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DOCK8: Regulator of Treg in response to corticotropin-releasing hormone

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Short title: DOCK8: Immune regulator in response to stress

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ABSTRACT

Background: Atopic dermatitis (AD) is exacerbated by psychological factors, such as stress. We previously reported that corticotrophin-releasing hormone (CRH) treatment in AD patients decreased the proportion of IL-10⁺ Tr1 cells, a subset of inducible regulatory T cells (Tregs). However, changes in the function of Tregs in response to CRH have yet to be studied.

Methods: We analyzed the total proteins taken from CRH treated- and untreated-Tregs from AD mice model (NC/Nga mice) by using a quantitative proteomic analysis for the different protein expressions.

Results: We found a statistically decreased protein level of DOCK8 in CRH treated Tregs from AD mice. In human, DOCK8 protein levels were also significantly decreased in CRH treated-Tregs from AD patients. Moreover, the expression of DOCK8 in Tregs was inversely correlated with the anxiety levels in the AD patients. In addition to the clinical correlation of DOCK8 with the stress level of AD patients, the knockdown of DOCK8 in Tregs reduced the inhibitory cytokines, IL-10 and TGF- β , and inhibited the regulatory function of Tregs to suppress the proliferation and TNF- α release of CD4⁺ T cells *in vitro*.

Conclusion: This study provides new insights on the mechanisms of stress induced AD aggravation by showing that CRH downregulated DOCK8 expression in Tregs that not only clinically correlates with anxiety levels of AD patients but also regulates suppressive function of Tregs on CD4⁺ T cells.

Key words: Corticotropin-releasing hormone, atopic dermatitis, regulatory T cell, dedicator of cytokinesis 8

INTRODUCTION

Stress is an important psychological factor that can trigger or exacerbate the symptoms of AD (1-3). AD patients suffering from psychological stress show increased Th2-polarized immune responses, increased IgE levels, and impairment of both the hypothalamic-pituitary-adrenal

(HPA) axis and the skin barrier function (4). In our previous study, values for various psychological parameters were found to be higher in AD patients compared with HCs (5). Supporting the relationship between AD and stress, progressive muscle relaxation therapy leads to significant decreases in the degree of pruritus and amount of lost sleep in AD patients (6). We also found that corticotropin-releasing hormone (CRH), a key regulator of the neuroendocrine response to stress (7-9), decreases IL-18 expression in dendritic cells (10) and decreases the number of IL-10⁺ Tr1 cells in T cells of AD patients (11).

Recently, accumulating data have shown that abnormal regulation of Tregs is an important factor in the pathogenesis of AD (12, 13). Tregs are a T-cell subset constituting 5–10% of peripheral CD4⁺ T cells, and Tregs constitutively express the IL-2 receptor α -chain (CD25) (14). Tregs interact with T effector cells via two distinct pathways: (1) a cell contact-dependent pathway; and (2) a contact-independent pathway mediated via inhibitory cytokines such as IL-10 and TGF- β (15). In allergic responses, Tregs can suppress Th2 cells, Th1 cells, mast cells, eosinophils, and basophils either directly or indirectly and also regulate antibody isotypes produced by B cells through secretion of the inhibitory cytokines IL-10 and TGF- β (16).

Dedicator of cytokinesis 8 (DOCK8) is a member of the DOCK180-related family of atypical guanine nucleotide exchange factors, which are involved in a diverse range of biological processes, including cell migration, polarization, phagocytosis, fusion, and morphogenesis (17-20). Loss-of-function mutations in the DOCK8 gene were found in patients who had been previously diagnosed with autosomal recessive hyper-IgE syndrome or combined immunodeficiency disorders characterized by repeated bacterial and viral infections and severe eczema (21, 22). Recently, it was reported that DOCK8 plays a critical

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role for Treg homeostasis and function and the enforcement of peripheral B-cell tolerance (23).

In this study, we hypothesized that stress-induced modulation of the immune response in AD might be mediated by functional changes in Tregs in response to CRH exposure. To test this hypothesis, we used a quantitative proteomic approach to identify proteins that are differentially regulated by CRH in Tregs. A total of 20 proteins were identified as candidates for further investigation. We then investigated the role of DOCK8, one of the down-regulated proteins, in regulating Treg function.

METHODS

Full details are described in Online Repository Materials.

AD mouse model

Female 6-week-old NC/Nga mice were purchased from SLC Japan (Shizuoka, Japan). The animals were housed in an air-conditioned room maintained at $24 \pm 2^{\circ}\text{C}$ and $55 \pm 15\%$ humidity. Mice were maintained for one week before experiments began.

The hair on the upper back of each mouse was shaved, and then 150 μl of a 4% (w/v) sodium dodecyl sulfate (SDS) (WELGENE, Daegu, Korea) solution was applied to the exposed dorsal skin and surfaces of each ear to disrupt the barrier function of the skin. After 2 h, 100 mg of *Dermatophagoides farinae* body extract (*DfE*) ointment (Biostir-AD, Biostir Inc., Kobe, Japan) was applied topically to the exposed skin areas twice weekly for 6 weeks.

Human samples

Human blood samples were obtained from 4 non-AD healthy controls (HCs) and from 10 AD patients who were diagnosed according to the criteria of Hanifin and Rajka.(24) We divided the patients with AD into 3 groups according to EASI scores: mild, less than 10; moderate, 10 to 20; and severe, greater than 20 (25). We also evaluated psychological stress by using the state-trait anxiety inventory, which comprises 40 questions: 20 questions regarding state anxiety and 20 questions regarding trait anxiety (26) (Supplementary Table E1). All subjects provided written informed consent to participate in the study, and the institutional review board approved this study.

Flow cytometry

Cells were washed with phosphate-buffered saline (PBS) and stained with a fixable viability dye. After washing, cells were labeled at 4°C for 30 min with anti-CD3, -CD4, and -CD25 antibodies conjugated with fluorescent dye (eBioscience, San Diego, CA). For intracellular labeling, cells were fixed and permeabilized with cytofix/cytoperm buffer (eBioscience) and labeled with anti-human DOCK8 antibody (Abcam, Cambridge, MA) and FITC-conjugated anti-rabbit secondary antibody (Abcam). Labeled cells were quantified using a BD FACSVerse flow cytometer, and the data were analyzed using FlowJo Software (BD Bioscience, San Jose, CA). For analysis of DOCK8 expression in Tregs, CD4⁺CD25⁺Foxp3⁺ Tregs were analyzed. The mean fluorescence Intensities (MFI) of the isotype control sample and the anti-DOCK8 stained sample were measured, and used to generate overlapping

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histograms. Δ MFI was calculated by subtracting the MFI of isotype control sample from that of the anti-DOCK8 stained sample.

