Surface protein marker of the induced regulatory T cells in NC/Nga mice

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ABSTRACT

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$CD25^+$ $CD4^+$ regulatory T cells (Tregs) are known as a key modulator of immune self-tolerance and also have been found in atopic dermatitis (AD) patients. Since it has been rarely known that the different functional role between naturally occurring Tregs (nTregs) in thymus and induced Tregs (iTregs), and their surface marker proteins, we designed the experiments to separate two different subpopulations of Tregs, nTregs from thymus of healthy control mice and iTregs from spleen from the AD model mice.

We initially observed AD-like skin lesions in NC/Nga mice sensitizing *D. farinae* ointments for six weeks and we measured their significant increases of serum IgE level and enhanced IL-4 production from Th2 subsets. $CD4^+$ $CD25^+$ Treg cells were separated from those AD-like mice and healthy control mice using MACS®, with high separation purity confirmed by flow cytometry analysis. Membrane proteins

were extracted from $CD4^+$ $CD25^+$ Tregs and labeled with TMT reagents for 1DLC-MS/MS analysis. As a result of TMT-labeling method, we obtained 533 protein, 63 membrane proteins and 16 plasma membrane proteins identification list based on database research. Remarkably, H-2 class II histocompatibility antigen has the highest ratio of iTregs to nTregs among those identified proteins, and other membrane proteins including receptor-type tyrosine-protein phosphatase C and leukocyte surface antigen CD47, also showed high expression levels in iTregs, compared with nTregs. Considering the increased ratios of selected proteins in iTreg cell population and their functional roles that have been elucidated from many research groups, we believed that they might be key marker proteins or functional regulators of iTregs.

key words : atopic dermatitis, NC/Nga mouse, regulatory T cells, nTreg, iTreg

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Ⅰ. INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic, chronic relapsing inflammatory skin disease characterized by dry itchy skin and immune responses caused by immune cells in the dermis of the skin and various antigens[1]. In terms of the immunology, it had been demonstrated that an IgE levels elevation caused by Th2-dominated immune response and the imbalance of Th1/Th2 cells, could be important factors in the pathogenesis of AD.[2-4]

$CD25^+$ $CD4^+$ regulatory T (Treg) cells are known as a key modulator of immune self-tolerance and homeostasis by suppressing excessive immune responses which can be hazardous to the host.[5,6] Treg cells have been reported to play an important role in many autoimmune disease and allergic diseases, such as AD pathogenesis and development.[7] The noticeable increase in Tregs has been found in AD patients as compared to healthy controls and increased percentage of these cells has been

also reported in the peripheral blood in AD. In addition, elevation of Treg population correlates with the severity of AD skin lesion expressed by SCORAD index.[8,9]

There are two large categories in Treg cell populations: Naturally occurring Tregs (nTregs) which is constitutively expressing the nuclear transcription factor–forkhead winged helix P3 (FoxP3) and induced Tregs (iTreg or adaptive Treg (aTreg)) in the periphery by different antigens and under tolerogenic conditions.[8,10] The population of iTregs expands and differentiates from nTregs or $CD4^+$ $CD25^-$ T cells[11] after the stimulation by cytokines, such as TGF-β and IL-10 inducing Tregs, IL-10 inducing T-regulatory type 1 cells (Tr1) and TGF-β inducing Th3 cells.[8,12]

Several experiments were conducted in many groups to find out the developmental and functional differences between nTreg and iTreg cells.[13,14] Haribhai and coworkers compared the gene expression of in vitro–generated iTreg cells which were induced by TGF-β and IL-2 with total Tregs originated from healthy Foxp3-GFP reporter mice, a population consisted mostly of nTreg cells. Their results of study proved that iTreg and nTreg cells were genetically distinct even though numerous genes were expressed by both Treg subsets.[14] From other studies, Ikzf2 (Helios) and Nrp1 (Neuropilin-1) expression were enhanced in nTreg cells compared with iTreg cells and they suggested that these genes, in particular, Helios, could potentially be used as specific markers for nTregs. [15,16]

Although Helios and other possible markers must be outstanding discoveries, we still need to know specific surface markers of iTreg cells in order to separate these two different kinds of cells definitely for further studies. It has been reported that not only the high-affinity IL-2 receptor chain (CD25), but cytotoxic T lymphocyte-

associated antigen (CTLA)–4, glucocorticoid-induced tumor necrosis factor receptor (GITR), and HLA-DR also represented on the surface of nTreg cells.[7,13] In contrast to nTregs, there has been no discovery about surface molecules of the iTreg cells.[13]

In these aspects, we assumed Tregs in the thymus of healthy control mice were regarded as nTregs and Tregs in the spleen of AD-like mice were iTregs so that surface markers of iTregs can be discovered in protein levels using proteomics techniques. Since, developing Tregs in the thymus are a relatively homogenous population of naive T cells and preferentially migrate to secondary lymphoid tissues,[17] Based on these results, we assumed that the increase of iTregs in peripheral lymphoid organs of *Dermatophgoides farinae* (*D. farinae*)-sensitized mice guarantees the quantitative differences in protein levels between two organs of two different conditioned mice.

Ⅱ. MATERIALS AND METHODS

1. Atopic dermatitis induction with NC/Nga mice

A. Animals and induction conditions.

Female NC/Nga mice (aged 6 weeks) were purchased from Central Lab Animal Incorporation (Seoul, Korea) and housed under Specific Pathogen-Free (SPF) conditions with temperature (22 ± 3℃), humidity (55 ± 15%) during the entire experiment. After a week of stabilization, hairs on the back of the mice

were removed using an electric clipper and hair removal cream. One day after removing hairs, 150 μl of 4% sodium dodecyl sulfate (SDS) was topically applied on the surface for disruption of skin barrier. Then, 2 hours later, 100 mg of Biostir AD cream consisting of *D. farinae* body extracts (AD Biostir, purchased from Central Lab Animal Incorporation) was applied onto the shaved dorsal surface. All procedure conducted twice a week during 8 weeks.

B. Evaluation of dermatitis

According to SCORAD index, mice were evaluated the severity of their AD skin lesion with respect to erythema/hemorrhage, scarring/dryness, excoriation/erosion and edema. Each symptom was scored as, 0 (none), 1 (mild), 2 (moderate), or 3 (severe) respectively. Dermatitis score was defined as the sum of these individual scores and examined every week during 8-week sensitization period. Additionally, the mice were photographed once per week.

