequently recognize those antigens and activate Th2 cells in response. Activated Th2 cells secrete cytokines such as IL-4, -5, -10, -13, -17, and -31. IL-4 and -13 can activate and make B cells secrete IgE. Eosinophils are induced by IL-5. IgE binds to IgE receptors on mast cells and basophils and they produce various inflammatory mediators such as histamine, neutral protease, and prostaglandin D2.[18,19] Th17 cells, secreting IL-17 and IL-22, are increased in the blood and skin of patients with acute AD. In chronic AD, IL-17 secretion is reduced by the cytokines secreted from Th2 cells.[20] Th22 cells, secreting IL-22 that causes epidermal proliferation mainly in the AD of the chronic phase, are also associated with disease activity.[21] Therefore, in acute AD, external antigens easily penetrate the damaged skin barrier and sensitize Langerhans cells, and thus the inflammatory reaction of Th2 and Th17 predominate. In contrast, in chronic AD, Th1, Th2, and Th22 are predominantly induced by the repeated encounters of infections,

irritants, and allergens resulting in epidermal proliferation and keratinocyte damage due to the relevant cytokine.

Because the mechanism of AD is not yet fully understood, it is impossible yet to eradicate AD entirely.[22] Therefore, the most widely used treatments today for chronic AD are systemic immune suppressing therapies such as the application of steroids, cyclosporine A, azathioprine, and mycophenolate mofetil.[23,24] However, the effects of these treatments are transient, and sometimes completely ineffective clinically. Although it is widely known that the presence of T cells in the skin is the most important factor in the pathogenesis of AD, most studies so far have focused on blood circulating T cells which are easy to access. In other words, there are only a few AD studies on T cells residing in the skin, and it is therefore essential to investigate skin-resident T cells. Given that there are twice as many T cells in the skin tissue than in the bloodstream, it seems especially obvious that investigating skin-resident T cells is more important than investigating systemic T cells.[25] For this reason, it is necessary to develop a novel method of specifically targeting skin-resident T cells which play an important role in the process of chronic recurrent AD.

2. Tissue-resident memory T cells

Memory T cells give the host effective immunity against previously met pathogens. Originally, memory T cells had been thought to be composed of two major subsets:

central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} cells express the chemokine receptor CCR7 and the vascular addressin L selectin (CD62L), which can enter the lymph nodes from blood. T_{EM} cells express low levels of CCR7 and CD62L but can access peripheral tissues.[26] However, it has recently become clear that there is another subset of memory T cells, called tissue-resident memory T (T_{RM}) cells. The best characterized are T_{RM} cells that bear the α-chain (CD103) of the integrin $\alpha_E\beta_7$ together with the activation marker CD69.[27] CD69 is a marker of early T cell activation in lymph node, but most T_{RM} cells in peripheral tissues have not been activated by antigen. It is thought that CD69 maintains T_{RM} cells in peripheral tissues.[28,29] The kruppel-like factor 2 (klf2) normally enhances sphingosine 1 phosphate receptor 1 (S1PR1), and both of them are down-regulated in T_{RM} cells.[30] The chemokine receptor CCR7 is a G protein-coupled receptor, and the expression of $CCR7^+$ is a population of T cells that migrate out of skin, called migrated memory T cells (T_{MM} cells).[31] As a marker of T_{RM} cells, the expression of CD103 is more predominant on $CD8^+$ T_{RM} cells than on $CD4^+$ T_{RM} cells.[32]

As a subset of memory T cells, T_{RM} cells persist long-term in peripheral tissues and populate epithelial barrier tissues such as gastrointestinal tract, gut, reproductive tract, and skin.[33] T_{RM} cells can be maintained in regional peripheral tissues because they lodge during the early phase of peripheral infection and then never return to circulation.[34,35] T_{RM} cells are also characterized by an individual differentiation program that can respond rapidly to pathogen challenges at their resided peripheral tissues for a long time,

thus mediating the rapid protective immunity distinguishing them from circulating T cells in the blood.[36] It is known that T_{RM} cells reside in tissues during the effector phase of the immune response, while memory precursor cells are present in the circulation. Such precursor cells can be defined by the differences in their expression of the KLRG1 receptor and the IL-7 receptor α–chain (CD127).[37] However, unlike circulation effector cells, $CD103^+CD8^+$ T_{RM} cells are particularly long lived, which means that they are more likely to originate from $KLRG1^-$ precursor cells that give rise to persisting memory cells, e.g., the T_{CM} cells found in the circulation.[38]

In generation of T_{RM} cells, upon first encountering a pathogen in a barrier tissue, DCs send antigen to tissue-draining lymph nodes and present it to naive T cells. Depending on the lymph nodes of each tissue, various trafficking molecules, including CLA or CCR4 in skin, are expressed on the expanding activated T cell population, and effector T cells with specific tissue-homing properties exit blood in peripheral tissues. Tissue-draining lymph nodes induce the expression of tissue-homing molecules on antigen-activated T cells.[39-41] After effector T cells exit a tissue-draining lymph node and enter the blood, those with tissue-homing markers are trapped by inflamed vessels in peripheral tissues.

T_{RM} cells are also currently becoming a focus of investigation due to the potential role they play in incurable inflammatory lung, gut, and skin diseases.[42-44] T_{RM} cells in lung were first considered with the identification of $CD8^+CD69^+$ T_{RM} cells that remained

after influenza infection.[45] Intranasal infections with influenza in mice results in the generation and maintenance of lung T_{RM} cells. Therefore there is evidence that $CD8^+$ T_{RM} cells can be protective against influenza infection.[46] T_{RM} cells in gut reside in the intestinal epithelium or the lamina propria.[47] Several pathogens, such as lymphocytic choriomeningitis virus (LCMV) and others, have been shown to induce the generation of long-lived T cells in mice.[48] One study also showed that long lived gut T_{RM} cells expressed the low level of KLRG-1, but the cell with high level of KLRG-1 entered gut.[49] Furthermore, immunization by skin leads to the generation of lung T_{RM} cells which can protect the immunized mice from the challenge with vaccinia virus (VACV).[50] In addition, in a recent study, T_{RM} cells were extracted from skin lesion of psoriasis patients. In epidermis, $CD8^+$ T cells that generate IL-17 and $CD4^+$ T cells that produce IL-22 were retained and supported by skin-T_{RM} cells in psoriasis.[51,52] As a result, skin-T_{RM} cells are considered to be key players in pathogenesis of chronic relapsing inflammatory skin diseases such as psoriasis and AD. Although, T_{RM} cells are supposed to be a major cause to the generation and degeneration of chronic relapsing skin disease, there is a dearth of research about skin-T_{RM} cells that reside in AD lesions.

3. CCCTC-binding factor

The timely realization of intricate nuclear functions (such as transcription, replication, DNA repair and mitosis) is facilitated by the three-dimensional interplay of protein-

DNA complexes.[53] A combination of microscopy and chromosome conformation capture (3C)-related approaches has revealed that CCCTC-binding factor (CTCF) is responsible for bridging the gap between nuclear organization and gene expression.[54,55] The 11-zinc finger protein CTCF is a ubiquitously expressed and highly conserved transcriptional regulator implicated in many key processes within the nucleus, including promoter activation and repression, hormone-responsive gene silencing, and genomic imprinting.[56] Of the CTCF-binding sites, ~5,000 are ultraconserved between mammalian species and tissues and correspond to high-affinity sites, whereas 30-60% show cell-type-specific distribution.[57-60] CTCF often binds to the vicinity of insulators, elements that affect gene expression by preventing the spread of heterochromatin, by acting as a chromatin barrier and inhibiting inappropriate interactions between regulatory elements on adjacent chromatin domains and also acting as an enhancer blocker.[61] Genome-wide mapping of CTCF binding sites (CBS) in the human genome identified ~14,000 sites. Domains with few or no CBS tend to include clusters of transcriptionally regulated genes, and those regions are often flanked by CBS.[62,63]

Recently, CTCF was found to control MHC class II gene expression and long-range chromatin interactions between MHC class II promoter regions. These data suggest an important role for CTCF in immune system, particularly in the regulation of gene transcription in complex loci.[64] CTCF also plays multiple roles in different cell types. CTCF regulates early thymocyte development[65] and cytokine production in helper T

cells.[66,67] Very recently, CTCF was also found to control the homeostatic maintenance and migration of Langerhans cells. This study, in particular, discovered that CTCF-deficient LCs expressed higher levels of cell adhesion molecules including CD69.[68] In B lymphocytes, CTCF-dependent chromatin organization modulates immunoglobulin gene rearrangement.[69-71] It has also been shown that overexpression of CTCF alters DC survival, proliferation, and differentiation.[72] However, the possible role of CTCF in T_{RM} cells as well as in AD is largely unknown. To that end, I performed the study to examine the role of skin-T_{RM} cells in chronic relapsing AD lesions as well as the role of CTCF in skin-T_{RM} cells in AD.

II. MATERIALS AND METHODS

1. Tissue and blood sample collection

Normal human skin samples were obtained as surgical discard and dermatology from 11 healthy volunteers (age range 39-56). Atopic dermatitis (AD) skin tissues from 5 AD patients (age range 24-48) were obtained from the Department of Dermatology at Severance Hospital (Seoul, Korea). Peripheral blood mononuclear cells (PBMCs) were obtained from 3 healthy donors (age range 26-46). All donors were female to avoid gender-based disparities. The protocols of this study were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the Yonsei University College of Medicine (Seoul, Korea). All the patients and volunteers provided written informed consent.

2. Preparation of cell suspensions

A. CLA^+ T cells

Cutaneous lymphocyte-associated antigen $(CLA)^+$ T cells were isolated by fluorescence activated cell sorting (FACS) from the human PBMCs with a BD FACSAria II (BD Bioscience). CLA^+ T cells were cultured using a complete RPMI1640 medium (LONZA, Walkersville, MD, USA) with 5% (v/v) FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco). Cells were activated

in the plates coated with 2 μg/ml of anti-CD3 and anti- CD28 (eBioscience, San Diego, CA, USA) in the presence of 25 ng/ml recombinant IL-2 and IL-15 (eBioscience) to induce proliferation of skin-resident T cells. To construct an AD-mimicking *in vitro* model, cells were treated with AD triggering factors, including recombinant thymic stromal lymphopoietin (TSLP) (50 ng/ml, R&D systems, Minneapolis, MN, USA), IL-4 (10 ng/ml, Peprotech, Rocky Hill, NJ, USA) and IFN-γ (50 ng/ml, R&D systems) for 48 hours at the 1^{st}, 2^{nd}, and 3^{rd} week of the culture, respectively.

B. Skin

Fresh human and mouse skin samples were dissociated into small pieces. Chopped skin samples were then incubated in 1 mg/ml collagenase A (Roche, Mannheim, Germany) with 40 μg/ml DNase (Roche) and 5mM Ca^{++} (Sigma, St. Louis, MO, USA) in an HBSS medium containing 5% (v/v) FBS 1%, 1M HEPES, 1% L-glutamine, 1% penicillin/streptomycin, and 1000X beta-mercaptoethanol (Gibco) in a shaking incubator for 1 hour at 37°C. After incubation, cell suspensions were tiltered with 70 μm cell strainer to collect single cells.

3. Mouse sensitization

Specific-pathogen-free 6-week-old female BALB/c mice were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea). Mice were reared in a

temperature-controlled room at 24 ± 2°C and 55 ± 15% humidity with a 12 hour light and dark cycle. Before experimentation began, mice underwent a one-week purification period. Ovalbumin (OVA) sensitization of the mice was performed as described by Spergel et al.[73] Briefly, mice were anesthetized with isoflurane. The hairs on the upper back were removed with an electric shaver and depilatory cream. After hair removal, 100 μg of OVA (grade V, Sigma) in 100 μl of PBS or control (100 μl of PBS) was placed on 2 X 2 cm sterile gauze, which was secured with a transparent dressing film (Tegaderm, St. Paul, MN, USA). The experiment comprised five OVA or PBS exposures per week with a 2-week interval between each exposure week during 7 weeks (Fig. 1).

Either 2,4-dinitrofluorobenzene (DNFB, Sigma) or 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, Sigma) was used to induce contact hypersensitivity. 25 μl of 0.5% (wt/vol) DNFB or 3% (wt/vol) oxazolone in acetone/olive oil (4:1) were applied to the mice's shaved abdomens for sensitization. After 5 days, challenges with 10 μl of 0.2% DNFB or 0.4% oxazolone on the right ear were performed three times per week for 2 weeks (Fig. 1). Application of all three antigens (OVA, DNFB and oxazolone) was used together to induce AD as described above. All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Yonsei University (Seoul, Korea).

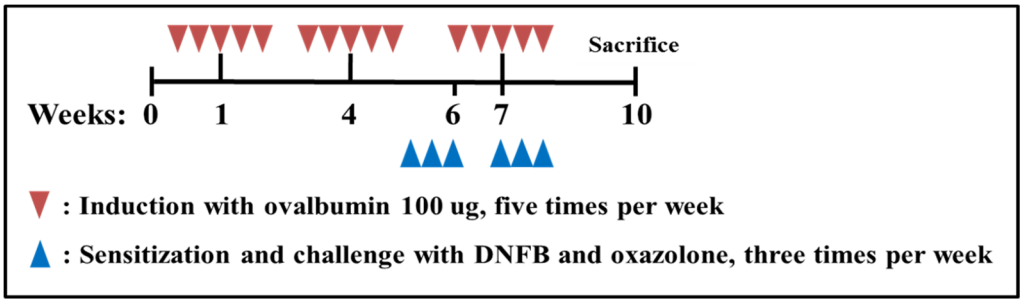


Figure 1. Application schedule of respective antigens for AD-like mouse models. OVA sensitization is applied to the shaved skin of the back on an occlusive patch (2 X 2 cm) with 100 μg of OVA or saline during three 1-week exposures to the patch separated from each other by 2-weeks intervals (red triangles). DNFB and oxazolone are applied as a challenge to the ears of mice three times per week for 2 weeks (blue triangles).

