A Doctoral Dissertation

# The effect and action mechanism of docosahexaenoic acid (DHA) in a mouse model of atopic dermatitis

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February, 2016

# 마우스 아토피피부염에 대한 docosahexaenoic acid (DHA)의 효과 및 작용기전

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# The effect and action mechanism of docosahexaenoic acid (DHA) in a mouse model of atopic dermatitis

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# ABSTRACT

Regulatory T cell (Treg) plays important role in the immune mechanism by suppressing the differentiation and proliferation of various immune cells. The anti-inflammatory effects of docosahexaenoic acid have been described for many diseases; however, the mechanism by which it modulates immune system is poorly understood. Therefore, the object of this research was to examine whether docosahexaenoic acid suppresses allergic reactions and up-regulates the generation of $CD4^+Foxp3^+$ T cells. We also examined the effects of transfusing IL-10/TGF-β–modified macrophages (M2 macrophages) treated with docosahexaenoic acid into a mouse model of atopic dermatitis. Here, we show that administration of docosahexaenoic acid up-regulates the generation of TGF-β-dependent $CD4^+Foxp3^+$ Tregs. Docosahexaenoic acid induced T cell hypo-responsiveness and down-regulated cytokines associated with T helper (Th)-1, Th2, and Th17 cells. The differentiation of $Foxp3^+$ Tregs into $CD4^+$ T cells was directly mediated by docosahexaenoic acid-M2 macrophages, which deactivated effector macrophages, and reduced $CD4^+$T cell differentiation and proliferation. Docosahexaenoic acid showed therapeutic effects in mice with experimental atopic dermatitis. These results show that docosahexaenoic acid enhances the function of M2 macrophages, and that the generation of Tregs effectively protects mice against an inflammatory immune disorder. Thus,

docosahexaenoic acid may be a useful therapeutic strategy for treating chronic inflammatory diseases.

**Key words**: docosahexaenoic acid; regulatory T cell; Macrophage; transforming growth factor-β; atopic dermatitis

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# 1. Introduction

Atopic dermatitis (AD), a type of chronic inflammatory skin disease, is linked to cutaneous hyper-reactivity, including edema and itching, which affects approximately 10-20% of children worldwide (Leung *et al*., 2004). This systemic disorder is caused by T cell hyper-proliferation, skin barrier dysfunction, severe skin dehydration, and mutations in the filaggrin gene, which plays a vital role in modulating epidermal homeostasis. The skin lesions in AD patients are characterized by the proliferation and infiltration of inflammatory cells (Li *et al*., 2010; Wahlgren, 1999; Jakasa *et al*., 2011).

Regulatory T cell (Treg) plays important role in the immune mechanism, including type 2 helper T cell (Th2)-mediated diseases such as AD, inflammatory bowel disease (IBD), and asthma. Tregs maintain peripheral immune homeostasis and tolerance to allergens, and protect against or attenuate the differentiation, proliferation, and function of immune cells through a process that is dependent on cell-to-cell contact, transforming growth factor-β (TGF-β), or interleukin-10 (IL-10) (Ziegler, 2006; Shevach, 2002; Tiemessen *et al*., 2006). The depletion of Tregs can exacerbate antigen-induced Th2 cell-mediated inflammation (Saito *et al*., 2008). Tregs express high levels of CD25, a well-known Treg marker, on the cell surface. They also express forkhead box protein 3 (Foxp3), which is a important regulator of Treg activation and

differentiation. $CD4^{+}Foxp3^{+}$T cells suppress the activation or proliferation of helper T cells, including Th1, Th2 and Th17 cells (Zheng and Rudensky, 2007; Shevach *et al*., 2006).

In chronic AD lesions, macrophage accumulation correlates closely with the degree of skin injury and immune dysfunction. Macrophages play a key role in immune responses. They assume a defensive role by phagocytosing parasites and microbes, and are essential for the antigen-induced activation and proliferation of T and B cells (Dokmeci and Herrick, 2008; Elhelu, 1983). Macrophages can be classified into several major functional subsets 1) Commonly-activated macrophages (M1 macrophages), which after stimulation by lipopolysaccharides (LPS) or interferon-gamma (IFN-γ), show characteristic anti-microbial and cytotoxic properties; and 2) alternatively-activated macrophages (M2 macrophages), which are defined by their anti-inflammatory and regulatory properties. The latter are further subdivided into M2a (induced by IL-4 or IL-13), M2b (induced by IL-1 or LPS), and M2c (induced by IL-10, TGF-β) macrophages (Martinez *et al*., 2008; Tjiu *et al*., 2009).