C. Enzyme-linked Immunosorbent Assay (ELISA) for total serum IgE

Total IgE levels in serum of the mice were measured with ELISA MAX™ Deluxe Set (BioLegend, San Diego, CA, USA), in accordance with the manufacturer's protocol. Blood specimens were obtained from the 0, 2, 4, 6, and 8 week sensitized mice by orbital puncture. One day prior to running the ELISA, IgE-specific monoclonal antibody was coated on a 96-well plate and incubated overnight at 4℃. After washing the wells, standards and serum samples were added to the plate and then incubated at room temperature for 2 hours with shaking. Captured IgE molecules were detected by biotinylated anti-mouse IgE

detection antibody which made antibody-antigen-antibody “sandwich”. Avidin-horseradish peroxidase is subsequently added, followed by TMB Substrate solution. Since the color of the solution changed in proportion to serum IgE levels, absorbance of each well could be measured at 450 nm with a microplate reader.

2. Isolation of regulatory T cells and their membrane proteins

A. Separation of splenocytes and thymocytes

The mouse spleen and thymus separation was carried out in sterile conditions. We put the spleen and thymus in 5 ml PBS solution, then placed it in 40 μm cell strainer (BD Bioscience, San Jose, CA, USA), repeated milling, filtered twice, and then spent 10 min to centrifuge the splenocytes at 1500 r/min under 4 ℃. Cell pellet from centrifugation was melted in red blood cell lysing buffer (Sigma, St. Louis, MO, USA) containing 8.3 g/L ammonium chloride in 0.01M Tris-HCl buffer (pH7.5±0.2) and centrifuged again.

B. Isolation of regulatory T cells using MACS®

The cell suspension of each organ was diluted in buffer for MACS analysis (containing PBS, 2 mM EDTA and 0.5% BSA) and incubated with a cocktail of lineage specific biotin-conjugated antibodies against CD8, CD11b, CD45R, CD49b, Ter-119, and Anti-Biotin MicroBeads for 10 min and 15 min, respectively. Non-$CD4^+$ T cells were depleted with LD column which place in the magnetic field of AutoMACS Pro Separator® (Medilab Korea Co, Seoul, Korea). Unlabeled $CD4^+$ T cell fraction obtained from previous separation steps was

incubated with CD25-PE antibody and Anti-PE MicroBeads in order to stain $CD25^+$ T cells. After staining, $CD25^+$ T cells were positively selected and collected into new tubes by AutoMACS Pro Separator®.

C. Flow cytometry

To check the purity of MACS® separation, isolated Treg cells were incubated with anti-CD4-FITC and anti-CD25-PE (eBioscience, San Diego, CA, USA) for 30 minutes and washed with PBS containing 1% BSA. Intracellular labeling of FOXP3 was performed using eBioscience fixation/permeabilization buffers, as per manufacturer's instructions.

The splenocytes were labeled with Anti-CD3-APC, anti-CD4-FITC and anti-IL-4-PE (eBioscience) to confirm the IL-4 levels in splenocytes of NC/Nga mice with a same method above.

Flow cytometry analysis was performed by using BD LSR II Flow Cytometer, then compensated and analyzed with FLOWJO software version 7.6.5 for Macintosh (TreeStar, San Carlos, CA, USA).

D. Membrane protein extraction

Isolated Treg cells were centrifuged at 850xg for 2 minutes and supernatant was removed. Reagent A (Thermo scientific, Waltham, MA, USA) was added to the pellet in order to lysis the cells. Two parts Reagent C with one part Reagent B (Thermo scientific) was also added and after centrifugation and incubation, hydrophilic layer (top layer) was discarded. Hydrophobic layer (bottom layer) was used for further steps.

3. TMT labeling proteomic analysis

A. TMT labeling

TMT sixplex reagents were purchased from Thermo Scientific. The sample containing 100 μg of proteins was equally splited into two and labeled with two different TMT reagents following the manufacturer's standard protocol. The protein samples from spleens of six-week sensitized mice were labeled with TMT-126 and TMT-130, whereas, those from thymus of normal mice with TMT-127 and TMT-131. Total four samples labeled with different TMT reagent were mixed and dried, then re-solubilized with water containing 0.5% formic acid for LC/MS/MS analysis.

B. Nano LC- MS/MS analysis

Resulted peptides were analyzed using one dimensional liquid chromatography/tandem mass spectrometry (1DLC-MS/MS). Peptides were identified using MS/MS with a nano-LC-MS system consisting of a Nano Acquity U7PLC system (Waters, Milford, MA, USA) and an LTQ Orbitrap elite mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray source. An autosampler was used to load 5 μl aliquots of the peptide solutions onto a C18 trap-column (i.d. 300 μm, length 5 mm, and particle size 5 μm; Waters). The peptides were desalted and concentrated on the column at a flow rate of 5 μL/min. Then, the trapped peptides were back-flushed and separated on a 200-mm home-made microcapillary column consisting of C18 (Aqua; particle size 3 μm) packed into 100-μm silica tubing with an orifice i.d. of about 6 μm. 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 5 min and was ramped to 15% B over 5 min, to 50% B over 100 min, to 95% B over 5 min, and remained at 95% B over 5 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 2.2 kV. During the chromatographic separation, the LTQ Orbitrap Elite was operated in a data-dependent mode and under direct control of the Xcalibur software (Thermo Scientific). The MS data were acquired using the following parameters; ten data-dependant collisional-induced-dissociation (CID) MS/MS scans per every full scan in label-free; ten data-dependant higher energy collision induced dissociation (HCD) MS/MS scans per every full scan in TMT; CID scans were acquired in LTQ with twomicroscan averaging; full scans and HCD scans were acquired in Orbitrap at resolution 30,000 and 15,000, respectively. With two-microscan averaging; 35% normalized collision energy in CID and in HCD; ±1.5 Da isolation window; dynamic exclusion enabled with ±1.5 Da exclusion window. All 1DLC-MS/MS analysis for TMT-labeling quantification performed duplicate for each sample.