Proteomic analysis

Protein samples from Tregs of each group were individually labeled using TMT-126 and -129 (control group); TMT-127 and -130 (24 h CRH treatment group), and TMT-128 and -131 (48 h CRH treatment group), following the manufacturer's protocol (Thermo Scientific, Rockford, IL). The six samples were then combined, speed-vacuum dried, and re-dissolved in 50 μ l of water containing 0.1% formic acid for 2D-LC-MS/MS analysis

RNA extraction and qPCR

Total RNA was extracted from Tregs using an RNeasy Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then subjected to reverse transcription. Quantitative PCR was performed at least three times for each sample using 2 μ l of cDNA supplemented with appropriate primers (Applied Biosystems, Foster City, CA). Primers specific for mouse IL-4 (Mm00445259_m1), DOCK8 (Mm00613802_m1), TGF- β 1 (Mm01178820_m1), IL-10 (Mm00439614_m1), and GAPDH (Mm99999915_g1) were mixed with cDNA and qPCR Master Mix in a 20- μ l reaction and then subjected to PCR using a StepOnePlus PCR system (Applied Biosystems).

Carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assay

Freshly collected Tregs were washed two times with PBS and then re-suspended at a density of 5×10^6 /ml in PBS. CFSE was added to the cells at final concentration of 1 μ M, and cells were then incubated for 10 min at room temperature in the dark. After incubation, the cells were washed with cold complete medium, incubated on ice for 5 min, and then washed three times with complete medium. Cell proliferation was measured using a BD FACSVerser flow cytometer, and the data were analyzed using FlowJo software (BD Bioscience).

Cytokine bead array

Cytokines in cell culture supernatants were measured using a BD CBA mouse Th1/Th2/Th17 cytokine kit (BD Bioscience) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were conducted using Graph Pad Prism, version 4.03 (GraphPad Software, Inc., San Diego, CA). The significance of differences between two groups was determined using a two-tailed Student's *t* test or Wilcoxon signed ranks test. When comparisons were multiple, one-way ANOVA with a Bonferroni test was used. A *P*-value < 0.05 was considered statistically significant. Significance is indicated in the figures as follows: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

RESULTS

Expression of CRHR in Tregs

The purity of isolated CD4⁺CD25⁺ Tregs was 80%, and over 90% of the CD4⁺CD25⁺ Tregs expressed Foxp3 (Fig. 1A). After sorting the Tregs, the expression of CRHRs was analyzed. The histograms shown in Fig. 1B demonstrate strong CRHR1 and CRHR2 expression on mouse spleen Tregs (Fig. 1B).

Tregs in AD mouse model and CRH-treated splenocytes

D. farinae induced AD mice showed AD like skin lesions, increased serum IgE levels, increased IL-4 expression in T cells (Supplementary Fig. E1A-E). The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs was increased in AD mice (12.00±0.20% in mice and 19.37±1.99% in AD mice) (Supplementary Fig. E1F). However, treatment of CRH did not alter the percentage of Tregs (Fig. 1C, 8.27±0.34% in the untreated group and 9.23±0.85% in the CRH-treated group).

Quantitative proteomic analysis using a TMT-labeling method

Proteins extracted from untreated CD4⁺CD25⁺ Tregs and those treated with CRH for 24 or 48 h were individually labeled with TMTs for quantitative proteomic analysis. Initially, we identified 1353 proteins in the CRH-treated and untreated Tregs. We then attempted to identify proteins that either increased or decreased in expression level over time after CRH stimulation. Proteins for which the log2 ratio was statistically significant ($P < 0.05$; ratio $\leq$

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-0.4 or ≥ 0.4) were considered to be differentially expressed. A total of 20 proteins were identified as candidates for further investigation. Among these proteins, 5 were upregulated (Table 1) and 15 were downregulated (Table 2). Of the downregulated proteins, we focused particularly on DOCK8 for further characterization because of its immunologic role and its association with AD in previous studies (21-23).

Expression of DOCK8 in CRH-treated Tregs

Splenocytes from AD mice were cultured with anti-CD3 and anti-CD28 antibodies for 48 h and treated with 10 nM CRH for 24 or 48 h. We found that the expression of DOCK8 was decreased in Tregs isolated from CRH-treated AD splenocytes in a time-dependent manner (32% reduction in cells treated for 24 hours and 46% reduction in cells treated for 48 hours) (Fig. 2A).

We next examined the expression of DOCK8 in human cells in response to CRH. PBMCs from 4 HCs and 5 AD patients were treated with CRH for 48 h. We determined the Δ MFI of DOCK8 between CRH-treated and untreated $CD4^+CD25^+Foxp3^+$ Tregs. CRH had no effect on DOCK8 expression in Tregs isolated from HCs (Δ MFI = 615.25 ± 183.52 in the untreated group and 676.25 ± 135.13 in the CRH-treated group). However, CRH treatment resulted in a decrease in DOCK8 expression in Tregs isolated from AD patients (Δ MFI = 526.80 ± 80.37 in the untreated group and 380.80 ± 82.05 in the CRH-treated group) (Fig. 2B).

Correlation of DOCK8 in Tregs with anxiety levels in AD patients

As CRH decreased DOCK8 expression in Tregs isolated from AD patients, we next questioned whether the expression of DOCK8 in AD patients was correlated with anxiety levels. First, we analyzed correlation between the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs and AD severity and we found statistically significant correlation between the percentage of Tregs and AD severity ($2.35 \pm 1.11\%$ in mild AD, $4.20 \pm 0.37\%$ in moderate AD, $4.39 \pm 0.70\%$ in severe AD, Spearman correlation $r=0.515$) (Fig. 3A). Severe AD patients showed increased percentage Tregs. Next, we analyzed the correlation of percentage of Tregs and the expression level of DOCK8 with the anxiety level. The level of anxiety was not correlated with the percentage of Tregs (Spearman correlation $r=0.000$ for state anxiety, $r=0.000$ for trait anxiety) (Fig. 3B). However, the expression of DOCK8 in Tregs showed negative correlation with state anxiety and trait anxiety in AD patients (Spearman correlation $r=0.402$ for state anxiety, $r=0.421$ for trait anxiety) (Fig. 3C). Higher the level of anxiety showed significantly lower expression of DOCK8.