Previous studies examined the anti-allergic effects of fish oil. Fish oil, which contains various omega-3 polyunsaturated fatty acids (n-3 PUFAs), can alleviate sensitization to allergens, weaken the severe AD, eczema, and asthma, and reduce the levels of IL-1, IL-4, and IL-13, and IFN-γ in serum (Kremmyda *et al*., 2011; Storey *et al*., 2005). The n-3 PUFAs showed protective effects in animal models of Alzheimer's and Parkinson's disease, and more recently in a model

of spinal cord and traumatic brain injury (Bailes and Mills, 2010; Bousquet *et al*., 2008; Lopez-Vales *et al*., 2010). The beneficial effects of docosahexaenoic acid (DHA) have been described for many diseases, but the mechanism by which it modulates immune responses and allergic reactions mediated by macrophages and $CD4^{+}$T cells is poorly understood. Therefore, the purpose of this research was to evaluate whether DHA suppresses allergic reactions and up-regulates the generation of $CD4^{+}Foxp3^{+}$T cells. We also examined the effects of transfusing IL-10/TGF-β/DHA–modified macrophages (DHA-M2 macrophages) into a mouse model of AD.

# 2. Materials and Methods

### 2-1. Experimental animals.

BALB/c mice (6 weeks old) were purchased from Orient Bio and maintained in the animal facility of Jeju National University. All procedures and animal experiments were approved by the Jeju National University Animal Care and Use Committee.

### 2-2. Disease models

To induce AD, mice were stimulated with 1 % dinitrochlorobenzene (DNCB; Tokyo Kasei Kogyo Co., Ltd) to the abdomen (on Day-7). On Day 0, mice were re-stimulated with 0.3 % DNCB to the ear every other day. From Day 12, the mice were fed DHA (100 mg/kg; Sigma-Aldrich, St Louis, MO, USA) via the drinking water everyday. The mice were sacrificed on Day 42.

### 2-3. Determination of Optimal Dosage and Treatment Duration of DHA

To determine the optimal dose of DHA, we tested the expression of Foxp3 by DHA in the DNCB-induced AD model. AD was induced by applying DNCB to the mice ear, and DHA was then orally administered with diverse doses (10, 50, and 100 mg/kg) in drinking water for 30

days. A minimum dosage of 50 mg/kg of DHA was required to induce Foxp3 expression, and a dosage of 100 mg/kg of DHA showed the most potent effects in DNCB-induced AD. To find the minimum duration of DHA treatment, DHA was differentially administrated for 10, 20, or 30 days. Results indicated that more than 30 days of DHA treatment was required to induce Foxp3 expression in DNCB-induced AD.

**2-4. Histology and immunohistochemistry (IHC) to detect TSLP and Foxp3 in tissues**

Tissues (ear, spleen (SP), and LN) were obtained from AD mice, fixed in 10% formalin, and then embedded in paraffin. Paraffin-embedded sections were stained with H&E and toluidine blue. To analyze the expression of TSLP and Foxp3 in each of the tissues, IHC staining was performed with rabbit anti-TSLP and rabbit anti-Foxp3 (Novus Biologicals, Cambridge, UK) antibodies and a rabbit-HRP/DAB detection IHC kit (Abcam, Cambridge, UK).

**2-5. Immunofluorescence (IF) Staining for Foxp3**

To analyze the $Foxp3^+$ population in the tissues (LN), consecutive sections were stained with rabbit anti-Foxp3 (1:500; Novus Biologicals) and then incubated with DyLight488-conjugated donkey anti-rabbit IgG (1:300; Biolegend, San Diego, CA, USA). An Isotype control IgG was also included. Tissue sections were analyzed under a confocal microscope (FV500 confocal laser scanning microscope system; Olympus, Tokyo, Japan).