C. Data analysis

Probability -based (and error-tolerant) protein database searching of MS/MS spectra against the latest IPI rat protein database (IPI rat v3.70) was performed with a local MASCOT server (2.3, Matrix Science, London, UK) to identify and quantify proteins. The rate of decoy hits in the combined forward and reversed database was less than 1% of the forward hits on both the peptide and the protein levels in each of

these experiments. Search criteria were set at: 20 ppm precursor ion mass tolerance, 0.5 Da product ion mass tolerance, two missed cleavages, trypsin as enzyme, TMT modification at the N-terminus and lysine residues and carbamidomethylation at cysteine residues as static modifications, oxidation at methionine, phosphorylation at serine, threonine, and tyrosine as a variable modifications, an ion score threshold of 20 and TMT-6 plex for quantification. Quantification was based on the averaged signal-to-noise ratio of TMT reporter product ions of more than two unique peptides. In TMT experiments, reporter ions for peptide identifications were extracted from small windows (±20 ppm) around their expected m/z in the HCD scan. Since the same sample was individually labeled with two TMT reagents, peptides showing the similar ratio in the comparison of the intensity of reporter ion within 30% were selected for protein quantitation. The abundance ratio of a protein was estimated using the ratio between the 12 protein's total intensities in different reporter ion channels. Considering distributions of protein log2 ratios, proteins showing $\leq$ -0.4 or $\geq$ 0.4 were considered as differentially expressed ones.

Ⅲ. RESULTS

1. Clinical features of *D. farinae* sensitized NC/Nga mice

During the eight-week sensitization period, AD-like lesion in the back skin of the mice were observed and mean value of the SCORAD score was measured by SCORAD index (Fig.1). House dust mite-induced dermatitis was primarily developed by *D. farinae* extracts application twice a week onto NC/Nga mice for

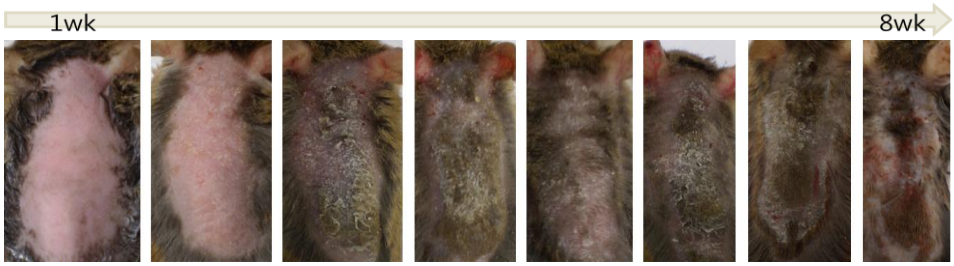
eight weeks prior to the further study. After sensitizing for one week, all experimental animals showed no symptom, but three weeks later, clinical scarring and erythema appeared on the back of the mice. After six weeks from sensitization, we observed the most severe AD-like symptoms such as erythema, hemorrhage, scarring, dryness, and excoriation on their dorsal skin during sensitization period (Fig. 1A and 1B).

When scoring the skin lesion of NC/Nga mice for each symptom factors, we also found out SCORAD score showed rapid and significant increment during sensitization period and the mice sensitized for six weeks had the highest score with respect to AD-like symptoms. The score increased 0 to 6.2 for three weeks, and although the clinical scores at 4 and 5 weeks from the *D. farinae* sensitization were slightly decreased (score = 5.6, respectively), the highest score was measured in six-week AD-like mice (score = 7.2). After 6 weeks, the score was inclined to decrease in their scarring factor number and showed clinical improvement (Fig. 1C). This SCORAD score result was also correlated with the photos from Fig. 1A.

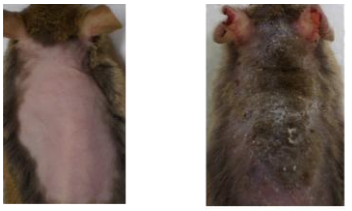
2. IgE levels in AD-like NC/Nga mouse

The hyperproduction of IgE is one of the most characteristic features of AD.[1,2] To investigate whether the IgE levels were increased in our model, we analyzed blood samples collected at interval of two weeks. As shown in Fig. 2, repeated topical application of hose dust mite caused a significant increase in serum IgE levels in NC/Nga mice. The mean value of IgE levels was only 53±8.8 ng/ml in two-week mite sensitized mice, but remarkably increased in two and four weeks later (525.4±12.4 and 679.2±20.1 ng/ml, respectively.)

A



B



C

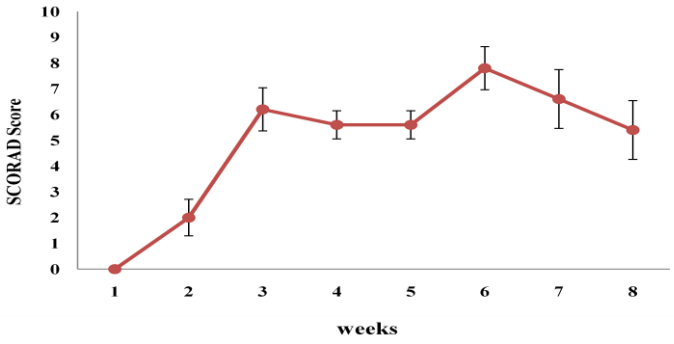


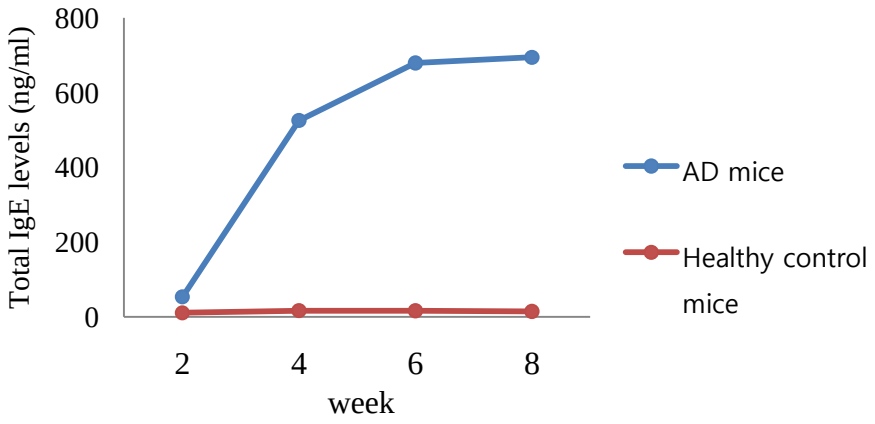
Figure 1. (A) The photos were taken from one mouse over 8 weeks, in order to check the severity of skin lesion. (B) Based on the photos of the mice, six-week *D. farinae* sensitized mouse was shown most severe AD skin. (C) Scores were accumulated and averaged for 8 weeks. The change of SCORAD score showed that AD-induced mice for six weeks had the most severe AD symptoms on their dorsal skins (n=5). (HC: Healthy Control, AD: Atopic Dermatitis)

Comparing with serum from normal mice, those from 6-week AD induced mice showed 40-folded increases in serum IgE levels and which means theses mice are enough to be considered an AD mouse model (Fig. 2B). Since it has little differences between 6 weeks and 8 weeks mice IgE levels (679.2±20.1 ng/ml and 694.2±13.5 ng/ml, respectively), we used the 6 weeks sensitized mice for further studies.