Down-regulation of inhibitory cytokines in Tregs after DOCK8 knockdown

Tregs suppress effector T cells through secretion of inhibitory cytokines such as IL-10 and TGF- β . Therefore, we monitored the levels of these cytokines in mouse CD4⁺CD25⁺ Tregs after DOCK8 knockdown using RNA interference. The transfection rate was 60% in both qPCR analysis and immunofluorescence staining (Fig. E2 A and B).

Interestingly, knockdown of DOCK8 in CD4⁺CD25⁺ Tregs caused decreases in the expression of both IL-10 and TGF- β (Fig. 4A). Secreted IL-10 and TGF- β were also

decreased in the supernatant of siRNA-transfected Tregs (Fig. 4B). This result suggests that knockdown of DOCK8 suppress the expression of inhibitory cytokines.

Abrogation of immunosuppressive function on CD4⁺ T cells by DOCK8 knockdown in Tregs

To determine whether DOCK8 in Tregs is involved in the immunosuppressive activities on CD4⁺ T cell proliferation and cytokine secretion, we used a coculture system of CD4⁺ T cells and CD4⁺CD25⁺ Tregs. The proliferation of CD4⁺ T cells cocultured with untransfected CD4⁺CD25⁺ Tregs was suppressed ($39.50 \pm 6.24\%$ proliferation in CD4⁺ T cells cultured alone; $16.45 \pm 6.49\%$ in CD4⁺ T cells cocultured with untransfected Tregs). However, knockdown of DOCK8 in CD4⁺CD25⁺ Tregs restored the proliferative capacity of the CD4⁺ T cells ($21.78 \pm 6.70\%$ in CD4⁺ T cells cocultured with SC siRNA-transfected Tregs and $34.85 \pm 5.24\%$ in CD4⁺ T cells cocultured with DOCK8 siRNA-transfected Tregs) (Fig. 4C and D).

Cytokine release into the supernatants of CD4⁺ T cells stimulated with anti-CD3/-CD28 antibodies on day 3 was determined to analyze whether DOCK8 knockdown in CD4⁺CD25⁺ Tregs inhibits cytokine release from CD4⁺ T cells. Coculture of CD4⁺CD25⁺ Tregs with CD4⁺ T cells inhibited TNF- α secretion and blockade of DOCK8 expression in CD4⁺CD25⁺ Tregs reversed the inhibitory effect of Tregs. There were no changes in IL-2 production after DOCK8 knockdown (Fig. 4E). This result suggests that inhibition of DOCK8 expression in Tregs not only restores the proliferative capacity of CD4⁺ T cells but also abrogates the inhibitory function on CD4⁺ T cells cytokine release.

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DISCUSSION

As imbalance in the Th1/Th2 immune responses is an important factor in the development of AD, treatment of this condition necessitates restoring the Th1/Th2 balance (27). Tregs are a subset of CD4⁺ T cells that play an important role in the Th1/Th2 balance. The cytokines expressed by Tregs control allergic diseases by suppressing Th2-type immune responses (28). As for the function of Tregs in AD, it has been reported that their immunosuppressive activity is similar to that of Tregs in HCs (29). However, staphylococcal enterotoxin B, which is an aggravating factor in AD, reverses the immunosuppressive function of Tregs (29). Reffer et al.(30) recently demonstrated that Th2-promoting Tregs are present in AD and that their function depends on the expression of skin-homing receptor CCR6. However, the role of Tregs in stressful condition in AD has yet to be investigated. Therefore, identifying and characterizing proteins that are differentially expressed in Tregs of AD patients affected by stress hormones will enhance our understanding of this disease and aid in the development of novel therapeutic approaches to alleviate stress-induced aggravation of AD symptoms.

The TMT isobaric chemical labeling method is often used for MS-based quantification and identification of biological macromolecules such as proteins, peptides, and nucleic acids and is known for its high accuracy and reproducibility (31, 32). Here, we used the TMT labeling method to identify proteins that are differentially expressed in Tregs isolated from CRH-treated and untreated splenocytes of AD mice. We found that the expression of 5 of the 1353 initially identified proteins was upregulated and the expression of 15 of the proteins was downregulated after stimulation with CRH. As previous reports indicated that one of the downregulated proteins we identified, DOCK8, not only regulates immunologic functions but is also involved in AD (21, 22), we then investigated the relationship between DOCK8 and stress-induced aggravation of AD in more detail.

Our present study is the first to report that DOCK8 expression in Tregs is down-regulated by CRH. DOCK8 expression in Tregs was inversely correlated with anxiety levels in AD patients, while the percentage of Tregs was not correlated with anxiety levels. Knockdown of DOCK8 in Tregs using siRNA transfection resulted in decreased production of the key inhibitory cytokines IL-10 and TGF- β . IL-10 is known to suppress allergen-specific effector T cells and block the B7/CD28, ICOS, and CD2 costimulatory pathways in T cells (33-35). TGF- β inhibits the proliferation, differentiation, and effector functions of T cells and also stimulates Foxp3 expression and peripheral generation of Tregs (36). Therefore, down-regulation of these inhibitory cytokines might result in enhanced effector T cell proliferation, activation, and function. Supporting these data, we found that decreased expression of DOCK8 in Tregs abrogated their immunosuppressive capacity on T cell proliferation and TNF- α production. Increased expression of TNF- α might contribute to the aggravation of inflammatory responses in AD under conditions of stress. Based on these results, we suggest that down-regulated DOCK8 by CRH may reduce the production of inhibitory cytokines and resulted in reduced suppressive activity on effector T cells.

DOCK8 is highly expressed by components of the immune system, especially lymphocytes, suggesting that the protein has a crucial function in these immune cells. In DOCK8-deficient patients, severe AD develops from infancy (21, 22) and the sensitization pattern is weighted to food allergens than aeroallergens (37). DOCK8-deficient mice exhibit T cell lymphopenia and decreased survival rate of peripheral T cells and a loss of memory T cells, especially CD8⁺ T cells (38, 39). With respect to the Th2 polarization that is characteristic of DOCK8-deficiency, the proportion of IL-4-secreting T cells is higher in DOCK8-deficient mice than wild-type mice under Th2 differentiation conditions (39). In addition, T cells from DOCK8-deficient patients show impaired Th17 differentiation (40).

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Apart from T cells, DOCK8 has also been investigated in other immune cells, such as NK cells, DCs, and B cells. DOCK8 mutations in humans affect NK cell-mediated cytotoxicity (41, 42) and DOCK8-deficient mice show impaired migration of DCs to the lymph nodes (43). Furthermore, DOCK8 mutations in mice affect immune synapse formation in B cells and long-lasting humoral immunity (18, 20).