4. Immunofluorescence staining

Paraffin-embedded human skin tissues were deparaffinized and rehydrated in xylene together 100%, 95% and 85% ethanol plus distilled water (DW) for 5 minutes. Antigen retrieval was carried out in 10 mM sodium citrate buffer (pH 6.0) (Sigma) using a 95°C water bath for 15 minutes followed by for 30 minutes of tissue cooling at room temperature. Tissues were incubated in a humidified chamber for 1 hour at room temperature with a blocking solution containing 5% bovine serum albumin (Thermo Scientific, Rockford, IL, USA). They were then stained with mouse anti-human CD4 (BC/1F6, abcam) or rabbit anti-human CD8 (EP1150Y, abcam) antibodies overnight at 4℃. After washing with PBST, sections were stained with Alexa-633-conjugated goat anti-mouse IgG (ThermoFisher Scientific) or Alexa-633-conjugated goat anti-rabbit IgG (ThermoFisher Scientific) for 1 hour at room temperature. Sections were then washed with PBST and stained with secondary fluorescence-conjugated primary antibodies—FITC-conjugated anti-human CD69 (FN50, Biolegend) or FITC-conjugated anti-human CD103 (Ber-ACT8, Biolegend)—for 1 hour 30 minutes at room temperature. After washing with PBST, slides were also stained with a third primary antibodies—FITC-conjugated anti-human IL-4, IL-13, IL-17, IL-22, and IFN-γ from Biorbyt (Cambridge, United Kingdom), or rabbit anti-human CTCF (D31H2, Cell signaling, Danvers, MA, USA)—for 2 hours at room temperature. Slides were also washed with PBST and stained with a third secondary antibodies—Alexa-555-

conjugated goat anti-rabbit IgG (ThermoFisher Scientific) for CTCF primary antibody—for 1 hour at room temperature. Slides were mounted in a VECTASHIELD mounting media containing 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Sections were visualized using confocal microscopy (LSM 780, Zeiss, Oberkochen, Germany).

5. Flow cytometry and antibodies

Human CLA^+ T cells and mouse skin single cell suspensions were incubated with Cell Stimulation Cocktail (500X) (eBioscience) for 6 hours at 37°C in order to observe intracellular cytokines. After incubation, cells were stained with cell surface markers for 30 minutes at 4°C. For intracellular staining, cells were incubated with a fixation buffer (Fixation/Permeabilization Solution Kit, BD Bioscience) for 30 minutes at 4°C and labeled with cytokine antibodies. Flow cytometry analysis was performed using BD FACSVerse (BD Bioscience) and BD LSRFortessa (BD Bioscience) flow cytometers, and then the data were analyzed using FlowJo (version 7.6.2, TreeStar, San Carlos, CA, USA). The following anti-human antibodies were obtained from eBioscience, Percp-Cy5.5-conjugated anti-CD8 (RPA-T8), PE-conjugated anti-IFN-γ (4S.B3), PE-conjugated anti-IL-4 (8D4-8), and APC-conjugated anti-IL-17 (eBio64DEC17). The following anti-mouse antibodies were also obtained from Biolegend, APC-Cy7-conjugated anti-CD4 (T4/Leu-3), FITC-conjugated anti-CD69

(FN50), PE-Cy7-conjugated anti-CD103 (Ber-ACT8), and APC-conjugated anti-IL-22 (2G12A41). Additional anti-human antibodies were obtained from eBioscience—eF450-conjugated anti-CD3 (17A2), FITC-conjugated anti-IFN-gamma (XMG1.2), and PE-conjugated anti-IL-22 (1H8PWSR)—and Biolegend—PE-Cy7-conjugated anti-CD4 (GK1.5), Percp-Cy5.5-conjugated anti-CD8 (53-6.7), APC-conjugated anti-CD69 (H1.2F3), PE-conjugated anti-IL-4 (11B11), and FITC-conjugated anti-IL-17 (TC11-18H10-1).

6. Human skin culture for analyzing T cell migration

Subcutaneous fat was removed from the skin. Each biopsy was placed and floated dermal-side down for 4 days in one well of a 6-well plate containing an RPMI1640 medium (LONZA) of 5% (v/v) and 1% penicillin/streptomycin and 2-mercaptoethanol (Gibco). Half of the medium was replaced every 2 days. Migrated cells from the skin were then defined as T_{MM} cells, and resident cells in skin were defined as T_{RM} cells.

7. Fluorescence activated cell sorting

For the isolation of T cells, the pooled cell suspensions were subjected to positive magnetic selection using human CD3 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell suspensions were stained with fluorescence-conjugated antibodies for 30 min at 4°C. Stained cells were sorted in an RLT lysis buffer (Qiagen,

Valencia, Calif) directly for RNA extraction using BD FACSAria II (BD Bioscience). Anti-human antibodies were obtained from Biolegend (Percp-Cy5.5-conjugated anti-CD8 [SK1]) and eBioscience (eF450-conjugated anti-human CD3 [OKT3], PE-conjugated anti-CD4 [OKT4], and APC-conjugated anti-CD69 [FN50]). Gating strategy: T_{MM} cells: $CD3^+CD4^+CD69^-$ or $CD3^+CD8^+CD69^-$, T_{RM} cells: $CD3^+CD4^+CD69^+$ or $CD3^+CD8^+CD69^+$.

8. RNA extraction and RNA quality check

Total RNA was extracted using the RNeasy Micro Kit (Qiagen), according to the manufacturer's protocol, with on-column DNase digestion, and then stored immediately at -80°C. RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA).

9. Affymetrix whole transcript expression arrays

The Affymetrix whole transcript expression array process was executed according to the manufacturer's protocol (GeneChip WT Pico Reagent Kit). cDNA was synthesized using the GeneChip WT Pico Amplification Kit according to the manufacturer's instructions. The sense cDNA was then fragmented and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip WT Terminal Labeling Kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix

GeneChip Human 2.0 ST Array at 45°C for 16 hours. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Affymetrix). Signal values were computed using the Affymetrix® GeneChip™ Command Console software.

10. Raw data preparation and statistical analysis

Raw data were extracted automatically through the Affymetrix data extraction protocol using the Affymetrix GeneChip® Command Console® software (AGCC). After importing CEL files, the data were summarized and normalized using the robust multi-average (RMA) method implemented through the Affymetrix® Expression Console™ software (EC). I exported the results with gene-level RMA analysis and then performed differentially expressed gene (DEG) analysis. The comparative analysis between test sample and control sample was carried out using fold change. For the DEG set, hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-Enrichment and Functional Annotation analysis for significant probe lists was performed using gene ontology (http://geneontology.org/) and KEGG (http://kegg.jp). All statistical tests and visualizations of differentially expressed genes were conducted using the R statistical language v. 3.1.2. (www.r-project.org).

11. Immunohistochemical staining

Immunohistochemistry staining was performed on paraffin-embedded human normal and AD skin tissues. Dissected tissues were deparaffinized in xylenes and rehydrated with a graded series of ethanol for 5 minutes. Antigen retrieval was carried out in a 10 mM sodium citrate buffer (pH 6.0) (Sigma) in a 95°C water bath for 15 minutes, after which tissues were cooled for 30 minutes at room temperature and washed with distilled water. CTCF antibody was stained using immunohistochemistry staining kit (EnVision™ G｜2 Double Staining Kit) in accordance with the manufacturer's protocol. Briefly, endogenous peroxidase activity was quenched using Dual Endogenous Enzyme-Blocking Reagent for 15 minutes at room temperature. Tissues were incubated with 5% bovine serum albumin (Thermo Scientific) to reduce nonspecific antibody binding. The primary antibody, CTCF (D31H2, Cell Signaling) was incubated overnight at 4℃. Slides were stained with a horseradish peroxidase (HRP)-labeled polymer solution for 30 minutes at room temperature. Slides were then incubated with 3,3′ -diaminobenzidine (DAB^+) substrate-chromogen for 3 to 10 minutes. Tissues were washed twice in distilled water before nuclear staining with hematoxylin (Vector, Burlingame, CA, USA) for 3 minutes. Slides were finally dehydrated in ethanol and immersed in xylene for 5 minutes and then mounted in a permanent mounting medium (Vector). Expression level of CTCF was quantitatively analyzed using Meta-Morph image analysis software (Universal Imaging Corp, Sunnyvale, CA, USA).

12. Real-time RT-PCR

Reserve transcription reactions were performed. cDNA was synthesized from total RNA using the AccuPower RT PreMix kit (BIONEER, Daejeon, Korea). Quantitative real-time PCR was performed at least three times for each sample using 4 μl of cDNA, a TaqMan master mix (Applied Biosystems, St. Austin, TX, USA) in a 20 μl reaction, and the StepOne Plus Real-Time PCR System (Applied Biosystems). mRNA expression of CTCF (Hs00902016_m1) was normalized to glyceraldehyde-3-phosphate dehydrogenase (Hs02758991_g1). Relative quantification was performed using the Applied Biosystems 7500 software (version 2.0.1).

13. Plasmid and stable transfection

Mission pLKO.1-puro plasmid was used to transfect the human $CD4^+$ T cells. Cells transfected with the pLKO.1-puro plasmid containing human CTCF shRNA (TRCN0000014550) and the non-mammalian shRNA control (SHC002), and these plasmids were purchased from Sigma. The cells were collected at 72 hours and concentrated using the Lipofectamine 3000 Transfection Kit (Invitrogen, CA, USA) according to the protocol. After 72 hours, puromycin (Sigma) was added to select transduced populations for 48 hours.

14. Statistical analyses

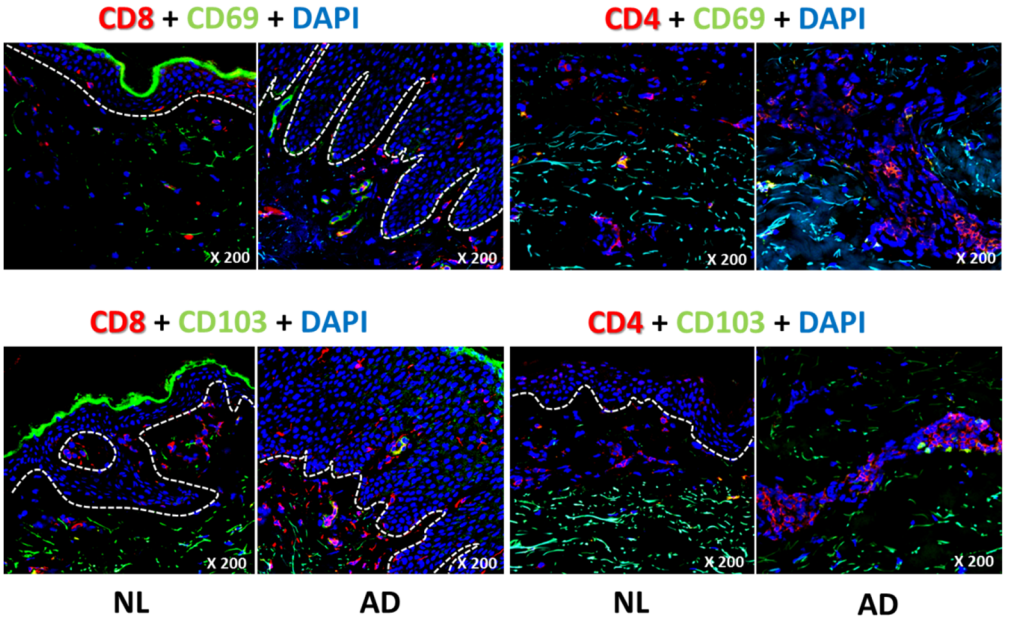
All assays were repeated at least three times, and data are displayed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 5 (version 4.03, GraphPad Software, Inc., San Diego, CA, USA). Differences between the two groups were determined using a two-tailed Student's t test. If comparisons were between more than two groups, a one-way ANOVA and a Tukey-Kramer test were used. Statistical significance was assigned to p-values < 0.05. Significance is indicated in the figures as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

III. RESULTS

1. The evidence of skin T_{RM} cells in AD patients

I analyzed the expression profiles of $CD4^+$ and $CD8^+$ skin-resident memory T cells (T_{RM} cells) using well-known T_{RM} cell markers (CD69 and CD103) associated with atopic dermatitis (AD) using immunofluorescence and compared them with normal skin. In AD, $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells infiltrated significantly more into AD skin compared to normal skin. $CD4^+CD103^+$ or $CD8^+CD103^+$ T_{RM} cells were also more prevalent in AD skin than in normal skin (Fig. 2A). The increase in $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cell expression and in $CD4^+CD103^+$ or $CD8^+CD103^+$ T_{RM} cell expression was quantitatively measured and was shown to be significantly different in AD skin. However, normal skin also has a number of $CD4^+CD69^-$ or $CD8^+CD69^-$ and $CD4^+CD103^-$ or $CD8^+CD103^-$ T cells (Fig. 2B).