3. Th2 cytokine changes in the spleen of AD mice.

When it comes to human patients with AD, it has been demonstrated that the number of Th2 cells and IL-4 levels are significantly enhanced[3,4]. When we performed the flow cytometry analysis with splenocytes of NC/Nga mice sensitized for six weeks, we gained same results with those of human (Fig. 3). IL-4 producing $CD4^+$ cells, determined by intercellular cytokine staining for IL-4, surface staining for CD3 and CD4, were statistically up-regulated in NC/Nga mice with AD (5.79±4.54%) compared with HC subjects (0.16%). In these results, six week sensitized mice were optimal animal model of Atopic dermatitis patients.

A



B

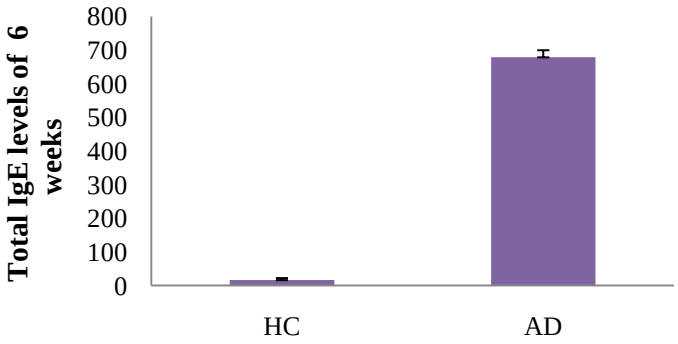
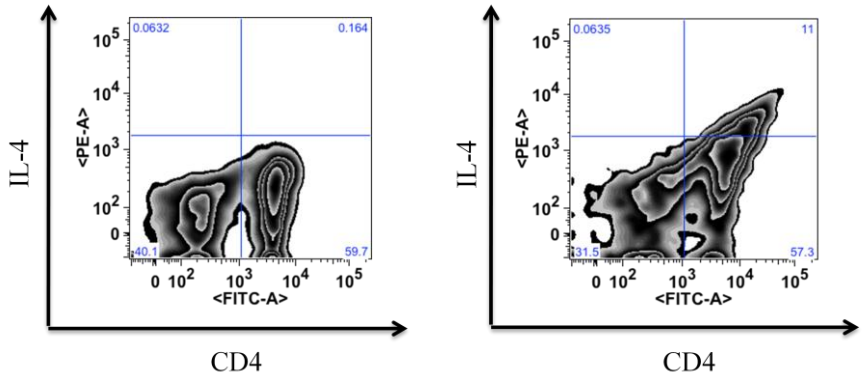


Figure 2. Increment of IgE levels in AD induced mice in a time dependent manner. Serum IgE was originated from blood samples of mice which were collected by orbital puncture and quantified by enzyme-linked immunosorbent assay (ELISA). (A) The ELISA result for serum IgE in blood of 0, 2, 4, 6, and 8 weeks sensitized mice, indicated that serum IgE levels were increased in a time dependent manner. (B) Six-week sensitized mice had much higher level of IgE levels, compared with HC mice. Serum IgE levels of HC mice were only 16.5±5.3 ng/ml, while those of AD were 679.2±20.1 ng/ml. (HC: healthy control, AD: atopic dermatitis)

A



B

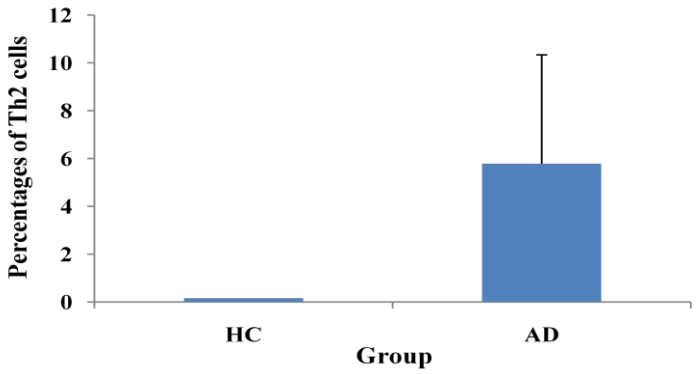


Figure 3. IL-4 cytokines increase in splenocytes of the AD-induced mice.

Results were analysed by surface staining and intracellular antibodies, using flow cytometry. (A) IL-4 levels in healthy control mouse (0.16% of splenocytes) and *D. farinae* sensitized mouse (11% of splenocytes) and (B) the mean percentage of IL-4 secreting $CD4^+$ T cells were examined in healthy subjects (0.16%, n = 1) and AD subjects (5.79±4.54%, n = 3)

4. Regulatory T cell separation using MACS®

$CD4^+CD25^+$ regulatory T cells were isolated from the splenocytes of AD mice using MACS® and we conducted flow cytometry analysis so as to confirm the purity of Tregs (Fig.4).

After magenetic bead sorting, we obtained $2\text{-}4\times10^5$ Treg cells from the thymus of one normal mouse and $2\text{-}3\times10^6$ Treg cells from the spleen of the house dust mite treated mouse with a high purity (79.5%) detected by flow cytometry staining. An electronic gate was drawn around all live $CD4^+$ $CD25^+$ cells and $Foxp3^+$ cells within this $CD4^+$ $CD25^+$ population examined for the expression of intracellular Foxp3. $Foxp3^+$ Treg ratio in collected Tregs from MACS flow was 94.7% which indicated that most of purified cells expressed Foxp3 as well as CD4 and CD25 marker proteins.