In conclusion, our TMT labeling quantitative proteomic analysis found that the expression of DOCK8 is down-regulated in CRH-treated AD-affected Tregs. Based on this finding, we then evaluated the functional role of DOCK8 using siRNA-treated Tregs. Our results suggest that decreased expression of DOCK8 in response to CRH might disturb the immunosuppressive function of Tregs and contribute to stress-induced aggravation of AD symptoms.

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FIGURE LEGEND

Figure 1. Effect of CRH on Treg population

(A) Purity of CD4⁺CD25⁺ Tregs after MACS sorting. The purity of isolated Tregs was 80%, and over 90% of the Tregs expressed Foxp3. (B) Flow cytometry showed that Tregs isolated from spleens of AD mice expressed both CRHR1 and CRHR2. Red lines indicate isotype controls and blue lines indicate CRHR1 and CRHR2. (C) Mouse splenocytes were treated with 10 nM CRH for 48 h. Treatment with CRH did not change the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the spleen of AD mice ($p > 0.05$).

Figure 2. Downregulation of DOCK8 expression in CRH-treated AD Tregs

(A) Splenocytes of AD mice were cultured with anti-CD3/-CD28 antibodies for 48 h and treated with 10 nM CRH for 24 or 48 h, and then CD4⁺CD25⁺ Tregs were isolated from splenocytes. DOCK8 expression on Tregs was evaluated by Western blotting. DOCK8 expression was decreased in 24 h and 48 h CRH treated Tregs than non-treated control Tregs. Relative DOCK8 expression was normalized to that of β -actin (***) ($p < 0.001$). (B) PBMCs from HCs and AD patients were isolated from whole

blood and treated with anti-CD3/-CD28 antibodies and 10 nM CRH for 48 h. In flow cytometry, the Δ MFI of DOCK8 was determined in CD4⁺CD25⁺Foxp3⁺ Tregs. Grey lines indicate isotype controls, blue lines indicate DOCK8 in the untreated group, and red lines indicate DOCK8 in the CRH-treated group. DOCK8 expression was down-regulated in Tregs isolated from CRH-treated PBMCs in AD patients; however, CRH had no effect on DOCK8 expression in Tregs of HCs (* p < 0.05).

Figure 3. Correlation of DOCK8 in Tregs with anxiety levels in AD patients

(A) The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs was positively correlated with AD severity (Spearman correlation $r = 0.515$). (B) Both state anxiety and trait anxiety were not correlated with the number of Tregs. (C) DOCK8 expression in Tregs was correlated with state anxiety (Spearman correlation $r = 0.402$) and trait anxiety (Spearman correlation $r = 0.421$) ($n=10$, * p < 0.05).

Figure 4. Decreased suppressive activity of Tregs after DOCK8 knockdown.

(A) Expression of IL-10 and TGF- β mRNAs was examined using qPCR in mouse Tregs, and relative expression was normalized to that of GAPDH mRNA. Expression of the inhibitory cytokines IL-10 and TGF- β was lower in Tregs transfected with DOCK8 siRNA compared with those transfected with SC siRNA (* p < 0.05). (B) Secretion of IL-10 and TGF- β in the supernatant was analyzed using ELISA. Both IL-10 and TGF- β release were significantly decreased with DOCK8-siRNA transfection (** p <

0.01). (C, D) CFSE-labeled CD4⁺ T cells were cultured independently or cocultured with Tregs, SC siRNA-transfected Tregs, or DOCK8 siRNA-transfected Tregs at a ratio of 2:1. Proliferation of CD4⁺ T cells was detected on day 3 after stimulation. Inhibition of DOCK8 expression in Tregs restored the proliferative capacity of CD4⁺ T cells (**p* < 0.05, ***p* < 0.01). (E) Culture medium of each group was collected and TNF- α and IL-2 were detected using a CBA kit. DOCK8 knockdown in Tregs increased secretion of TNF- α (**p* < 0.05, ***p* < 0.01). For each group, mice *n* = 5.

Table 1. Upregulated proteins identified using the TMT labeling method.

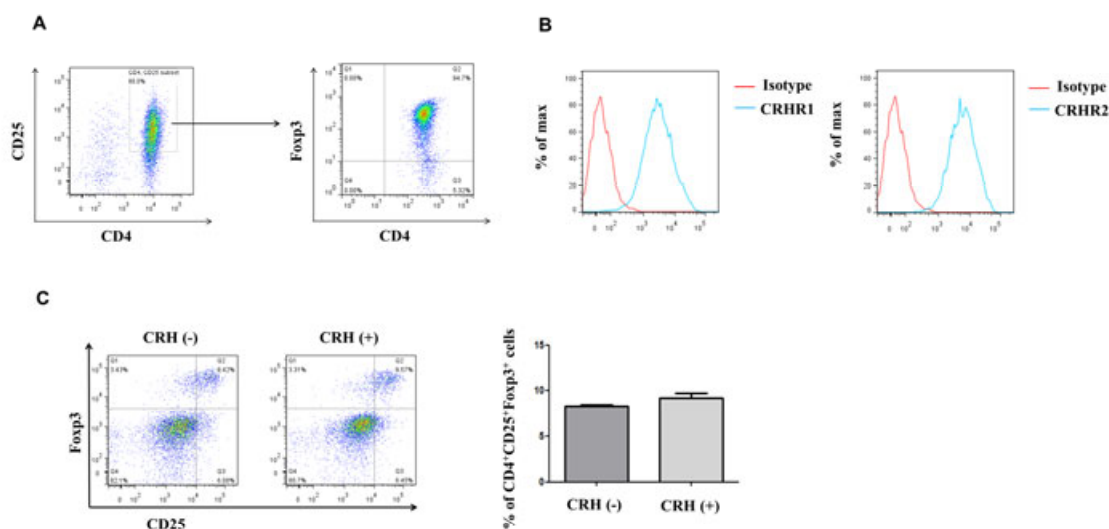
NO.	IPI NO.	DESCRIPTION	ratio log2	ratio log2
			(CRH24:Cont)	(CRH48:Cont)
1	IPI00109044.8	2900073G15Rik myosin light chain, regulatory B-like	0.5635	0.6461
2	IPI00132705.1	Myl12b Myosin regulatory light chain 12B	0.5738	0.6546
3	IPI00403540.4	Slc9a7 Isoform 2 of Sodium/hydrogen exchanger 7	0.5580	0.5971
4	IPI00750595.2	Myl9 Myosin regulatory light polypeptide 9	0.4514	0.5334
5	IPI00677454.2	Cbx3;Gm10068 Chromobox homolog 3	0.4660	0.5293