(A)



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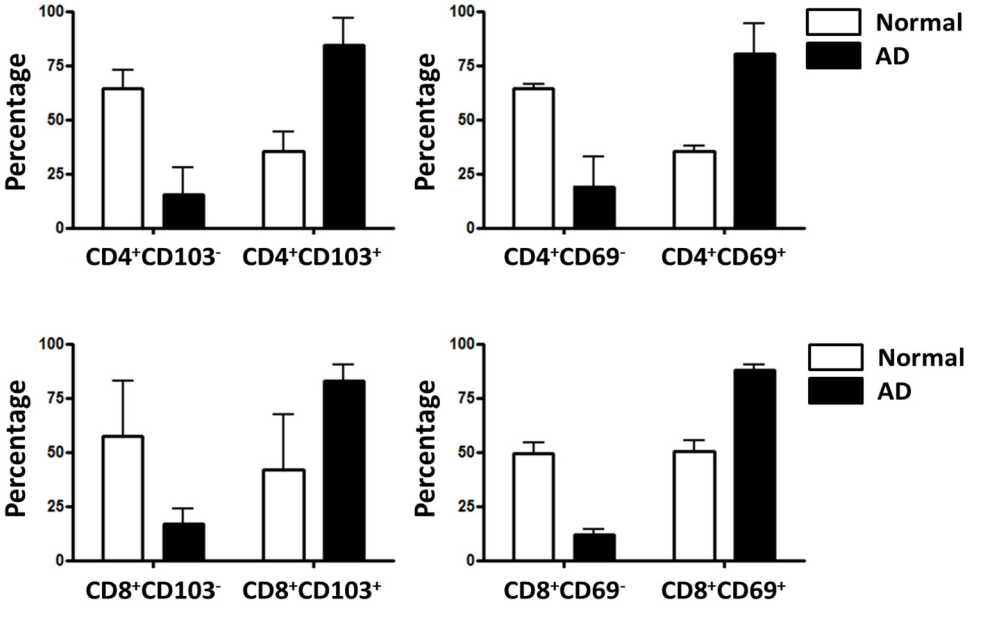
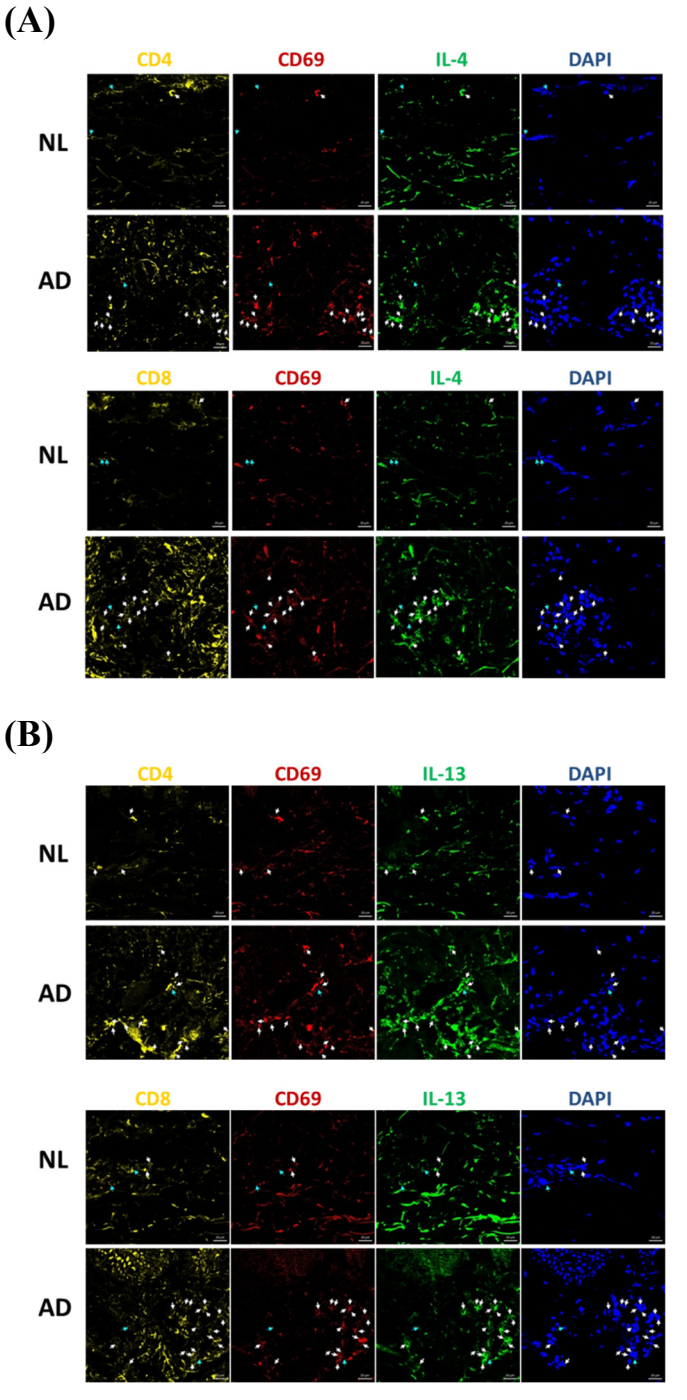


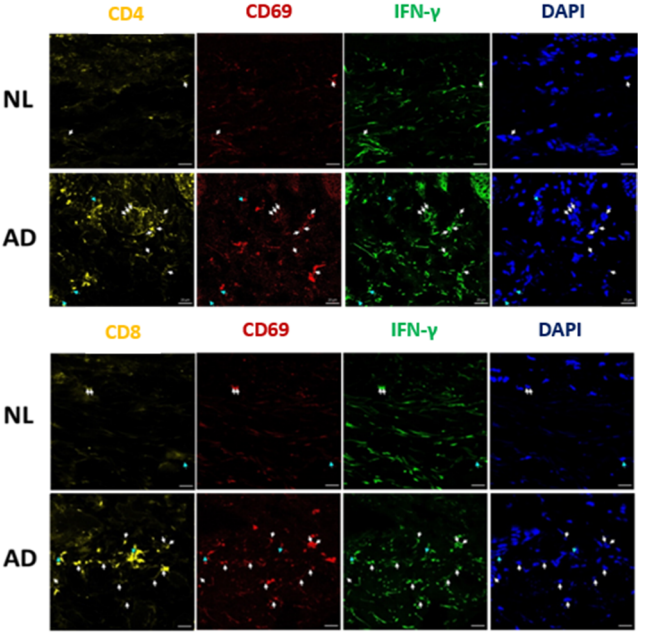
Figure 2. Increased $CD4^+$ and $CD8^+$ skin T_{RM} cells in AD patients. Expression of $CD4^+$ and $CD8^+$ T_{RM} cells are examined in AD and normal skin tissues. (A) $CD4^+$ or $CD8^+$ $CD69^+$ and $CD103^+$ T_{RM} cells are significantly increased in AD skin compared to normal skin. Original magnification x 200. (B) Percentage of $CD69^+$ or $CD103^+$ T_{RM} cells is measured by microscope and is shown to be up-regulated in AD skin in contrast with normal skin. However, the percentage of $CD69^-$ and $CD103^-$ cells is increased in normal skin tissues. Data are representative of three normal tissues and three AD tissues. Data are displayed as mean ± standard deviation SD. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

2. Expression of cytokines on $CD69^+$ T_{RM} cells in human skin

I examined which cytokines were expressed in T_{RM} cells in human AD skin tissues compared to normal skin using immunofluorescence. Typical Th2 cytokines (IL-4 and IL-13), Th1 cytokines (IFN-γ), Th17 cytokines (IL-17) and Th22 cytokines (IL-22) were stained with $CD4^+$ or $CD8^+$ T cells and $CD69^+$ T_{RM} cells. Triple-localization of cytokines (green), $CD4^+$ or $CD8^+$ (yellow) and $CD69^+$ T_{RM} cells (red) is noted by a white arrow, and cytokines (green), $CD4^+$ or $CD8^+$ (yellow), and $CD69^-$ cells are indicated with a blue arrow. Nuclei were stained with DAPI (blue). All cytokines were more strongly expressed in AD tissues compared to normal skin, and the expression of various cytokines was significantly up-regulated in $CD69^+$ T_{RM} cells compared to $CD69^-$ T cells in AD skin (Fig. 3A-E). Next, to evaluate cytokines expression in $CD69^+$ T_{RM} cells and $CD69^-$ non-T_{RM} cells in normal and AD skin, I quantified cytokines stained with $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells and $CD4^+CD69^-$ or $CD8^+CD69^-$ T cells using a microscope. According to those graphs, $CD4^+CD69^+$ AD T_{RM} cells produced significant amounts of IL-4, IL-13, IL-17, and IL-22 and lower levels of Th1 cytokines (IFN-γ), while $CD4^+CD69^-$ T cells only produced lower levels of IL-22 and IFN-γ. $CD8^+CD69^+$ AD T_{RM} cells also produced IL-4, IL-13, IL-17 and IL-22 (Fig. 3F).



(C)



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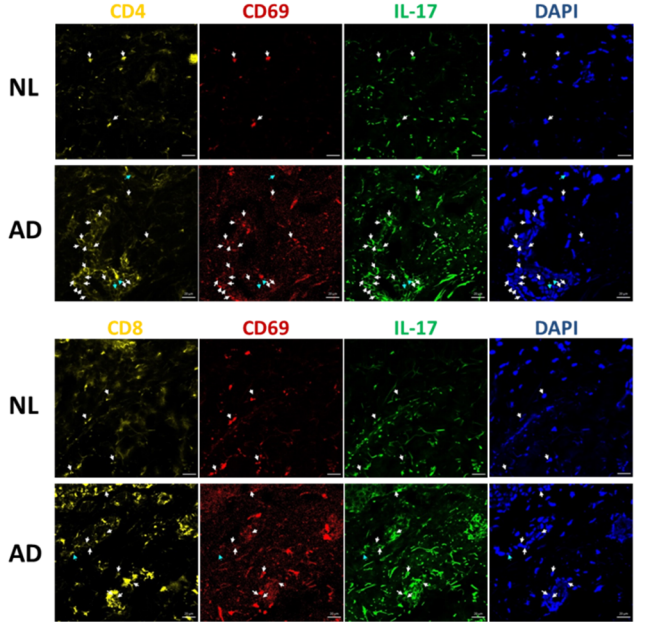

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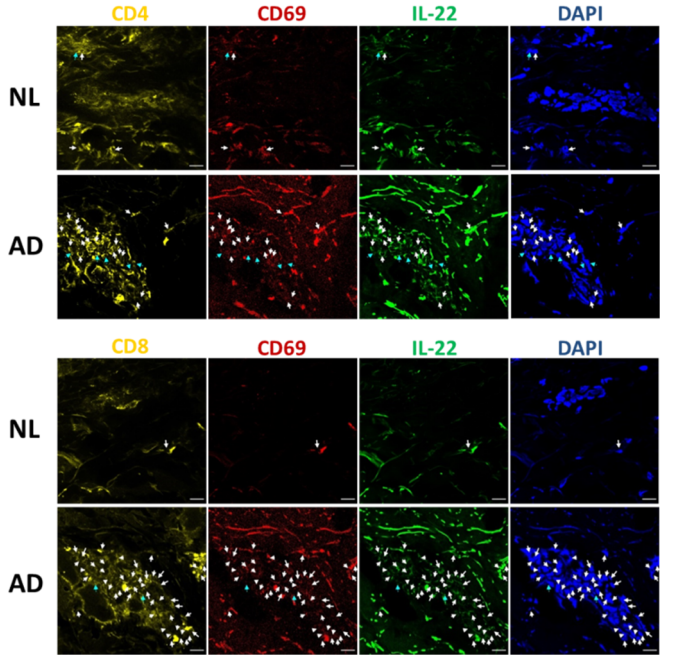