5. Protein Identification using a TMT-labeling proteomic analysis

Membrane proteins extracted from the $CD4^+CD25^+$ Tregs of spleens and thymuses were dissected and labeled with TMT isobaric tags separately for quantitative proteomic analysis. A total of 510 proteins were identified and quantified across all two LC-MS/MS runs (Data not shown).

A B

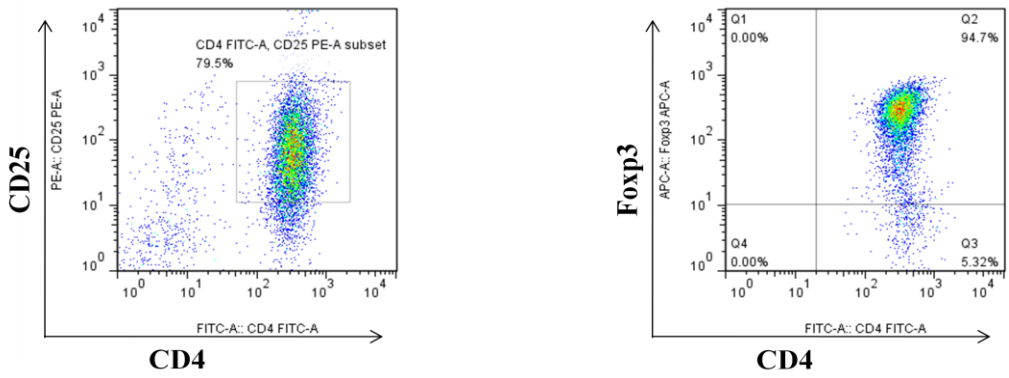


Figure 4. Purity of isolated regulatory T cells.

Tregs were sorted by magnetic beads and MACS® and their efficiency was measured by FACS. (A) $CD4^+CD25^+$ Treg population was 79.5% of total eluted Treg cells from MACS® and (B) $CD4^+Foxp3^+$ Treg cells was 94.7% of the $CD4^+CD25^+$ T regs.

Among those proteins, 63 proteins were located in membrane fraction of the cells (Table 1) and 13 plasma membrane proteins were identified from database research (Table 2). The proteins listed in Table 1, were upregulated (47 proteins) or downregulated (16 proteins) in iTreg membrane protein samples. Considering distributions of protein log2 ratios, proteins showing $\leq$ -0.4 or $\geq$ 0.4 were considered as differentially expressed ones. From those statistic cutting on Table 2, total six plasma membrane proteins (H-2 class II histocompatibility antigen, h-2 class I histocompatibility antigen K-W28 alpha chain-like isoform 1, D-P alpha

chain-like isoform 4, protein tyrosine phosphatase receptor type C-associated protein, isoform 3 of Receptor-type tyrosine-protein phosphatase C and Isoform 1 of Leukocyte surface antigen CD47) were identified using TMT-labeling and they were considered to be significantly increased in iTregs, compared with nTregs. Among those six proteins, we selected three intriguing proteins (H-2 class II histocompatibility antigen, receptor-type tyrosine-protein phosphatase C and leukocyte surface antigen CD47) that were correlated with the diseases and immunologic functions based on the researches have been reported. Remarkably, H-2 class II histocompatibility antigen not only has the highest ratio of iTregs to nTregs (Fit ratio of $\log_2$ (iTreg/nTreg) = 1.43) among those identified proteins, but also has been studied related to Tregs and Foxp3 expression.