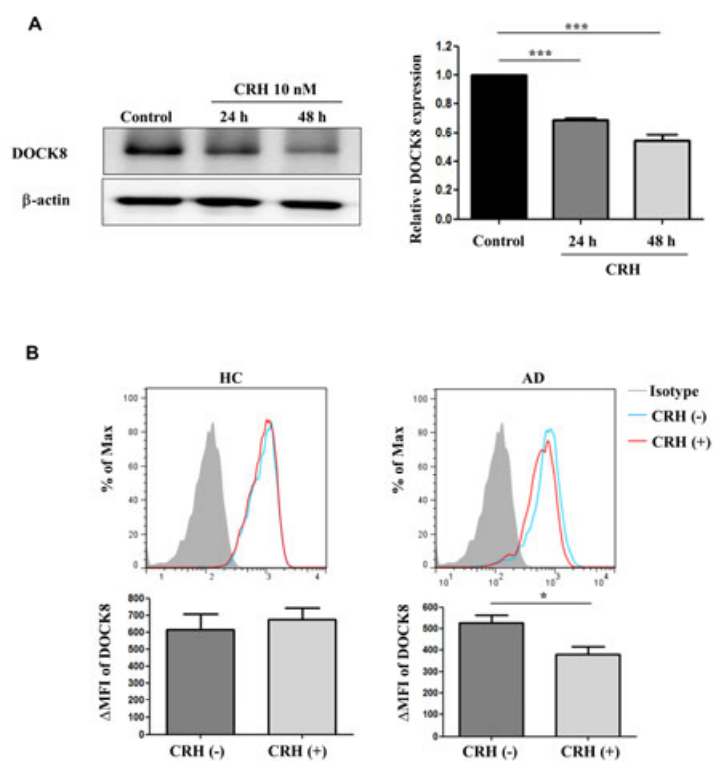
Table 2. Downregulated proteins identified using the TMT labeling method.

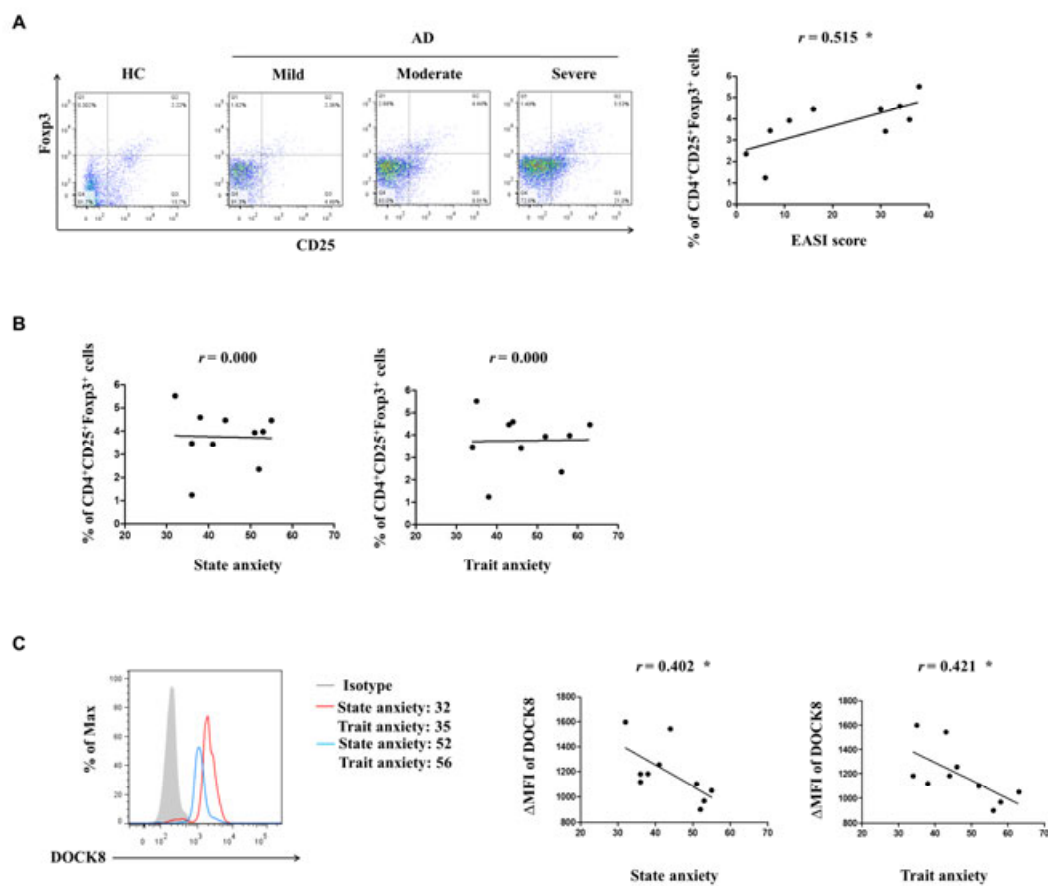
NO.	IPI NO.	DESCRIPTION	ratio log2	ratio log2
			(CRH24:Cont)	(CRH48:Cont)
1	IPI00465568.5	Rps24;LOC677113 40S ribosomal protein S24	-0.6743	-0.7587
2	IPI00321170.9	Rpl3 60S ribosomal protein L3	-0.6487	-0.6525
3	IPI00881253.3	Rpl5 60S ribosomal protein L5-like isoform 3	-0.6097	-0.6812
4	IPI01027593.1	H2-Eb1 I-E beta MHC class II protein (Fragment)	-0.5633	-0.6608
5	IPI00353563.4	Fscn1 Fascin	-0.5618	-0.7275
6	IPI00172243.1	H2-Ab1 H-2 class II histocompatibility antigen, A-K beta chain	-0.5224	-0.5576
7	IPI00331361.2	Mybbp1a Myb-binding protein 1A	-0.5153	-0.6038
8	IPI01027719.1	Rps6 40S ribosomal protein S6	-0.5108	-0.6600
9	IPI00555113.2	Rpl18 60S ribosomal protein L18	-0.4776	-0.4812
10	IPI01019147.1	H2-Ab1 MHC class II beta chain A-beta-7 (Fragment)	-0.4593	-0.5020
11	IPI00114493.11	H2-Ea-ps H-2 class II histocompatibility antigen, E-K alpha chain	-0.4358	-0.5689
12	IPI00626312.1	Gm6136 60S ribosomal protein L6-like	-0.4358	-0.5401
13	IPI00330363.8	Rpl7a 60S ribosomal protein L7a	-0.4358	-0.4893
14	IPI00122223.1	Top2a DNA topoisomerase 2-alpha	-0.4358	-0.4364
15	IPI00475383.2	Dedicator of cytokinesis protein 8	-0.4358	-0.5886