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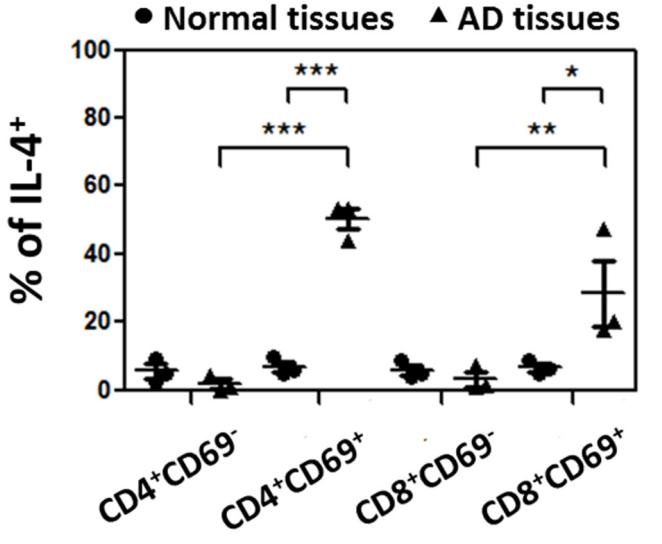
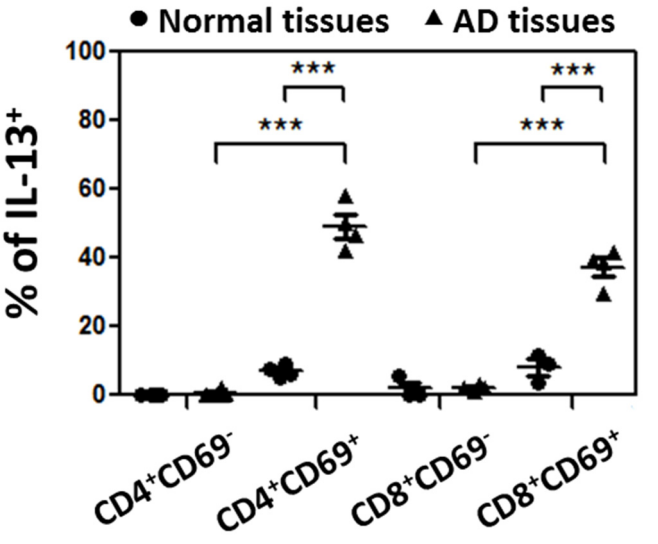
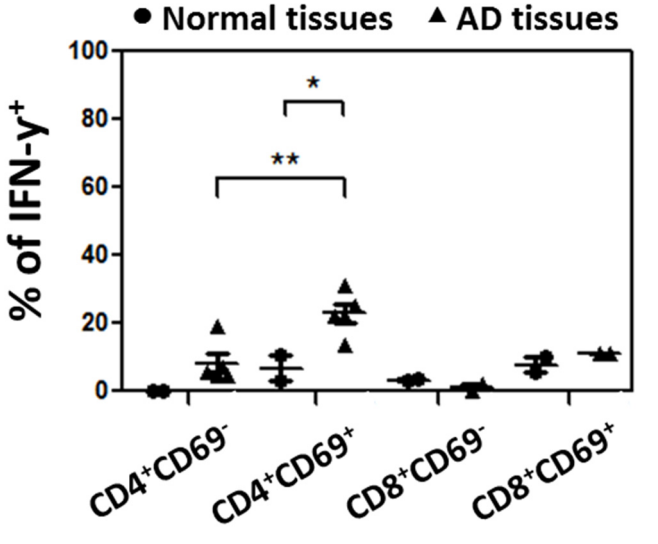
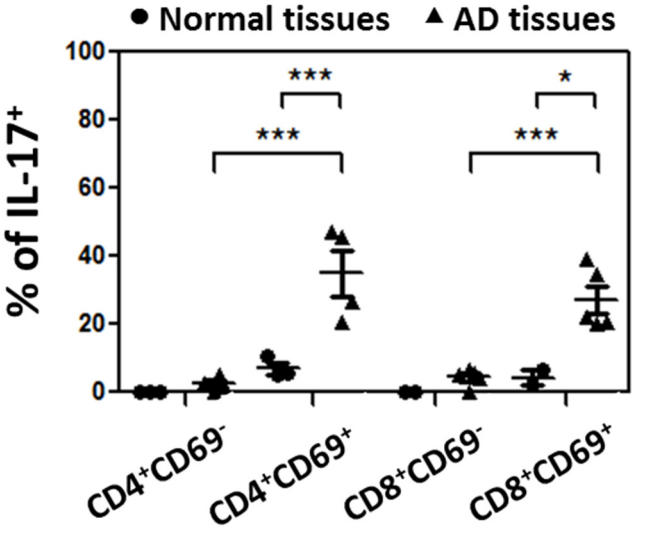








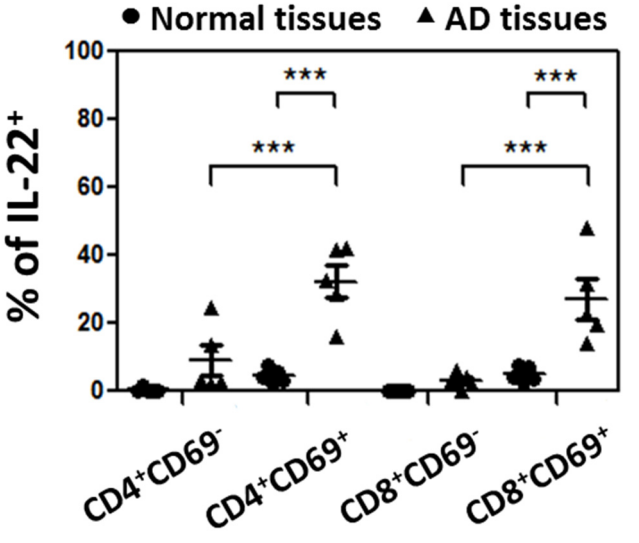
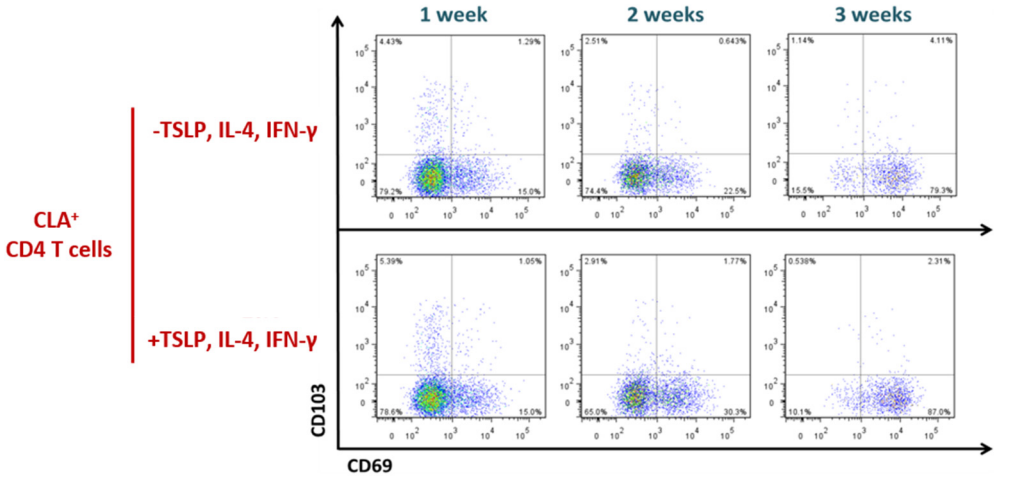


Figure 3. AD skin T_{RM} cells demonstrate increased expression of cytokines. Immunofluorescence confocal images are obtained for CD4 or CD8 (yellow), CD69 (red), and cytokines such as (A) IL-4, (B) IL-13, (C) IFN-γ, (D) IL-17, and (E) IL-22 (green) in the skin of normal and AD patients. Scale bar, 20 μm. Original magnification x 400. The image is representative of at least three skin lesions. (F) The percentage of each cytokine is measured on $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells and $CD69^-$ T cells in normal and AD skin using a microscope. Data are representative of at least four normal tissues and four AD tissues. Data are displayed as mean ± standard deviation SD. ***P < 0.001, **P < 0.01, *P < 0.05.

3. Identification of $CD69^+$ T_{RM} cells in AD circumstances

To study the development of T_{RM} cells *in vitro*, I obtained peripheral blood mononuclear cells (PBMCs) from human normal blood. Using purified CLA^+ T cells from normal PBMC, I determined that the AD triggering factors TSLP, IL-4 and IFN-γ affect the development of T_{RM} cells. Flow cytometry showed that $CD4^+CD69^+$ T_{RM} cells and $CD8^+CD69^+$ T_{RM} cells increased considerably in a time dependent manner at each week (Fig. 4A and 4B). After treated AD triggering components, $CD69^+$ T_{RM} cells were more up-regulated than non-treated group at 3 weeks.

(A)



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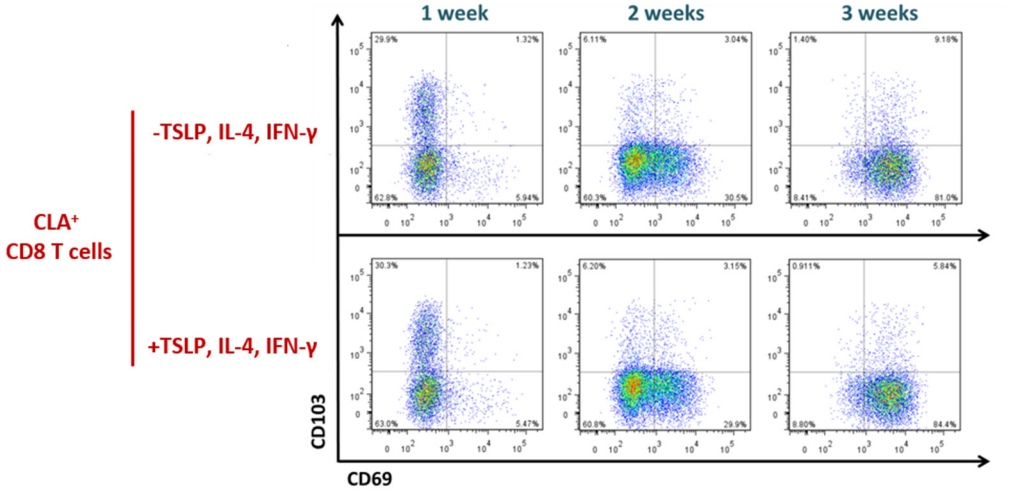
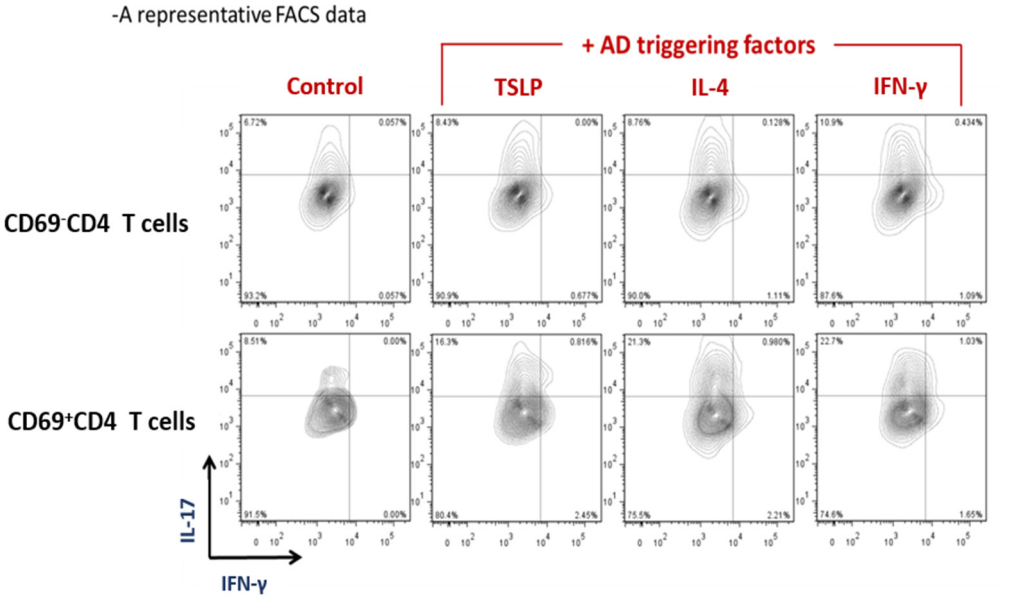


Figure 4. Up-regulated CD69$^+$ T_{RM} cells in AD triggering condition. I sort cutaneous lymphocyte-associated antigen (CLA)$^+$ T cells, skin homing memory T cells, from human normal PBMCs using flow cytometric sorting. CLA$^+$ T cells are incubated in the complete RPMI1640 media including recombinant IL-2 (25 ng/ml), IL-15 (25 ng/ml) in the plates coated with anti-CD3/CD28 antibodies plus with or without AD triggering factors, i.e., TSLP (50 ng/ml), IL-4 (25 ng/ml), and IFN-γ (25 ng/ml) for 48 hours at the 1st, 2nd, and 3rd week, respectively. During the incubation of CLA$^+$ T cells over 3 weeks, (A) CD69$^+$ in CD4$^+$ T cells continuously increase in a time-dependent manner, while CD103$^+$ expression do not significantly changed. (B) CD69$^+$ in CD8$^+$ T cells also continuously increase in a time-dependent manner, while CD103$^+$ expression do not significantly changed.

4. Production of cytokines on CD69$^+$ T_{RM} cells in CLA$^+$ T cells

To confirm the expression levels of cytokines in T_{RM} and non-T_{RM} cells, I examined over 3 weeks whether the AD triggering elements TSLP, IL-4 and IFN-γ induce production of cytokines in CD69$^+$ T_{RM} cells using purified CLA$^+$ T cells from normal PBMC. IFN-γ, IL-4, IL-17 and IL-22 were significantly increased in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ T_{RM} cells compared to CD69$^-$ non-T_{RM} cells, and cytokines were also up-regulated in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ T_{RM} cells after treated AD triggering factors compared to normal control at 3 weeks, of course, (Fig. 5A). Next, I quantified cytokines in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ T_{RM} cells or non-T_{RM} cells in AD mimicking groups at 3 weeks, and then I checked the percentage of cytokines in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ T_{RM} cells and CD4$^+$CD69$^-$ or CD8$^+$CD69$^-$ non-T_{RM} cells using a microscope. According to our results, all cytokines (IFN-γ, IL-4, IL-17 and IL-22) increased substantially in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ AD T_{RM} cells compared to CD69$^-$ AD T cells at 3 weeks (Fig. 5B).