Table1. Membrane protein list using a TMT labeling method

NO.	IPI NO.	DESCRIPTION	iTreg Log2(126+130	nTreg Log2(127+131	Fit_Ratio (iTreg/nTreg)
1	IPI00225390.5	Tax=10090 GS=Cox6b1 Cytochrome c oxidase subunit 6B1	13.2891	13.6851	-0.5458
2	IPI00626994.3	Tax=10090 GS=Ipo5 Isoform 1 of Importin-5	8.6202	8.9498	-0.4795
3	IPI00116877.5	Tax=10090 GS=Tnpo2 transportin-2	10.0495	10.2952	-0.3955
4	IPI00798527.1	Tax=10090 GS=Tnpo1 Isoform 2 of Transportin-1	8.9195	9.1111	-0.3415
5	IPI00109727.1	Tax=10090 GS=Thy1 Thy-1 membrane glycoprotein	12.6675	12.7869	-0.2693
6	IPI00165694.1	Tax=10090 GS=Tomm34 Isoform 1 of Mitochondrial import receptor subunit TOM34	10.8784	10.9784	-0.2498
7	IPI00230540.1	Tax=10090 GS=Vdac1 Isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1	11.7840	11.8828	-0.2487
8	IPI00938467.1	Tax=10090 GS=Lck proto-oncogene tyrosine-protein kinase LCK isoform a	11.7831	11.8807	-0.2475
9	IPI00229935.3	Tax=10090 GS=Ptprb Receptor-type tyrosine-protein phosphatase beta	11.5368	11.5056	-0.1187
10	IPI00120466.1	Tax=10090 GS=Cd5 T-cell surface glycoprotein CD5	8.0688	7.9730	-0.0541
11	IPI00121309.2	Tax=10090 GS=Ndufs3 NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	8.9542	8.8549	-0.0505
12	IPI00130344.3	Tax=10090 GS=Clic1 Chloride intracellular channel protein 1	11.6859	11.5823	-0.0463
13	IPI00125929.2	Tax=10090 GS=Ndufa4 NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	13.1399	13.0186	-0.0286
14	IPI00118986.1	Tax=10090 GS=Atp5o;LOC100047429 ATP synthase subunit O, mitochondrial	11.6007	11.4692	-0.0184
15	IPI00311682.5	Tax=10090 GS=Atp1a1 Sodium/potassium-transporting ATPase subunit alpha-1	11.5598	11.4171	-0.0072
16	IPI00930882.1	Tax=10090 GS=Slc3a2 4F2 cell-surface antigen heavy chain isoform a	9.1199	8.9730	-0.0029
17	IPI00119138.1	Tax=10090 GS=Uqcrc2 Cytochrome b-c1 complex subunit 2, mitochondrial	10.6442	10.4834	0.0110
18	IPI00323881.2	Tax=10090 GS=Kpnb1 Importin subunit beta-1	11.0277	10.8536	0.0243
19	IPI00845575.2	Tax=10090 GS=Atl3 atlastin-3 isoform 1	9.6760	9.4848	0.0413
20	IPI00404182.2	Tax=10090 GS=Rhob Rho-related GTP-binding protein RhoB	11.4687	11.2678	0.0511
21	IPI00133240.1	Tax=10090 GS=Uqcrfs1 Cytochrome b-c1 complex subunit Rieske, mitochondrial	9.7465	9.5288	0.0679
22	IPI00129792.1	Tax=10090 GS=Kpna4 Importin subunit alpha-4	11.9586	11.7313	0.0775
23	IPI00111885.2	Tax=10090 GS=Uqcrc1 Cytochrome b-c1 complex subunit 1, mitochondrial	11.6341	11.4064	0.0778
24	IPI00133706.1	Tax=10090 GS=Rab1b Ras-related protein Rab-1B	12.6260	12.3771	0.0991
25	IPI00121288.5	Tax=10090 GS=Ndufb10 NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	13.8617	13.6027	0.1091
26	IPI00467062.2	Tax=10090 GS=Tapbp Isoform Long of Tapasin	11.0743	10.8151	0.1093
27	IPI00116770.1	Tax=10090 GS=Rab18 Ras-related protein Rab-18	9.8218	9.5597	0.1122
28	IPI00130280.1	Tax=10090 GS=Atp5a1 ATP synthase subunit alpha, mitochondrial	13.2884	13.0150	0.1235
29	IPI00122548.4	Tax=10090 GS=Vdac3 Voltage-dependent anion-selective channel protein 3	10.8050	10.5292	0.1260
30	IPI00622837.1	Tax=10090 GS=Gm6265 cytochrome c oxidase subunit 6C-like	13.0492	12.7682	0.1311
31	IPI00989100.1	Tax=10090 GS=Rab1 22 kDa protein	12.8755	12.5816	0.1440
32	IPI00411115.1	Tax=10090 GS=Rab8b Ras-related protein Rab-8B	10.2913	9.9681	0.1733
33	IPI00990016.1	Tax=10090 GS=Rab14 RAB14 protein variant	9.3363	8.9936	0.1928
34	IPI00127983.1	Tax=10090 GS=Tmed2;Gm10698 Transmembrane emp24 domain-containing protein 2	9.7858	9.4415	0.1944
35	IPI00119618.1	Tax=10090 GS=Canx Calnexin	14.1173	13.7627	0.2048
36	IPI00987138.1	Tax=10090 GS=LOC100047577 cytochrome b5 type B-like	14.9090	14.5461	0.2130
37	IPI00224518.2	Tax=10090 GS=Rab5c Ras-related protein Rab-5C	10.0028	9.6327	0.2203
38	IPI00407954.2	Tax=10090 GS=Rap1b Ras-related protein Rap-1b	13.2249	12.8544	0.2207
39	IPI00759999.1	Tax=10090 GS=Prdx5 Isoform Cytoplasmic+peroxisomal of Peroxiredoxin-5, mitochondrial	10.2007	9.8120	0.2389
40	IPI00110849.1	Tax=10090 GS=H2-Aa H-2 class II histocompatibility antigen, A-K alpha chain	8.9564	8.5546	0.2519

41	IPI00131176.1	Tax=10090 GS=mt-Co2 Cytochrome c oxidase subunit 2	15.1602	14.7242	0.2861
42	IPI00408892.2	Tax=10090 GS=Rab7 Ras-related protein Rab-7a	9.3238	8.8811	0.2928
43	IPI00555000.2	Tax=10090 GS=Uqcrb Cytochrome b-c1 complex subunit 7	13.7627	13.3125	0.3003
44	IPI00341282.2	Tax=10090 GS=Atp5f1 ATP synthase subunit b, mitochondrial	14.4398	13.9238	0.3661
45	IPI00403079.4	Tax=10090 GS=Cd47 Isoform 1 of Leukocyte surface antigen CD47	14.8106	14.2506	0.4102
46	IPI00128856.2	Tax=10090 GS=Ptprc Isoform 3 of Receptor-type tyrosine-protein phosphatase C	12.3368	11.7648	0.4221
47	IPI00114377.1	Tax=10090 GS=Cox7a2 Cytochrome c oxidase subunit 7A2, mitochondrial	15.4368	14.8582	0.4287
48	IPI00466570.4	Tax=10090 GS=Tmed10 Isoform 1 of Transmembrane emp24 domain-containing protein 10	14.3041	13.6975	0.4567
49	IPI00230241.6	Tax=10090 GS=Atp5e ATP synthase subunit epsilon, mitochondrial	11.4311	10.8022	0.4791
50	IPI00117978.1	Tax=10090 GS=Cox4i1 Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	13.5709	12.9405	0.4805
51	IPI00985828.1	Tax=10090 GS=LOC100045864 h-2 class I histocompatibility antigen, D-P alpha chain-like isoform 4	13.5130	12.8470	0.5161
52	IPI00316976.3	Tax=10090 GS=Ptprcap Protein tyrosine phosphatase receptor type C-associated protein	11.7475	11.0731	0.5245
53	IPI00850057.1	Tax=10090 GS=LOC100044874 h-2 class I histocompatibility antigen, K-W28 alpha chain-like isoform 1	13.4310	12.7493	0.5319
54	IPI00108844.1	Tax=10090 GS=M6pr Cation-dependent mannose-6-phosphate receptor	12.1324	11.4145	0.5680
55	IPI00953773.1	Tax=10090 GS=Ssr4 translocon-associated protein subunit delta isoform 1 precursor	12.3014	11.5799	0.5716
56	IPI00857192.1	Tax=10090 GS=Fis1 mitochondrial fission 1 protein isoform 2	12.1282	11.4031	0.5752
57	IPI00120719.4	Tax=10090 GS=Cox5a Cytochrome c oxidase subunit 5A, mitochondrial	14.0156	13.2366	0.6292
58	IPI00674006.3	Tax=10090 GS=Tmem173 Isoform 1 of Transmembrane protein 173	10.2513	9.4440	0.6575
59	IPI00323897.3	Tax=10090 GS=Rab11a Ras-related protein Rab-11A	10.9208	10.0480	0.7230
60	IPI00759904.1	Tax=10090 GS=Cyb5r3 Isoform 2 of NADH-cytochrome b5 reductase 3	12.5679	11.6410	0.7771
61	IPI00128450.1	Tax=10090 GS=Esyt1 Isoform 1 of Extended synaptotagmin-1	10.7710	9.8392	0.7820
62	IPI00120346.3	Tax=10090 GS=Rab27b Ras-related protein Rab-27B	10.8696	9.8760	0.8437
63	IPI00172243.1	Tax=10090 GS=H2-Ab1 H-2 class II histocompatibility antigen, A-K beta chain	11.5565	9.9744	1.4322