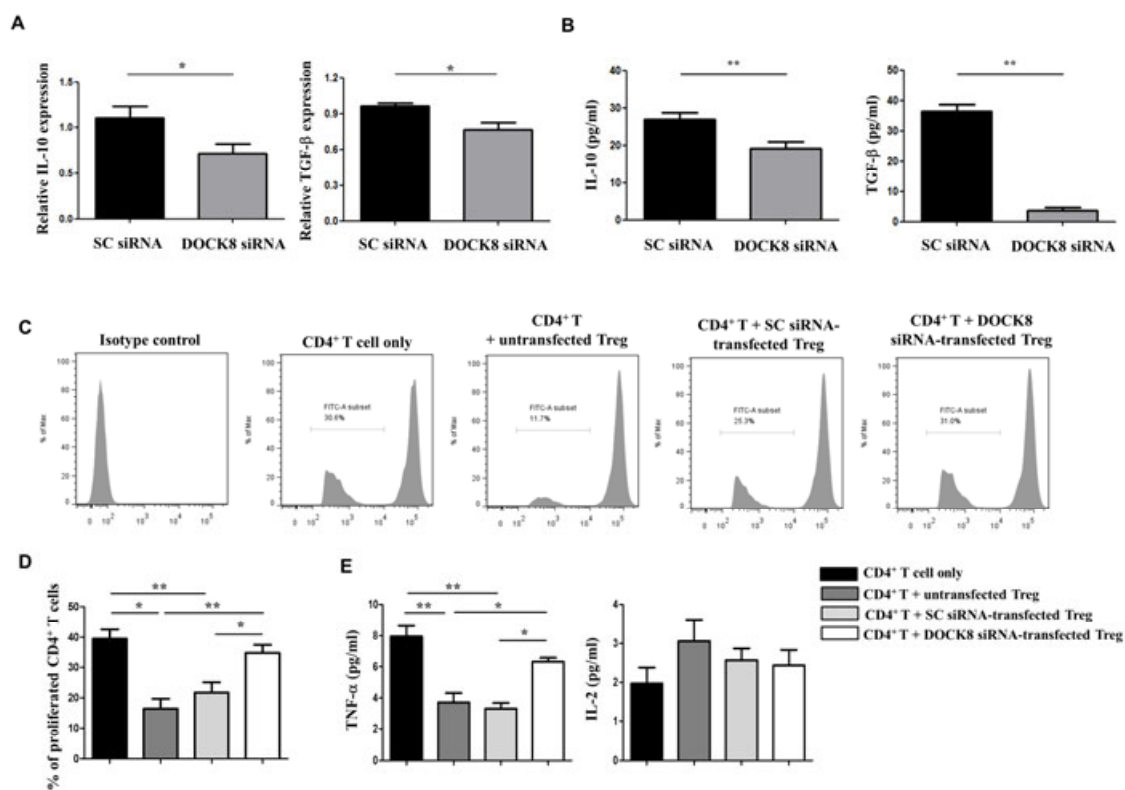
Author contributions

Shan Jin and Jung U Shin designed and performed experiments, analyzed the data, and drafted the manuscript. Chang Ook Park and Ji Yeon Noh designed experiments, interpreted data, and assisted in drafting the manuscript. Jin Young Kim performed proteomic analyses. Seo Hyeong Kim and Nara Lee performed the in vivo study and analyzed the data. Hyeran Kim and Ji Hye Kim supported the in vitro experiments. Hemin Lee and Jung Soo Lee performed the in vitro study and analyzed the data. Kwang Hoon Lee conceived the project, designed experiments, interpreted data, and assisted in drafting and editing the manuscript. All authors read and approved the final version of the manuscript.









Supplementary table

Supplementary Table E1. Characteristics of the study subjects.

	Mild AD	Moderate AD	Severe AD
No. of patients (M/F)	3 (1/2)	2 (1/1)	5 (3/2)
Age (y)	27.0 ± 4.0	27.5 ± 9.2	31.0 ± 10.9
Total IgE (kU/L)	1120.1 ±1358.4	1752.4 ±1874.0	2852.2 ±1947.6
EASI score	5.0 ± 2.6	13.5 ± 3.5	33.8 ± 3.3
State anxiety	41.3 ± 9.2	53.0 ± 2.8	41.3 ± 7.8
Trait anxiety	42.7 ± 11.7	57.5 ± 7.8	45.2 ± 8.3

*Age, total IgE level, EASI score, state anxiety and trait anxiety are presented as means ± SDs.

Supplementary Figure legends

Figure E1. *Dermatophagoides farinae* body extract (DfE) induces AD-like skin inflammation in NC/Nga mice. (A) Photographs of DfE-treated NC/Nga mice showing AD-like skin lesions, including hemorrhage, dryness, and excoriation on the skin of the back after 6 weeks of application. (B) The level of severity before and after induction was scored by the dermatitis area ($***p < 0.001$). (C) Histologic analysis showed hypertrophy and hyperkeratosis in the epidermis and infiltration of inflammatory cells in the dermis after induction. (D) The serum total IgE level was increased after induction of AD-like skin lesions ($***p < 0.001$). (E) mRNA level of IL-4 was increased in the CD4⁺ T cells of AD mice compared with normal mice ($*p < 0.05$). (F) AD mice showed an increased percentage of CD4⁺CD25⁺ Foxp3⁺ Tregs ($**p < 0.01$).