(A)



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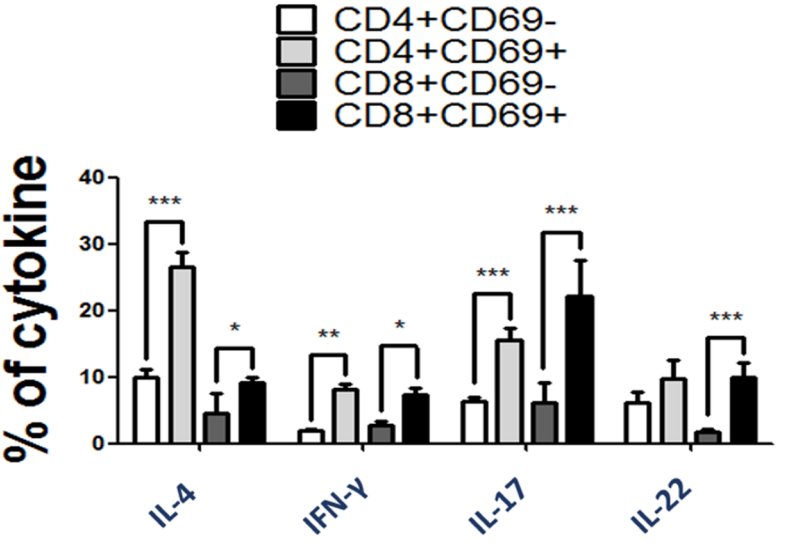
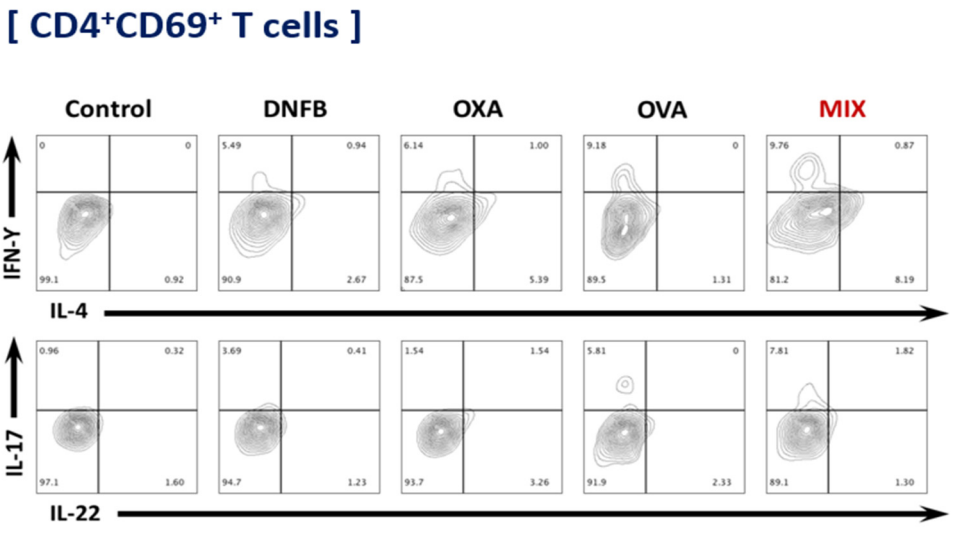


Figure 5. AD triggering factors induce production of cytokines in CD69$^+$ T_{RM} cells over 3 weeks. (A) Representative contours and (B) quantification of cytokine (IL-4, IFN-γ, IL-17 and IL-22) secretion by $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells and $CD69^-$ T cells in AD mimicking CLA^+ T cells at 3 weeks. Quantification is measured by FACS graphs at 3 weeks after treatment with AD triggering elements. Data are displayed as mean ± standard deviation SD. ***P < 0.001, **P < 0.01, *P < 0.05.

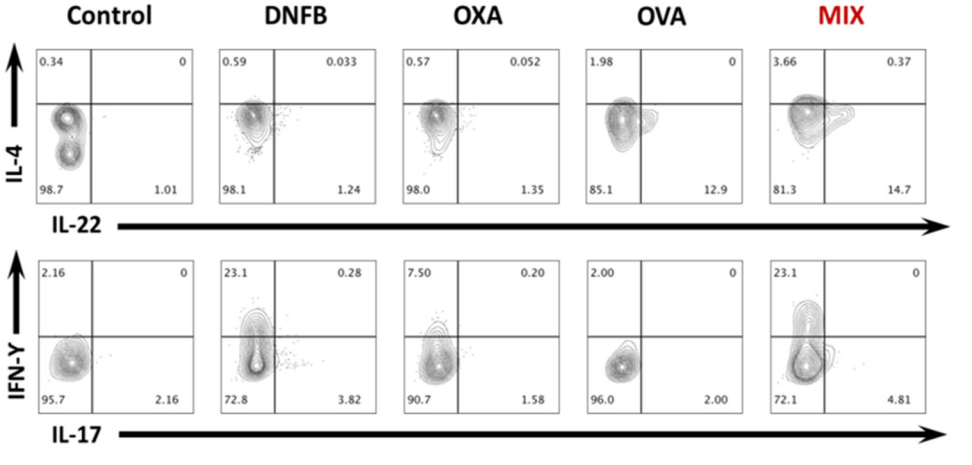
5. Increased expression of cytokines by CD69$^+$ T_{RM} cells in AD-like mouse models

I have observed that CD69$^+$ T_{RM} cells, not CD69$^-$ non-T_{RM} cells, develop various cytokines in AD patients (Fig. 3). To support this observation, I examined whether numerous cytokines would be secreted by skin T_{RM} cells in AD-like mouse models similarly to human AD T_{RM} cells. I first assessed 2,4-dinitrofluorobenzene (DNFB), 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)-induced contact hypersensitivity (CHS) responses, and also assessed ovalbumin (OVA)-induced AD-like mouse models. In addition, I applied both allergens to induce AD-like mouse model which resembled human AD. Likewise, I examined the expression of cytokines in CD4$^+$CD69$^+$ or CD8$^+$CD69$^+$ T_{RM} cells obtained from mouse lesion skin. According to the data, CD4$^+$CD69$^+$ T_{RM} cells produced considerable amounts of IFN-γ, IL-4, and IL-17 in a AD-like mouse model induced by mixture of allergens more similar to the processes in human AD, compared to the other substance-induced AD-like mouse model (Fig. 6A). CD8$^+$CD69$^+$ T_{RM} cells also produced significant IFN-γ, IL-4, IL-17 and IL-22 in the AD-like mouse model induced by mixture of allergens, compared to the other mouse models (Fig. 6B).

(A)



(B)



(C)

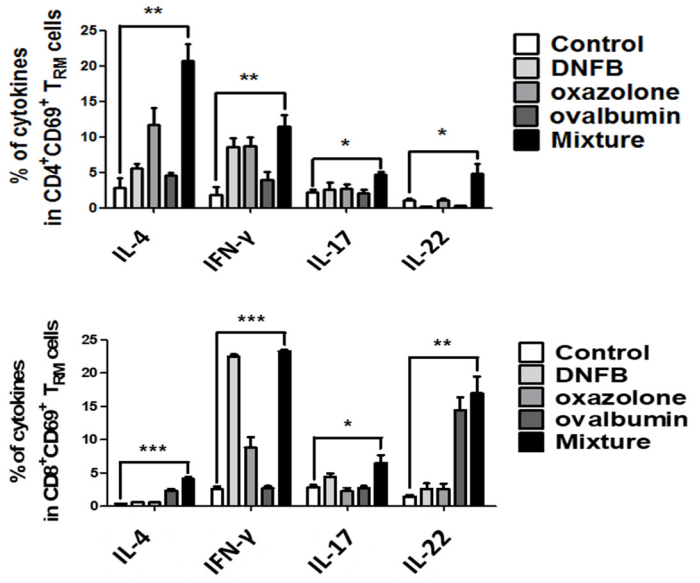


Figure 6. Production of cytokines in $CD4^+CD69^+$ and $CD8^+CD69^+$ skin T_{RM} cells in AD mouse models. Mice (n=3/group) are treated with DNFB, oxazolone, ovalbumin, or a mixture of all of them according to their experimental schedule as described in Fig. 1. Cytokines are secreted in (A) $CD4^+CD69^+$ T_{RM} cells and (B) $CD8^+CD69^+$ T_{RM} cells from mouse lesional skin, assessed by flow cytometry. (C) Cytokines (IL-4, IFN-γ, IL-17 and IL-22) produced by $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells are quantified by FACS graphs. Data are displayed as mean ± standard deviation SD. ***P < 0.001, **P < 0.01, *P < 0.05.

6. Distinct transcriptional profile of T_{RM} cells

To distinguish between T_{RM} cells and non-T_{RM} cells, I attempted to generate human normal and AD skin tissues following culture for 5 days. Then, I respectively sorted T cells which migrated from the skin (T_{MM}) such as $CD4^+CD69^-$ or $CD8^+CD69^-$ T_{MM} cells and remained on skin (T_{RM}) such as $CD4^+CD69^+$ or $CD8^+CD69^+$ skin T_{RM} cells (Fig. 7).

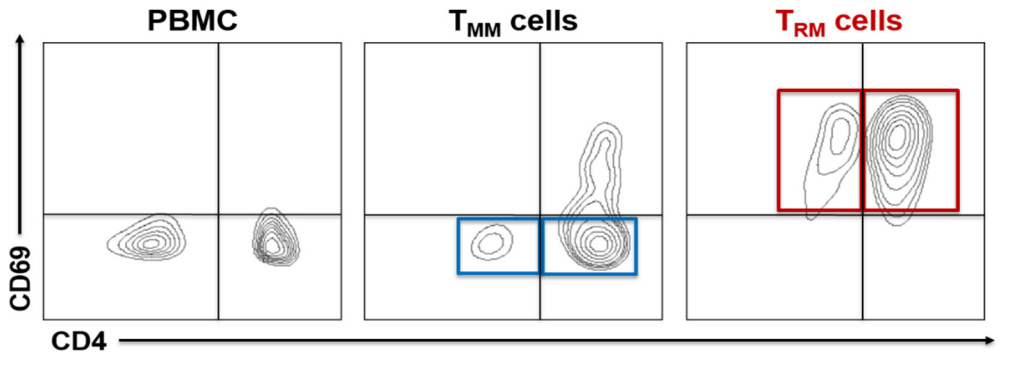


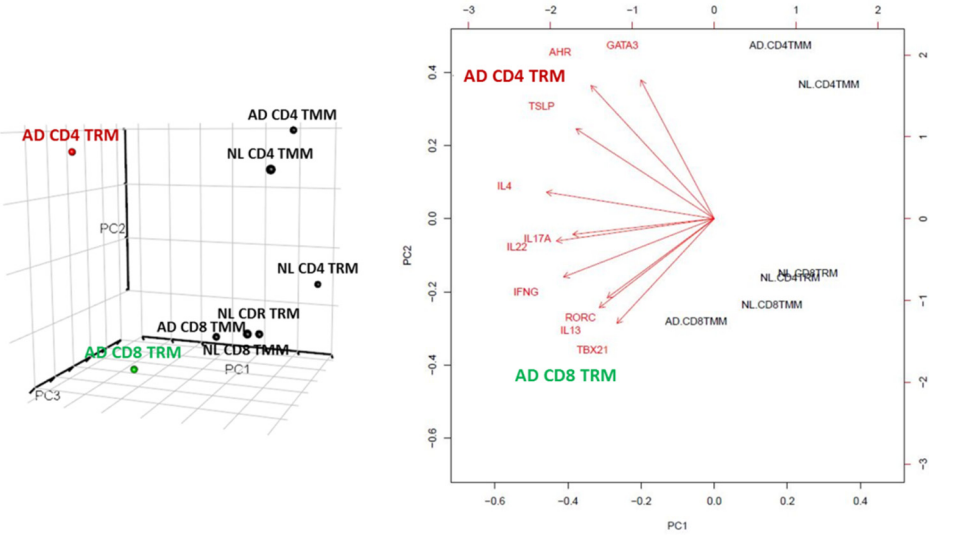
Figure 7. Gating strategy for FACS sorting of CD69$^+$ T_{RM} cells. $CD4^+CD69^-$ and $CD8^+CD69^-$ T_{MM} cells (blue box) and $CD4^+CD69^+$ and $CD8^+CD69^+$ T_{RM} cells (red box) are sorted by FACS from the human skin culture. After preparation of RNA, microarrays are performed.

Then I performed microarray-based gene expression profiling thereof after RNA preparation. The relative amounts of transcripts of genes whose expression was specifically up- or down-regulated in $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells were compared with those in $CD4^+CD69^-$ or $CD8^+CD69^-$ T_{MM} cells (Fig. 8). First, I validated the expression of Th1, Th2, Th17 and Th22-related cytokines and their transcription factors in $CD4^+CD69^+$ or $CD8^+CD69^+$ T_{RM} cells compared to those in $CD4^+CD69^-$ or $CD8^+CD69^-$ T_{MM} cells, while supported our previous data from human normal and AD skin samples. Three dimensional principal component analysis (3D PCA) reveals that AD $CD4^+CD69^+$ and $CD8^+CD69^+$ T_{RM} cells are expressed as a specific tendency in multiple cytokine genes distinct from other AD $CD69^-$ T_{MM} cells and normal T cells (Fig. 8A). In addition, heat map analysis reveals that many cytokines are differentially expressed in each group. In particular, AD $CD4^+CD69^+$ and $CD8^+CD69^+$ T_{RM} cells produce significant levels of Th1 cytokines (IFN-γ), Th2 cytokines (IL-4, IL-13 and TSLP), Th17 cytokines (IL-17), Th22 cytokines (IL-22), and the relevant transcription factors (T-bet, GATA3, RORγt and AhR) which were important for AD pathology, as compared to AD $CD69^-$ T_{MM} cells and normal skin $CD69^+$ T_{RM} cells (Fig. 8B). These results suggest that $CD4^+CD69^+$ and $CD8^+CD69^+$ T_{RM} cells produce multiple cytokines and play a key role in the worsening of AD.