Table2. Plasmamembrane protein list using a TMT-labeling method

NO.	IPI NO.	DESCRIPTION	iTreg Log2(126+130)	nTreg Log2(127+131)	Fit_Ratio (iTreg/nTreg)
1	IPI00109727.1	Tax=10090 GS=Thy1 Thy-1 membrane glycoprotein	12.6675	12.7869	-0.2693
2	IPI00938467.1	Tax=10090 GS=Lck proto-oncogene tyrosine-protein kinase LCK isoform a	11.7831	11.8807	-0.2475
3	IPI00229935.3	Tax=10090 GS=Ptprb Receptor-type tyrosine-protein phosphatase beta	11.5368	11.5056	-0.1187
4	IPI00120466.1	Tax=10090 GS=Cd5 T-cell surface glycoprotein CD5	8.0688	7.9730	-0.0541
5	IPI00130344.3	Tax=10090 GS=Clic1 Chloride intracellular channel protein 1	11.6859	11.5823	-0.0463
6	IPI00311682.5	Tax=10090 GS=Atp1a1 Sodium/potassium-transporting ATPase subunit alpha-1	11.5598	11.4171	-0.0072
7	IPI00930882.1	Tax=10090 GS=Slc3a2 4F2 cell-surface antigen heavy chain isoform a	9.1199	8.9730	-0.0029
8	IPI00404182.2	Tax=10090 GS=Rhob Rho-related GTP-binding protein RhoB	11.4687	11.2678	0.0511
9	IPI00127983.1	Tax=10090 GS=Tmed2;Gm10698 Transmembrane emp24 domain-containing protein 2	9.7858	9.4415	0.1944
10	IPI00110849.1	Tax=10090 GS=H2-Aa H-2 class II histocompatibility antigen, A-K alpha chain	8.9564	8.5546	0.2519
11	IPI00403079.4	Tax=10090 GS=Cd47 Isoform 1 of Leukocyte surface antigen CD47	14.8106	14.2506	0.4102
12	IPI00128856.2	Tax=10090 GS=Ptprc Isoform 3 of Receptor-type tyrosine-protein phosphatase C	12.3368	11.7648	0.4221
13	IPI00985828.1	Tax=10090 GS=LOC100045864 h-2 class I histocompatibility antigen, D-P alpha chain-like isoform 4	13.5130	12.8470	0.5161
14	IPI00316976.3	Tax=10090 GS=Ptprcap Protein tyrosine phosphatase receptor type C-associated protein	11.7475	11.0731	0.5245
15	IPI00850057.1	Tax=10090 GS=LOC100044874 h-2 class I histocompatibility antigen, K-W28 alpha chain-like isoform 1	13.4310	12.7493	0.5319
16	IPI00172243.1	Tax=10090 GS=H2-Ab1 H-2 class II histocompatibility antigen, A-K beta chain	11.5565	9.9744	1.4322

IV. DISCUSSION

The NC/Nga mouse has been proposed as a suitable model of human AD, since the skin lesions that developed in these mice resemble with those of human AD in a specific pathogen-free (SPF) condition.[18,19] We initially sensitized NC/Nga mice with SDS and *D. farinae* ointments for 8 weeks and performed ELISA experiments to find optimal period of sensitization. After sensitizing *D. farinae* for six weeks, severe AD-like skin lesion was appeared on their dorsal skin marked high level of IgE was also observed. It is well known that IgE levels are elevated in most patients with AD[2-4], and this phenomenon is caused by the high secretion of IL-4, an inducer of IgE production.[20] When we checked the IL-4 levels on splenocytes of 6-week sensitization mice using flow cytometry analysis, we could confirm the elevation of the cytokines levels, similar results with those in human. Based on these findings, 6-week *D. farinae* treated mice could be regarded as a suitable AD animal model.

Several approaches have been reported to discover the marker proteins that distinguishing nTregs and iTregs. As we mentioned before, some groups attempted to microarray analysis to compare the gene expression of in vitro–generated iTreg cells with nTregs originated from healthy Foxp3-GFP reporter mice[13,14] and they found out Ikzf2 (helios) and Nrp1 (neuropilin-1) expression were enhanced in nTreg cells compared with iTreg cells. Another microarray was performed with the following pairs of cell populations: fresh $CD25^{+}CD4^{+}$ T cells versus fresh $CD25^{-}$ $CD4^{+}$ T cells, activated $CD25^{+}$ $CD4^{+}$ T cells versus activated $CD25^{-}$ $CD4^{+}$ T cells and retrovirally Foxp3-transduced $CD25^{-}$ $CD4^{+}$ T cells versus mock-transduced $CD25^{-}$ $CD4^{+}$ T cells.[15] As a result of their study, helios could potentially be used as

specific markers for nTregs based on the facts that all immature thymocytes expressed helios and only a small fraction of mature T cells maintain helios expression in the periphery.[15,16,21] Despite significant findings that recently reported, there has been no research to find surface marker proteins, using fresh in-vivo nTregs and iTregs.

Now that iTregs are originated from the periphery in inflammatory conditions for human, we assumed that iTregs will be appeared and do their functions in an atopic condition for NC/Nga mice. We considered most Tregs from thymus of HC mice as nTregs and those from spleen of AD mice as iTregs, although there is a possibility for nTregs to move and circulate into the spleen of AD mice. We could not affirm the whole Tregs are clearly separated into two different subpopulations, However, quantitative differences would be observed between Tregs from thymus of HC mice and spleen of AD mice, since there has been reported $FOXP3^{+}$ regulatory T cells were expanded in PBMC of patients with AD and the number and the percentage of T regulatory type 1 (Tr1) cells, a subpopulation of iTregs, were significantly elevated while the ratio of Tregs to whole T cells had no changes in the peripheral blood of AD parients compared with non-AD controls.[9,22]

With the relatively high purity of $CD4^{+}CD25^{+}$ regulatory cells which confirmed by flow cytometry analysis, we performed the membrane protein identification using TMT-labeling method. Through the identification process, we detected 63 membrane proteins and several proteins on the list showed the quantitative differences in two different groups.