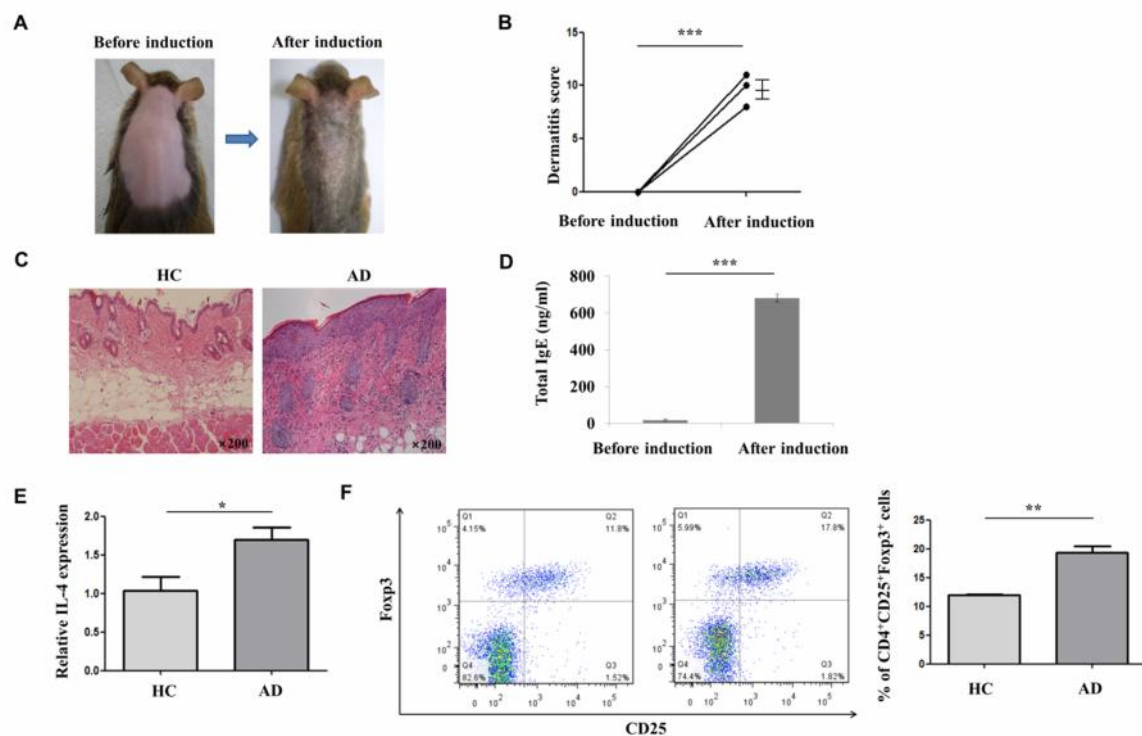
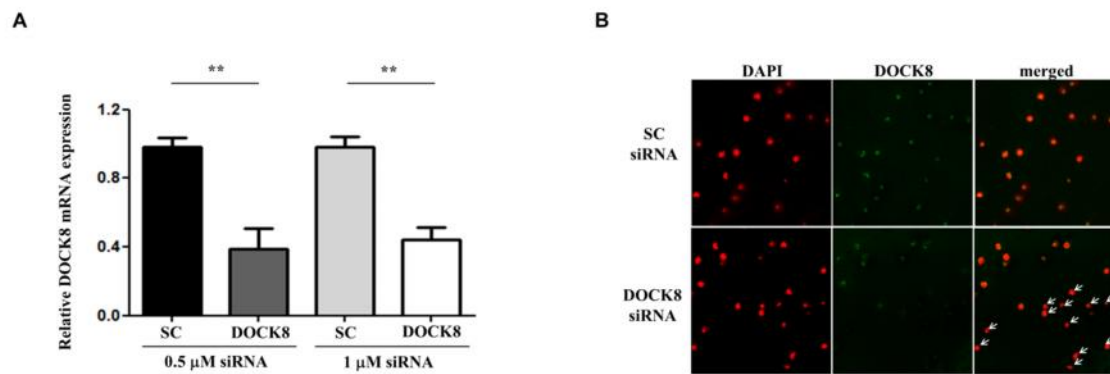


Figure E2. Downregulation of DOCK8 expression in Tregs treated with DOCK8 siRNA.

(A) Compared with the SC siRNA-treated group, DOCK8 mRNA expression was downregulated in Tregs treated with 0.5 or 1 μ M DOCK8 siRNA. The transfection efficiency was up to 60% (** $p < 0.01$). (B) Upper panels show Tregs transfected with 0.5 μ M SC siRNA, whereas lower panels show Tregs transfected with 0.5 μ M DOCK8 siRNA. Arrows indicate Tregs not expressing DOCK8 protein. The transfection efficiency was up to 60%. Nuclei labeled with DAPI appear red and DOCK8 labeled with Alexa fluor 633 appears green.



Supplementary Materials and Methods

Preparation of splenocytes

The spleen was removed from each mouse and disrupted using a syringe plunger to prepare a cell suspension. Blood cells associated with splenocytes were lysed using blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Cells were activated with 1 µg/ml of anti-CD3 and anti-CD28 (eBioscience) antibodies and treated with 10 nM recombinant CRH (Sigma-Aldrich) for 24 or 48 h.

Isolation of CD4⁺CD25⁺ Tregs using MACS[®]

Murine CD4⁺CD25⁺ T cells were sorted from splenocytes using a CD4⁺CD25⁺ Regulatory T cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The cells obtained were about 80% pure, as determined by FACS analysis.

Isolation of CD4⁺CD25⁺ Tregs using FACS Aria[™]

Cells were labeled with FITC or APC-conjugated CD4 and PE-conjugated CD25 and sorted using a FACS Aria[™] cell sorter (BD Bioscience). Treg purity was greater than 95% in all cases.

Human samples

Human blood samples were obtained from 5 non-AD healthy controls (HCs) and from 15 AD patients who were diagnosed according to the criteria of Hanifin and Rajka (1). We divided the patients with AD into 3 groups according to EASI scores: mild, less than 10; moderate, 10 to 20; and severe, greater than 20 (2). We also evaluated psychological stress by

using the state-trait anxiety inventory, which comprises 40 questions: 20 questions regarding state anxiety and 20 questions regarding trait anxiety (3). The patients did not receive any systemic or topical treatment with immunosuppressive drugs for at least 4 weeks before blood sample collection. This study was approved by the Institutional Review Board of Yonsei University Health System, Severance Hospital (No.2014-0952-001) and informed consent was obtained from all subjects before they participated in the study.

Proteome sample preparation

Mixtures of protein from each cell population were reduced with 500 mM Tris (2-carboxyethyl) phosphine at room temperature for 60 min and then alkylated with 500 mM IAA at room temperature in the dark for 60 min. The protein samples were then desalted using membrane filters (10-kDa molecular weight cutoff) and dissolved in 200 mM triethylammonium bicarbonate (TEAB) buffer to a final concentration of 1 µg/µl. Protein concentration was measured using a bicinchoninic acid assay (Thermo Scientific), following the manufacturer's protocol. Sequencing-grade trypsin (Promega, Madison, WI, USA) was added at an enzyme to protein ratio of 1:20 (wt/wt) in TEAB buffer and incubated overnight at 37°C. Samples were individually labeled using TMT-126 and -129 (control group); TMT-127 and -130 (24-h CRH treatment group), and TMT-128 and -131 (48-h CRH treatment group), following the manufacturer's protocol (Thermo Scientific). Aqueous hydroxylamine solution (5% w/v) was added to quench the reaction. The six samples were then combined, speed-vacuum dried, and re-dissolved in 50 µl of water containing 0.1% formic acid for 2D-LC-MS/MS analysis.