To directly identify individual genes in differential expression, I searched through many genes associated with tissue egress and tissue residency in the skin.[38, 74-76] Heat

map analysis shows that the genes involved in tissue egress are considerably increased in $CD4^+CD69^-$ and $CD8^+CD69^-$ T_{MM} cells. Meantime, the genes related to tissue residency are also substantially up-regulated in $CD4^+CD69^+$ and $CD8^+CD69^+$ T_{RM} cells. This signature includes genes required for tissue egress such as S1pr1, Klf3 and CTCF, tightly down-regulated in resident populations. Similarly, PRDM1, NR4A1, and TRIM63 are specifically up-regulated in the tissue resident gene set. Among down-regulated genes, I pay special attention to the gene encoding CCCTC-binding factor (CTCF) for further validation because of its role in immune response in hematopoietic cell lineages (Fig. 8C). CTCF is also associated with a decrease in CTCF expression, and CTCF-deficient mice have auto-inflammation and increased clinical features.[68] For this reason, I assume that CTCF may play a key role in homeostatic maintenance in the skin and in the inflammatory skin diseases such as AD. I subject the transcriptional data to principal component analysis (PCA) to delineate variations in the data. Transcriptomes of $CD4^+CD69^+$ and $CD8^+CD69^+$ normal and AD T_{RM} cells are distinct from those of $CD69^-$ T_{MM} cells (Fig. 8D). Scatter plots with highlighted genes known to be important for tissue egress and residency are shown in Fig. 8E. Interestingly, CTCF, a predicted target of $CD69^+$ T_{RM} cells is substantially down-regulated in $CD69^+$ AD T_{RM} cells.

(A)



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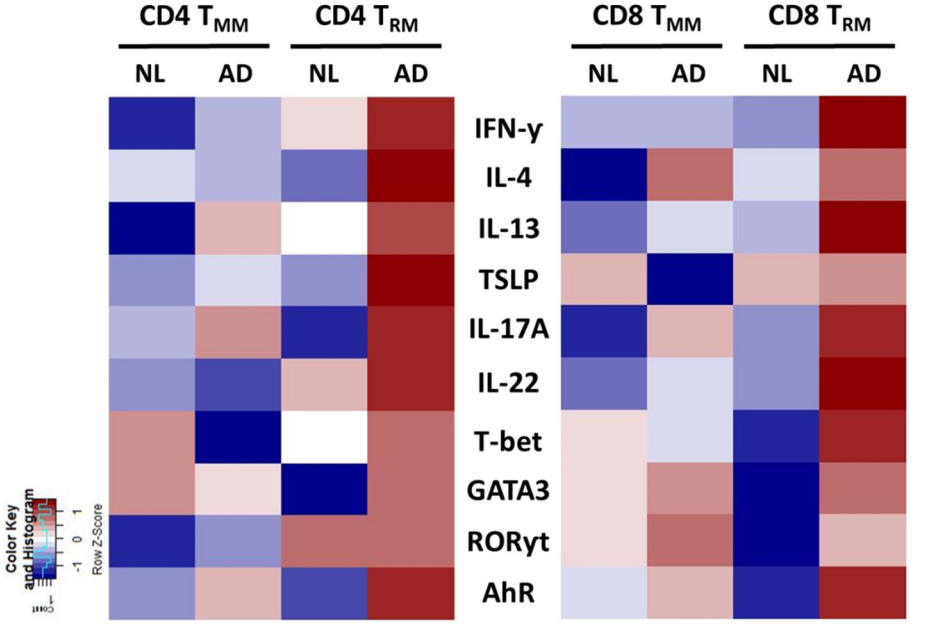

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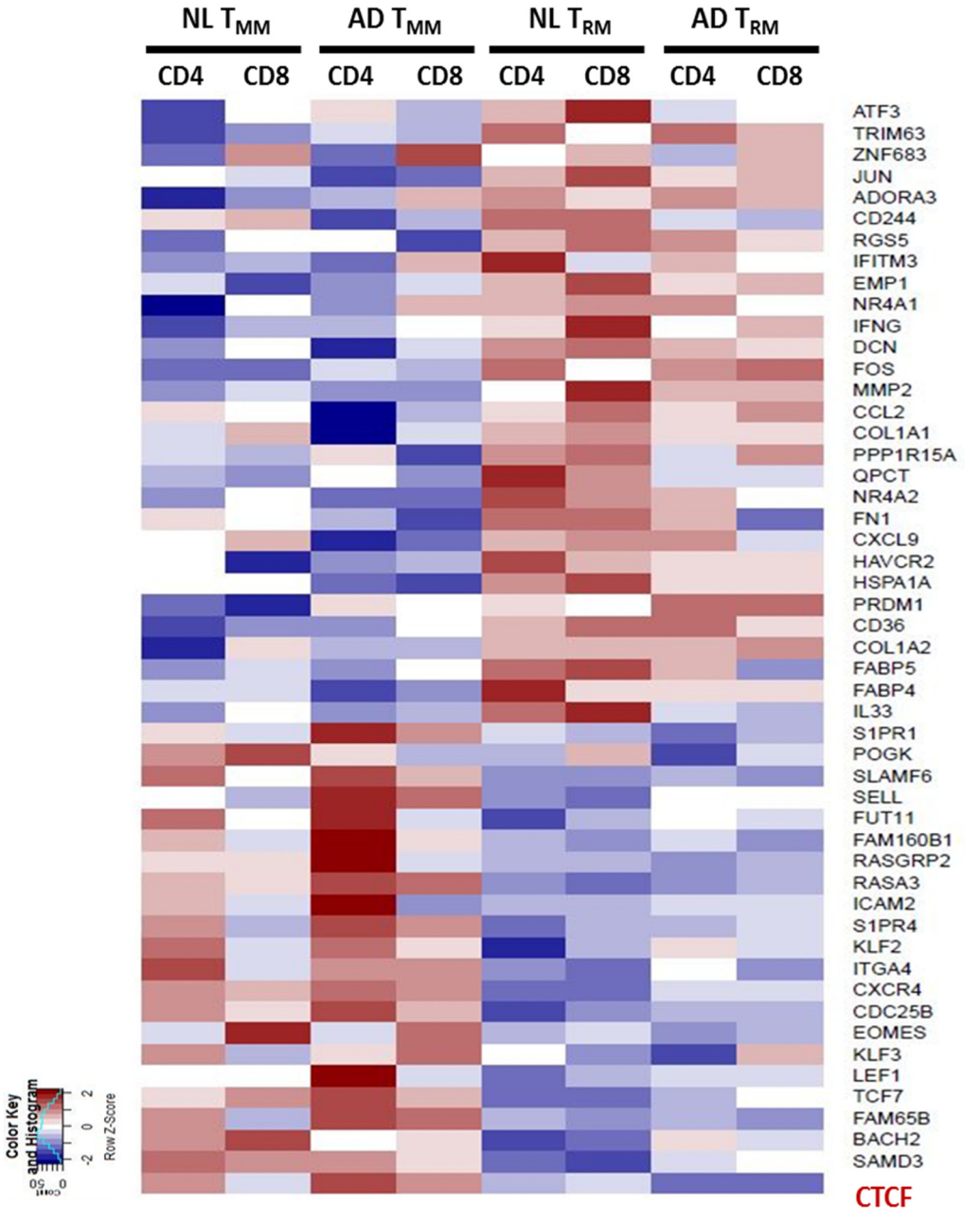

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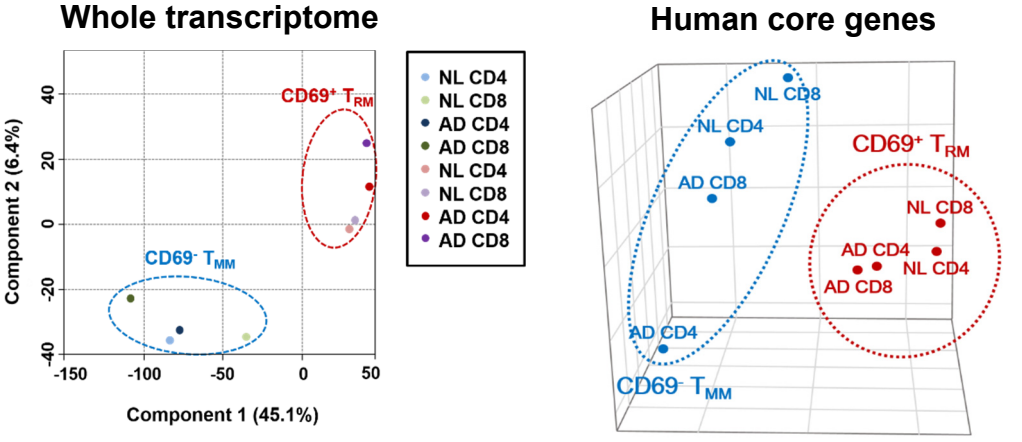


(E)

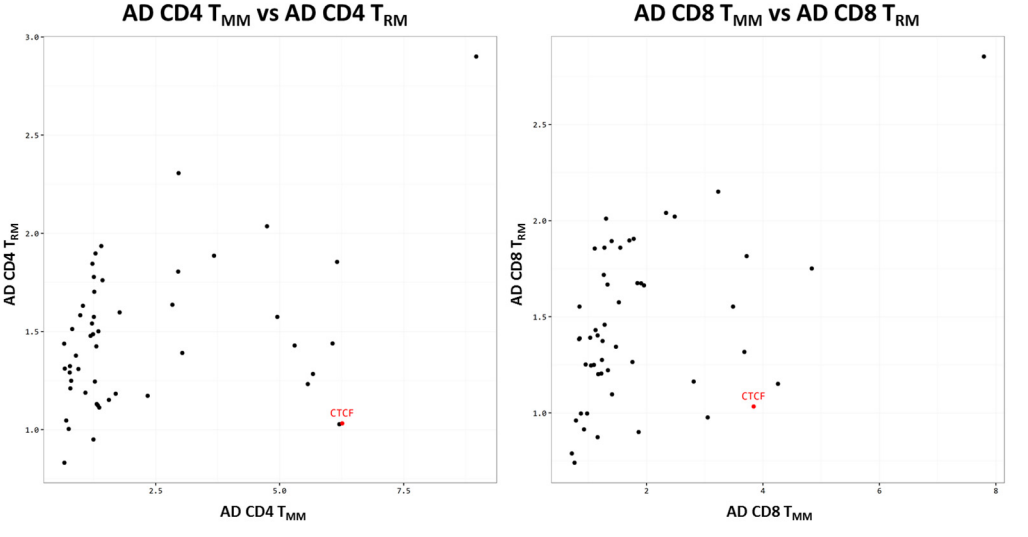
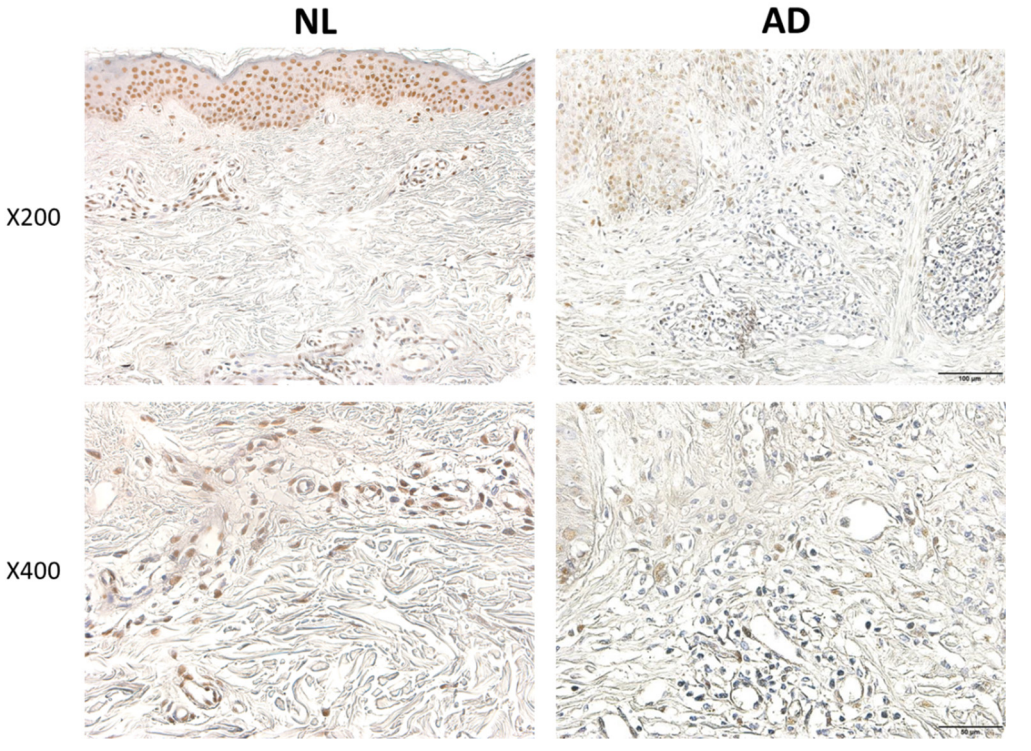


Figure 8. Gene expression profile of CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells. Principal component analysis (PCA) of (A) gene-expression data for multiple cytokines and (D) tissue egress and residency-related genes is analyzed for CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells. Heat map analysis is performed for differentially expressed genes such as (B) multiple cytokine genes and transcription factors, and (C) tissue egress and residency-related genes selected from CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells. (E) Scatter plot reveals the distribution of CTCF gene between CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells.