In our study, H-2 class II histocompatibility antigen seemed the most prospecting surface molecules in terms of their high iTregs to nTregs ratio. There are no

evidence that H-2 class II histocompatibility antigen play a role in Tregs, however, Baecher-Allan et al,[23] investigated expression of MHC class II (MHC-II) determinants (DR) on $CD4^{+}CD25^{high}$ Tregs and their functions. They insisted that the DR expressing Treg express higher levels of Foxp3, compared with the DR negative Treg population. In these aspects, H-2 MHC class II might have similar functions with MHC class II DR or might play a role as a candidate marker, distinguishing iTregs from nTregs.

Receptor-type tyrosine-protein phosphatase C and their associated proteins also found on iTreg population. Receptor protein tyrosine phosphatases (RPTPs), as the enzymatic counterparts to tyrosine kinases, serve an important role in regulating tyrosine phosphorylation during activation of B- and T-lymphocytes.[24,25] Although RPTPs are transmembrane proteins, intracellular domains of most of the RPTPs are involved in intracellular tyrosine phosphorylation-dependent signal transduction in response to extracellular ligands, such as growth factors and cytokines.[25-27] CD45 is the first RPTP to be identified based on the sequence homology of its PTP domain[28] and dynamically exist within lipid rafts and the immune synapse during T cell activation. The fine study of RPTPs in Treg cells have not conducted yet, however, there has been a report that raft-localized CD45 antagonizes IL-2 production, whereas raft-excluded CD45 promotes ERK-dependent polarized synaptic lipid raft clustering and IL-2 production.[29] Since IL-2/ STAT5 axis also have been demonstrated in nTregs and iTregs maturation and development,[30-32] we think PRTP and IL-2 might have an influence on nTregs and iTregs.

One of possible marker proteins on the list, CD47 (originally named integrin-associated protein (IAP)) has been known as a cell surface protein of the

immunoglobulin (Ig) superfamily and an important regulator of integrin function. CD47 and its two ligands, thrombospondin 1 (TSP-1) and SIRP-α, are known to serve an inhibitory role for dendritic cells and T cells.[33,34] There has been a study that CD47 expression was transiently regulated on Ag-specific CD4 T cells during the course of the in vivo immune response. The report insisted that $CD47^{high}$ status marked central memory CD4 T cell precursors at an early time point of the IR.[35] Recent study conducted from Van VQ et al, suggested that CD47 expression negatively regulated CD103+Foxp3+Treg proliferation and expansion under steady –state conditions.[36] They observed the augmentation of activated phenotype ($CD44^{high}$) $CD4^{+}CD25^{+}$ T cells and more specifically, that of $CD103^{+}$ Treg cells in CD47-deficient mice.[36] Considering the previous studies about CD47 expression on various T cells, the result of our study that peripheral induced Tregs more expressed CD47 rather than naturally occurring Tregs, possibly elucidated that suppressive function of iTregs on conventional effector T cells and their own population.

Our study is based on the proteomic analysis only, so we have to confirm these results in vitro and in vivo for further studies. We used Tregs from thymus in healthy control (HC) mice and Tregs from spleen in mice with AD in this experiment, but it was difficult to prove whether they are truly pure nTreg and iTreg each. In these aspects, we will confirm the expression of potential marker proteins in thymus and spleen of HC and AD mice.

Ⅴ. CONCLUSION

This study was carried out to discover candidate molecules as iTreg specific marker proteins using proteomic analysis. We isolated the CD4+CD25+ nTregs and iTregs each from thymus of normal mice and spleen of AD-induced mice and confirmed their purity using FACS. These Tregs were analyzed and identified in protein levels and we finally obtained several membrane proteins of iTregs including H-2 class II histocompatibility antigen, receptor-type tyrosine-protein phosphatase C and leukocyte surface antigen CD47.

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ABSTRACT (in Korean)

아토피피부염 마우스 모델에서 induced regulatory T 세포 표면 단백질의 발굴

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이 나 라

CD25+ CD4+ regulatory T 세포(Treg)는 면역 자가 억제의 중요한 조절인자로 알려져 있으며, 아토피피부염 환자에서 증가 되어있는 것으로 보고되어있다. Treg의 두가지 종류인 natural Treg(nTreg)과 adaptive Treg(iTreg)은 최근 활발히 연구되고 있는 주제로, 표면 막 단백질이 알려진 바가 없어 연구에 많은 어려움이 있다. 이러한 관점에서, 우리는 먼저 NC/Nga 마우스에 D.farinae 연고로 감작하여 6주째에 심한 아토피 유사 병변을 관찰하였고, 혈장 IgE의 증가와 Th2 사이토카인인 IL-4의 증가도 확인하였다. nTreg과 iTreg을 추출하기 위하여, 정상 마우스의 흉선과 아토피가 유발된 마우스의 비장에서 각각 Treg을 MACS®을 이용하여 분리였고, 높은 수득율로 분리가 됨을 FACS를 이용하여 확인하였다. 분리된 세포에서 막단백질을 분리하고 TMT 물질로 표시하고 1DLC-MS/MS 분석을 실시하였다. 그 결과 우리는 533개의

단백질을 동정하였고, 그 중 63개가 막 단백질이었으며, 데이터베이스 조사를 통하여 16개의 세포 표면 막 단백질을 찾아 내었다. 특징적으로 H-2 class II histocompatibility antigen은 nTreg보다 iTreg에서 많은 양의 단백질이 발현되는 것으로 밝혀졌으며, 다른 단백질인 receptor-type tyrosine-protein phosphatase C와 leukocyte surface antigen CD47 역시, iTregs에서 많은 양이 발현됨이 확인되었다. 이러한 양적 증가와 그들의 알려진 기능을 고려할 때, 이들이 iTreg과 nTreg을 분리하는 마커 단백질이나, iTreg에서의 기능적인 역할을 담당할 것으로 추측할 수 있었다.

핵심이 되는 말 : 아토피피부염, NC/Nga 마우스, regulatory T cells, nTreg, iTreg