2D-LC-MS/MS

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

An 11-step salt gradient was performed using 3 μl each of 0, 25, 50, 100, 250, and 500 mM ammonium acetate (in 0.1% formic acid/5% acetonitrile [ACN]) and 4, 5, 9, and an additional 9 μl of 500 mM ACN, (0.1% formic acid/30% ACN). Mobile phases A and B consisted of 0 and 100% ACN, respectively, each containing 0.1% formic acid. The LC gradient began with 5% B for 1 min and was ramped to 20% B over 5 min, to 55% B over 90 min, to 95% B over 1 min, remaining at 95% B for 13 min and then returning to 5% B over 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. The electrospray voltage was set to 2.0 kV. During the chromatographic separation, the LTQ Orbitrap Elite was operated in data-dependent mode. MS data were acquired using the following parameters: five data-dependent collision-induced dissociation–high-energy collision dissociation (CID-HCD) dual MS/MS scans per full scan; CID scans were acquired in the linear trap quadrupole (LTQ) with two-microscan averaging; full scans and HCD scans were acquired in the Orbitrap at resolution 60,000 and 15,000 respectively, with two-microscan averaging; 35% normalized collision energy (NCE) in CID and 45% NCE in

HCD; and ± 1 -Da isolation window. Previously fragmented ions were excluded for 60 s. In CID-HCD dual scans, each selected parent ion was fragmented first by CID and then by HCD.

Protein identification and quantification

MS/MS spectra were analyzed using the following software analysis protocols with searching against the IPI mouse database (IPI.MOUSE. 7.26.2012). Reversed sequences of all proteins were appended into the database for calculation of false-discovery rates. ProLucid (4) software was used to identify the peptides using precursor and fragment ion mass errors of 25 and 600 ppm, respectively. Trypsin was selected as the enzyme, with three potential missed cleavages. TMT modification (+ 229.1629) at the N-terminus, addition of a lysine residue by the labeling reagent, and carbamidomethylation of cysteine were chosen as static modifications. Oxidation of methionine was chosen as a variable modification. CID and HCD tandem MS spectra from the same precursor ion are often combined by the software to allow for better peptide identification and quantification (5). We used software developed in-house in which reporter ions from an HCD spectrum were inserted into the CID spectrum with the same precursor ion as the previous scan. Reporter ions were extracted from small windows (± 20 ppm) around their expected m/z value in the HCD spectrum. Output data files were filtered and sorted to compose the protein list using DTASelect(6) (The Scripps Research Institute, La Jolla, CA, USA), with ≥ 2 peptide assignments required for protein identification and a false-positive rate of < 0.01 .

A quantitative analysis was conducted using Census in the IP2 pipeline (Integrated Proteomics, San Diego, CA, USA). The intensity at a reporter ion channel for a protein was calculated as the average of the reporter ion's intensities from all constituent peptides derived from the identified protein (7). The measured intensity ratios of proteins were

transformed to the log₂ scale. Ratios were averaged and proteins showing differences with $P < 0.05$ were defined as differentially regulated.

Western blotting

Whole-cell protein extracts were prepared by lysing mouse Tregs using Pro-Prep lysis buffer (Intron, Seoul, Korea). The concentration of cellular protein was determined using copper (II) sulfate solution in the bicinchoninic acid assay (Sigma-Aldrich). Equal amount of cellular proteins mixed with 5× sample buffer were heated at 100°C for 5 min and then the proteins were resolved on an 8% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto an ECL nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using Tris buffer (0.025 M Tris-HCl, 0.192 M glycine, and 20% MeOH). The membrane was blocked for 1 h at room temperature with 5% skim milk in TBS-Tween 20, incubated overnight at 4°C with anti-mouse DOCK8 antibody (1:500; Santa Cruz Biotechnology) and anti-β-actin antibody (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Finally, the membrane was developed using enhanced chemiluminescence Western blotting detection reagents (Santa Cruz Biotechnology) according to the manufacturer's protocol.

Nucleofection

Nucleofection was performed according to Amaxa's optimized protocol for T-cell nucleofection using a primary T cell Nucleofection kit (Lonza, Cologne, Germany). Cells were suspended in P3 nucleofector solution and supplement. After suspension, cells were nucleofected using a 4D-nucleofector system (Lonza) and re-suspended in pre-warmed medium.

Immunofluorescence staining

Cytospin preparations of Tregs treated with SC siRNA and DOCK8 siRNA were air-dried and stored at -20°C until labeling. For labeling, slides were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing and permeabilization with 0.2% Triton X-100, cytopins were labeled with 5 $\mu\text{g/ml}$ of anti-mouse DOCK8 antibody for 1 h, washed in PBS, and then DOCK8 protein was detected by incubating the cytopins with 2 $\mu\text{g/ml}$ of anti-rabbit Alexa fluor 633 (Invitrogen, Camarillo, CA, USA) for an additional 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) for 5 min and then mounted with mounting medium. Slides were observed and imaged using an LSM 700 microscope (Carl Zeiss, Jena, Germany).

Cytokine bead array

Cytokines in cell culture supernatants were measured using a BD CBA mouse Th1/Th2/Th17 cytokine kit (BD Bioscience) according to the manufacturer's instructions. This array kit provides a mixture of seven capture beads of distinct fluorescence intensities coated with capture antibodies specific for each cytokine of interest. The operations were performed according to the manufacturer's instructions. The seven different bead types were mixed, and then 50 μl of the mixed captured beads, 50 μl of unknown sample or standard dilution, and 50 μl of PE detection reagent were added consecutively to each assay tube and incubated for 2 h at room temperature in the dark. The beads were then washed with 1 ml of wash buffer and centrifuged. The resulting bead pellet was resuspended in 300 μl of buffer after discarding the supernatant. The beads were then sorted by fluorescence on the BD FACSVerse flow cytometer, and the data were analyzed using FCAP ArrayTM software (BD Bioscience).

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants from scrambled siRNA-treated group and DOCK8 siRNA-treated group

were used to analyze IL-10 and TGF- β levels by using ELISA kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instruction. Serum samples from HC mice and AD mice were used to analyze IgE levels by using ELISA kit (Biolegend) according to the manufacturer's instruction.

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