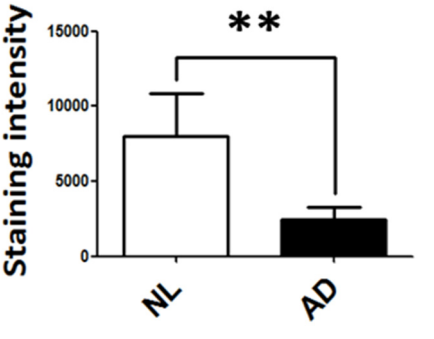
7. CTCF expression in CD69$^+$ T_{RM} cells in AD patients

To validate CTCF expression in normal and AD patients, I first evaluated whether CTCF protein is expressed in AD skin using immunohistochemical staining. I confirmed that CTCF was significantly down-regulated in skin lesions of AD patients compared to normal skin (Fig. 9A). Decreased CTCF expression was quantitatively measured using MetaMorph image analysis and was significantly lower in AD skin (Fig. 9B). To confirm expression levels of CTCF in CD4$^+$ and CD8$^+$ T_{RM} and T_{MM} cells in the skin lesions of AD patients, I performed immunofluorescence staining. I found that CTCF expression was considerably decreased in both CD4$^+$ and CD8$^+$ T_{RM} cells compared to T_{MM} cells in AD skin (Fig. 9C). The expression of CTCF on CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells in AD skin was quantitatively measured using a microscope and was found to be significantly lower in CD69$^+$ T_{RM} cells in AD skin (Fig 9D). To reinforce the microarray analysis, CD4$^+$ or CD8$^+$ T_{MM} cells and CD4$^+$ or CD8$^+$ T_{RM} cells were sorted from the lesional skin of AD patients and analyzed to detect CTCF expression by qRT-PCR was performed. The qRT-PCR analysis showed that the relative expression of CTCF was significantly decreased in CD69$^+$ T_{RM} cells compared to CD69$^-$ T_{MM} cells (Fig. 9E). These results suggest that CTCF might be closely associated with T_{RM} cells in the pathogenesis of AD.

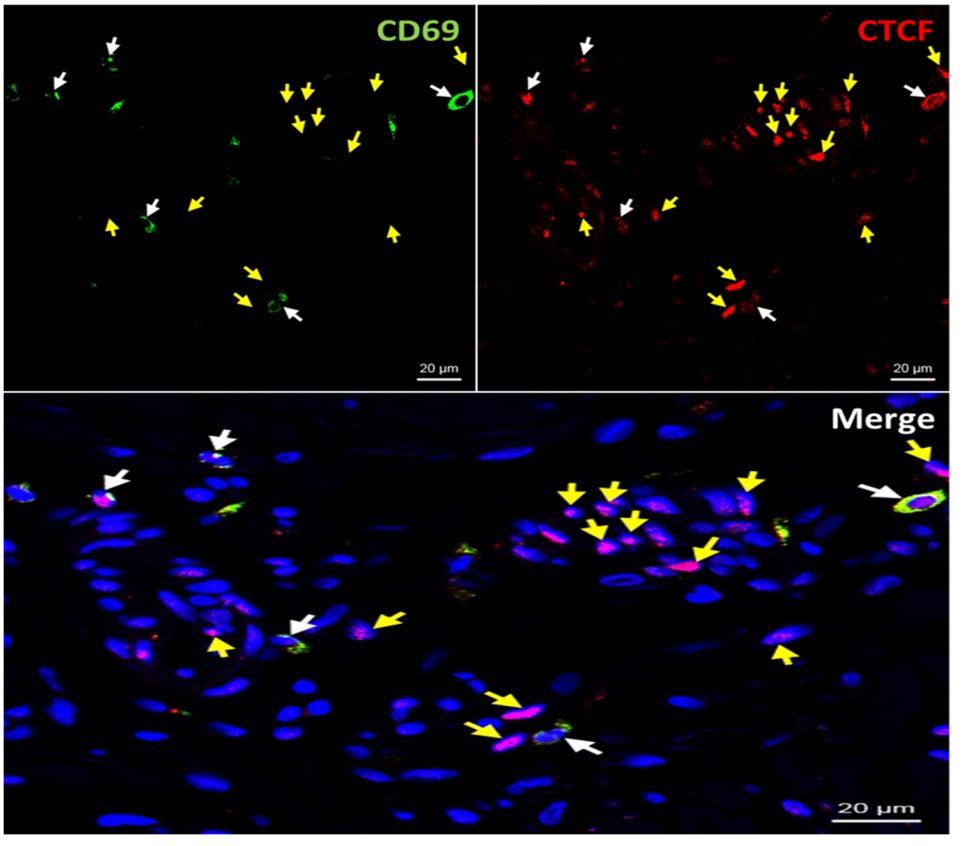
(A)



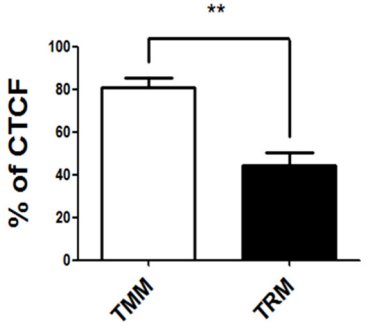
(B)



(C)



(D)



(E)

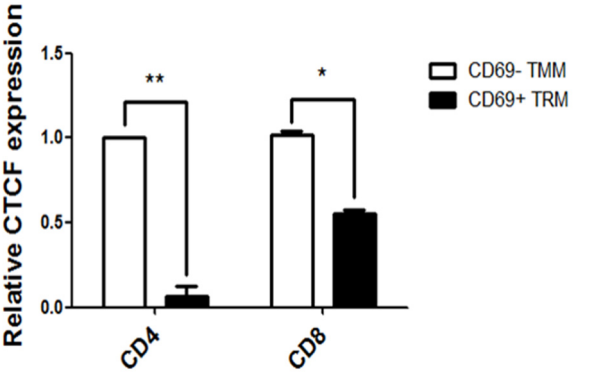
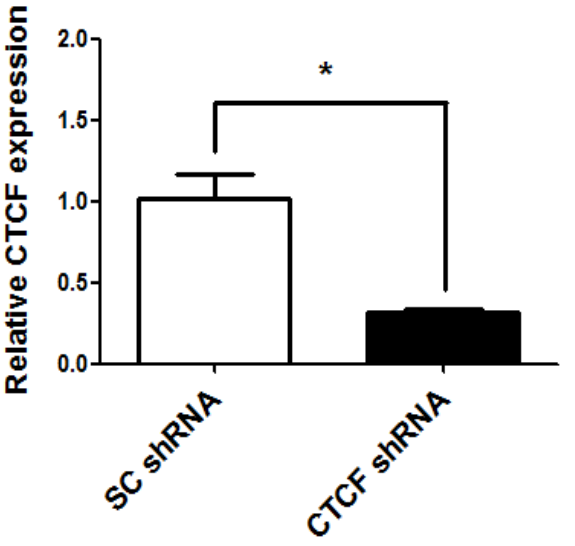


Figure 9. Decreased CTCF expression in CD69$^+$ T_{RM} cells in AD patients. (A) Immunohistochemical staining of CTCF is obtained in AD patients and normal skin. Scale bar, 20 μm. Original magnification x 400. (B) Decreased expression of CTCF is quantitatively measured using MetaMorph image analysis software. (C) Immunofluorescence confocal images are obtained for CD4 or CD8 (red), CD69 (green), and CTCF (yellow) in lesions from AD skin. White arrows indicate expression of CTCF in CD69$^+$ T_{RM} cells, and yellow arrows show CTCF merged with CD69$^-$ T_{MM} cells. Scale bar, 20 μm. Original magnification x 400. (D) Down-regulated expression of CTCF in CD69$^+$ T_{RM} cells and CD69$^-$ T_{MM} cells is quantitatively measured using a microscope. (E) qRT-PCR analysis of relative CTCF expression is examined in CD69$^-$ T_{MM} cells and CD69$^+$ T_{RM} cells on AD skin. Data are representative of three normal tissues or three AD tissues. Data are displayed as mean ± standard deviation SD. ***P < 0.001, **P < 0.01, *P < 0.05.

8. Functional significance of CTCF in human $CD4^+$ T cells

To study the role of CTCF in human $CD4^+$ T cells, I first suppressed the CTCF expression in $CD4^+$ T cells using shCTCF-based transfection technique. $CD4^+$ T cells were isolated from human normal PBMCs. Because $CD4^+$ T cells are suspension cells and have low transfection efficiency, I transferred the CTCF shRNA instead of using traditional siRNA based transfection. The expression level was normalized to GAPDH. CTCF expression in shRNA–treated group were suppressed up to 70% (Fig. 10A). I hypothesized that the inhibitions of CTCF would facilitate the expression of $CD69^+$ T_{RM} cells and then these cells would be expected to secrete various multiple cytokines, such as IFN-γ, IL-4, IL-17, and IL-22. So, I observed the changes of CD69, a marker of T_{RM} cells, and the relevant cytokines in $CD4^+$ T cells after knockdown of CTCF. Interestingly, knockdown of CTCF in $CD4^+$ T cells caused significant increase of CD69 and multiple cytokines, such as IFN-γ, IL-4, IL-17, and IL-22 (Fig. 10B). These results suggest that the down-regulations of CTCF is directly involved in the induction of $CD69^+$ T_{RM} cells and secretions of multiple cytokines.

(A)



(B)

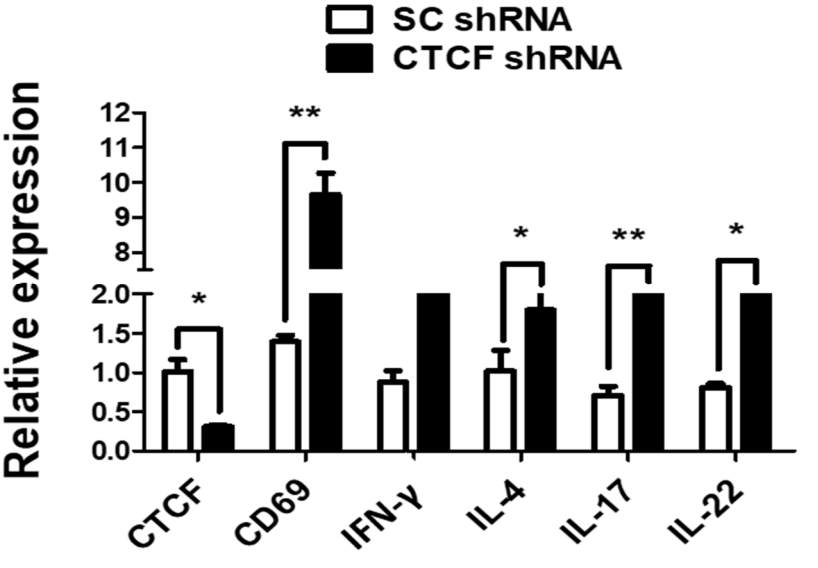


Figure 10. $CD69^+$ T_{RM} cells and multiple cytokines are up-regulated in CTCF shRNA-treated $CD4^+$ T cells. At 72 hours after transfection, I apply antibiotic selection to the cells with puromycin. After 48 hours, total RNA is extracted from scrambled (SC) shRNA-treated group and CTCF shRNA-treated group, and then mRNA levels of CD69, IFN-γ, IL-4, IL-17, and IL-22 are analyzed using qRT-PCR and relative expression is normalized to that of GAPDH. (A) Compared with scrambled (SC) siRNA-treated group, mRNA level of CTCF are down-regulated in $CD4^+$ T cells treated with CTCF shRNA. The suppression efficiency is up to 70%. (B) Open bar graph indicates SC shRNA-treated $CD4^+$ T cells and closed bar graph indicates CTCF shRNA-treated $CD4^+$ T cells. Result shows that CD69 and cytokines IFN-γ, IL-4, IL-17, and IL-22 are considerably increased in CTCF shRNA-treated group compared to SC shRNA-treated group. Data are displayed as mean ± standard deviation SD. ***P < 0.001, **P < 0.01, *P < 0.05.

IV. DISCUSSION

The manifestation of atopic dermatitis (AD) is determined by the complex interaction of genetic, immune and environmental factors.[10] Unaffected AD skin contains a spare perivascular T cell infiltrate not seen in normal skin. Affected AD skin activates keratinocytes to produce pro-inflammatory cytokines and chemokines and releases the extravasation of inflammatory cells into the skin. Inflammation in AD is associated with the increase of Th2 cells in acute skin lesions, but chronic AD results in the infiltration of inflammatory dendritic epidermal cells (IDECs), macrophages, and eosinophils.[1] Among the multiple factors contributing to AD, T cells are especially important immunological factors with potential to aggravate symptoms.[77,78] The important role of immunologic T cells in AD is supported by the fact that primary T cell immunodeficiency disorders show elevated serum IgE levels and eczematous skin lesions. Furthermore, the presence of Th1 and Th2 cytokines in the skin during inflammatory responses has been demonstrated in the experimental models of allergen-induced allergic skin inflammation from the mice carrying the targeted gene deletion or overexpression of those cytokines.[79,80] Interestingly, S Kannanganat et al. demonstrate that cytokine coexpression profiles indicate a strong positive association between the multiple cytokine production capacity of virus-specific CD4 T cells and their other functions. This strong association suggests that vaccines utilizing viral

infection would elicit CD4 T cells capable of producing more than one cytokine.[81] These findings suggest that T cells, a key player in AD, can produce various kinds of multiple cytokines, thereby contributing to the development and aggravation of AD.

The immunologic responses in AD is predominantly carried out by the T cells existing in the skin. Most studies so far have focused on circulating T cells because it is more difficult to prepare skin T cells than blood T cells. Recent studies have also shown that normal resting human skin contains twice as many T cells as blood does, and most (though not all) of these T cells are tissue resident.[25] For this reason, it is necessary to examine T cells resident in skin. Tissue-resident memory T cells (T_{RM} cells) are a subset of memory T cells that persist long term in peripheral tissues. Protective T cells are highly specific to the pathogens commonly encountered by each tissue. Pathogen-specific T_{RM} cells differentiate and accumulate in peripheral tissues after infection of a pathogen. However, dysregulation of T_{RM} cells can contribute to human autoimmune and inflammatory diseases. T_{RM} cells can induce and develop fixed, recurrent, and chronic inflammatory skin lesions.[36] In human skin tissues, transcriptomes of T_{RM} cells from skin, lung, and gut have similar features to those found in mice. One study showed that the localization of $CD8^+$ T_{RM} cells and $CD103^+$ T_{RM} cells were induced in the epidermis by TGF-β.[38] Recently, several studies have shown that T_{RM} cells play a key role in human chronic inflammatory skin diseases such as psoriasis and AD. Another study showed that cells extracted from clinically resolved psoriatic lesions contained

$CD8^+$ T_{RM} cells producing IL-17 and $CD4^+$ T_{RM} cells producing IL-22.[82] However, the role of T_{RM} cells in AD, which is a recurrent chronic inflammatory skin disease, has not yet been studied previously. Therefore, in my study, the characteristics and functions of T_{RM} cells are identified in AD lesional skin.

In this study, I demonstrate that the skin of AD lesions contains large numbers of skin-T_{RM} cells compared to normal skin, and they are as effective in producing multiple cytokines in AD lesions as T_{MM} cells. To verify the functions of T_{RM} cells in AD lesional skin, I used transcriptional analysis, which features high accuracy and reproducibility, as a tool of high-throughput analysis. I compared between AD skin T_{RM} cells resident in skin and AD skin T_{MM} cells migrated from the skin and sequentially identified considerably changed genes. Among the initially screened 47322 genes, I was able to identify significantly differentially expressed genes that defined a core set of Th1, Th2, Th17, and Th22-related cytokines and transcription factors. Our present study is the first to report that $CD69^+$ T_{RM} cells are significantly increased in the skin of AD patients compared to normal skin, and they produce more multiple-cytokines, compared to $CD69^-$ T_{MM} cells, in AD skin *in vivo* and *in vitro*. Supporting these data, I generated AD-like mouse model which is treated with DNFB, oxazolone, and ovalbumin. In mouse skin lesions, there was also significantly more expression of multiple-cytokines by $CD69^+$ T_{RM} cells than $CD69^-$ T_{MM} cells. T_{RM} cells contain a diverse TCR repertoire, and they express CD4+ and CD8+ T cells that produce IL-13, IL-17, and IFN-γ.[83-85] In

the lung T_{RM} cells, there are a host of diverse TCR repertoires. A recent study shows that lung contains a large number of repertoires in immunocompetent T_{RM} cells.[85] One of the most interesting findings in that study is that a large number of skin-T_{RM} cells produce multiple cytokines in the skin of AD lesions. Based on those, I suggest that $CD69^+$ T_{RM} cells in AD skin produce multiple cytokines, which aggravate the symptoms of AD.

Gene expression would be limited if the binding of proteins to one-dimensional DNA sequences explains the complex gene regulation networks. Mammalian chromatin, including in humans, maintains a three-dimensional structure in the nucleus and is distinguished by a specific "Topological Association Domain" (TAD) according to its function and expression.[53] This organization is established by architectural proteins, of which CTCF is the best identified. CTCF is a highly conserved, 11-zinc finger protein which contains a DNA-binding domain.[54] CTCF function is assigned according to its specific ability to mediate long-range DNA interactions that affect the 3-dimensional chromatin structure. It also functions as a boundary to distinguish each TAD and as a mediator between the enhancer and the target gene in TAD.[86,87] CTCF regulates early thymocyte development, cell differentiation,[65] and cytokine production in helper T cells.[67] CTCF also positively regulates the homeostatic pool and the efficient migration of LC, which are required for immune responses in the skin. One study demonstrated that after treating LC CTCF-KO mouse with DNFB, ear swelling and contact dermatitis

continued for a longer time than in DNFB-induced WT mice. CTCF-deficient LCs also express genes that promote cellular adhesion, such as CD69, Cadm3 and Cdh17, while tissue egress-related genes, such as S1pr3 and Ccr7, are down-regulated in these cells.[68] Furthermore, gene silencing mediated by epigenetic mechanisms is important for regulating the proper gene expression in cell differentiation. Chromatin modifications such as DNA methylation and acetylation, which are regulated by CTCF control the accessibility of transcriptional activators and repressors.[88] However, since the specific role and expression of CTCF in AD T_{RM} cells was unclear, I investigated the expression of CTCF by transcriptional analysis of AD T_{RM} cells, and also analyzed the cell adhesion and egress-associated genes. Among initially screened 47322 genes, I demonstrated more than 50 genes that designated a gist set of tissue egress and residency-related genes. CD69 and CD103 are well-known markers for T_{RM} cells. CD69 is related to down-regulated expression of the G protein-coupled receptor for sphingosine 1 phosphate (S1P1). Actually, there is a concentration gradient of sphingosine 1 phosphate which is a ligand of S1P1 in mice and human body, with the highest concentration in blood and the lowest in peripheral tissues.[29] Therefore, with the increased expression of CD69 in T_{RM} cells, it leads to lowering the expression of S1P1, and thus causing the T_{RM} cells to remain only in the specific tissue, not to go out circulating in bloodstream. Even though the role of CD103 is not completely elucidated, it is considered to be one of the differentiation markers in T_{RM} cells. Specifically, genes

required for tissue egress, such as Klf2 and Ccr7 are also down-regulated in tissue-resident populations.[47]

In my microarray experiments the expression levels of cell adhesion molecules, including Prdm1, NR4A1 and Trim63, are significantly higher in $CD69^+$ T_{RM} cells. However, tissue egress-related genes, including S1pr1, Slamf6 and Fam65b are considerably increased in $CD69^-$ T_{MM} cells. In this study, I also show for the first time (to my knowledge) that CTCF expression in T_{RM} cells is regulated in AD. Interestingly, CTCF expression is not decreased in AD T_{MM} cells. Therefore, this implies the functional alteration of T_{RM} cells in AD. To verify this transcriptional analysis, I stain CTCF expression in AD skin and confirm the relative expression of CTCF in AD $CD69^+$ T_{RM} cells and $CD69^-$ T_{MM} cells. Down-regulation of CTCF in AD skin is shown for $CD69^+$ T_{RM} cells compared to $CD69^-$ T_{MM} cells. Moreover, to identify a putative functional role of CTCF in human $CD4^+$ T cells, I inhibit CTCF expression in $CD4^+$ T cells with CTCF-specific shRNA. In parallel with the down-regulation of CTCF, $CD4^+$ T cells exhibit the significantly increased expression of CD69 and multiple cytokines, including IFN-γ, IL-4, IL-17, and IL-22. Depending on these data, I would suggest that CTCF regulates the enhancers of the genes in $CD69^+$ T_{RM} cells in the AD skin of the patients.

In summary, the current study demonstrates the down-regulation of CTCF in AD T_{RM} cells by transcriptional microarray analysis and evaluates the functional role of CTCF.

The results suggest that the decreased expression of CTCF in response to AD T_{RM} cells might facilitate the function of $CD69^{+}$ T_{RM} cells through transcriptional control and contribute to the production of various multiple-cytokines that induce worsening of AD.

V. CONCLUSION

This study is carried out to discover and validate the role of CTCF in AD $CD69^+$ T_{RM} cells. I show that AD $CD69^+$ T_{RM} cells produce multiple cytokines which exacerbate AD symptoms. At this time, CTCF plays a crucial role in AD $CD69^+$ T_{RM} cells. When CTCF expression is decreased, CD69 expression is regulated and T_{RM} cell expression is promoted. As a result, the number of T_{RM} cells increases and the symptoms of AD worsens.

In conclusion, CTCF may play an important role in $CD69^+$ T_{RM} cells by contributing to the production of multiple cytokines that induce the aggravation of AD symptoms. Further study is required to fully understand the underlying mechanisms of CTCF function in AD $CD69^+$ T_{RM} cells. I also anticipate that our findings will provide a clue to develop future AD therapies that aim to modulate CTCF activity.

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ABSTRACT (in Korean)

아토피피부염의 피부거주 T 세포에서 CCCTC-결합 인자의 역할

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김 서 형

아토피피부염은 소양증을 동반한 만성 재발성의 염증성 질환으로, 외부환경으로부터 침입한 항원 물질과 진피의 면역세포에 의해 야기되는 면역반응과 피부의 같은 자리에 계속해서 다시 발생하는 것을 특징으로 한다. 피부 거주 T 세포 (T_{RM} 세포)는 기억 T 세포의 한 종류로 말초 조직에 오랜 기간 동안 계속해서 존재하는 세포로 알려져 있으며, 위장관, 기도, 피부와 같은 숙주와 환경 사이의 접점의 상피 막 조직에 존재한다. CCCTC-binding factor (CTCF)는 DNA 결합 zinc-finger 단백질로서, 고차원 크로마틴 구조 결정에 중요하게 관여하여 다양한 분자생물학적 기전을 통해 유전자 발현을 조절한다고 알려져 있는 유전자이다. 본인은

아토피피부염에서의 피부 거주 T 세포의 역할과 성상을 확인하고자 하였고, 피부 거주 T 세포가 아토피피부염을 악화시키는데 있어서 CTCF 단백질의 발현 및 기능을 연구하였다.

$CD69^+$ 및 $CD103^+$ T_{RM} 세포가 정상 피부조직에 비해 아토피피부염 환자의 피부조직에 현저히 침투하는 것을 관찰하였고, 정상 피부조직에는 비교적 $CD103^-$ 및 $CD69^-$ 세포가 많이 발현되는 것을 확인하였다. 또한, 면역형광염색법을 통해 $CD69^+$ AD T_{RM} 세포가 Th2 사이토카인 (IL-4, IL-13), Th17 사이토카인 (IL-17, IL-22) 및 Th1 사이토카인 (IFN-γ)과 같은 다양한 사이토카인들을 분비하는 것을 확인하였다. 아토피피부염과 유사한 마우스 모델 (DNFB, 옥사졸론, 오발부민) 피부조직의 $CD69^+$ T_{RM} 세포에 비해 다양한 알러젠에 노출되는 아토피피부염 환자와 유사하게 이 알러젠들을 모두 혼합하여 유발한 마우스 모델 피부조직의 $CD69^+$ T_{RM} 세포에서 역시 다양한 사이토카인들을 많이 분비하는 것을 확인하였다. 3주간의 CLA^+ T 세포 배양 동안, $CD4^+$ 및 $CD8^+$ T 세포의 $CD69^+$ T_{RM} 세포의 발현은 시간이 지날수록 지속적으로 증가 하였고, 아토피피부염과 유사한 환경을 조성해 주었을 때, 더 많은 $CD69^+$ T_{RM} 세포 및 다양한 사이토카인들이 생성되는 것을 확인하였다. 이를 증명하기 위해 AD $CD69^-$ T_{MM} 및 $CD69^+$ T_{RM} 세포에서 RNA를 얻어 microarray 분석을 진행하였다. 그 결과 $CD69^+$ T_{RM} 세포에서 Th1, Th2, Th17 및 Th22 사이토카인과 그 전사인자 (T-bet, GATA3, RORγt 및 Ahr)들이 높은 수준을 나타내는 것을

확인하였다. 전체 47322 개의 유전자 중 AD $CD69^-$ T_{MM} 세포와 비교하였을 때 AD $CD69^+$ T_{RM} 세포에서 CTCF 유전자가 뚜렷하게 감소되었다. 이를 검증하기 위해, 본인은 CTCF 단백질의 발현이 정상인의 피부조직에 비해 아토피피부염 환자의 피부조직에서, 또한 $CD69^-$ T_{MM} 세포에 비해 $CD69^+$ T_{RM} 세포에서 현저하게 감소됨을 면역화학염색법과 면역형광염색, real-time PCR 및 shRNA를 통해 확인하였다.

결론적으로, 본 연구를 통해 아토피피부염 유발 원인에 반복적으로 노출되면 CTCF 유전자 발현의 감소되어 아토피피부염의 $CD69^+$ T_{RM} 세포를 조절하게 됨을 확인하였고, 이로 인해 $CD69^+$ T_{RM} 세포가 다양한 사이토카인들을 생성하게 되어서 결론적으로 아토피피부염의 악화를 유도하게 됨을 확인하였다.

핵심이 되는 말 : 아토피피부염, 피부거주 T 세포, 다양한 사이토카인, CCCTC-결합 인자