**2-6. Extraction of RNA and real-time PCR**

Total RNA was extracted using TRIzol reagent (Molecular Research Center Inc., Oxford, UK) according to the manufacturer's instructions. Reverse transcription was conducted with a First-Strand cDNA Synthesis kit (Promega). Real-time quantitative PCR was performed with a KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Woburn, MA, USA) and the iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) to measure gene expression levels. The results were analyzed using iQ™5 optical system software. The data were normalized using GAPDH. The following primers were used for RT-PCR: mIFN-γ (forward: 5'-TCA AGT GGC ATA GAT GTG GAA GAA-3'; reverse: 5'-TGG CTC TGC AGG ATT TTC ATG-3'), mIL-4 (forward: 5'-ACA GGA GAA GGG ACG CCAT-3'; reverse: 5'-GAA GAA CTA CAG ACG AGC TCA-3'), mIL-5 (forward: 5′-AGC ACA GTG GTG AAA GAG AC-3′; reverse: 5′-TCC AAT GCA TAG CTG GTG ATT T-3'), mIL-10 (forward: 5'-ATA ACT GCA CCC ACT TCC CA-3'; reverse: TCA TTT CCG ATA AGG CTT GG-3'), mIL-13 (forward: 5'-GCA ACA TCA CAC AGG ACC AGA-3'; reverse: 5'-GTC AGG GAA TCC AGG GCTAC-3'), mIL-17A (forward: 5′-TTC ATC TGT GTC TCT GAT GCT-3′; reverse: 5′-TTG ACC TTC ACA TTC TGG AG-3'), mIL-31 (forward: 5'-CGG TGC CCC AAT ATC GAA-3'; reverse: 5'-GAT GCC TGC TTT ATG CTA TAG TTG TT-3'), mTGF-β (forward: 5'-GAA GGC AGA GTT CAG GGT CTT-3'; reverse: 5'-GGT TCC TGT CTT TGT GGT GAA-

3’), mCTLA-4 (forward: 5′-AGA ACC ATG CCC GGA TTC TG-3′; reverse: 5′-CAT CTT GCT CAA AGA AAC AGC AG-3′) and mFoxp3 (forward: 5’-CCC ATC CCC AGG AGT CTTG-3’; reverse: 5’-CCA TGA CTA GGG GCA CTG TA-3’)

**2-7. Western blot analysis to detect Foxp3 and TSLP expression in tissues**

Total protein was isolated from tissues using lysis buffer. The protein was transferred onto a polyvinylidene difluoride membrane using an iBlot gel transfer device (Invitrogen). The membrane was then incubated with rabbit anti-TSLP (1:500; Novus Biologicals) and rabbit anti-Foxp3 (1:1500; Novus Biologicals). The membrane was responded to horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Cell Signaling Technology Inc., Beverly, MA, USA). The blot was visualized with an ECL detection system (iNtRON Biotechnology, Seoul, Korea).

**2-8. Isolation of $CD4^+$T cells, $CD4^+CD25^+$T cells and macrophage**

$CD4^+$T cells were isolated from the total lymphocyte population in the LN using a Dynabeads® Untouched™ Mouse CD4 cell kit (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, cells were incubated with an antibody mixture for 20 min. Bead-bound cells were then incubated for 15 min in Dynabeads buffer. The bead-free cells were then transferred to a new tube and resuspended in fresh medium.

$CD4^+CD25^+$T cells were isolated from the $CD4^+$T cells using a purified anti-mouse CD25 antibody (eBioscience, San Diego, CA, USA). To further purify $CD4^+CD25^+$ T cells, cells were labeled with the DSB-X$^{TM}$ Biotin Protein Labeling kit (Invitrogen) according to the manufacturer's instructions. Briefly, cells were responded to DSB-X-labeled antibody-coated beads for 10 min. Bead-bound cells were incubated for a further 10 min in FlowComp release buffer. The supernatant containing bead-free cells was removed and the cell pellet was resuspended in medium. Macrophages were isolated using two different methods: (1) Spleens were removed aseptically from normal mice and single cell suspensions prepared by removing red blood cells using red blood cell lysis buffer; (2) normal mouse peritoneal macrophages were harvested after intraperitoneal injection (8 ml per mouse) of PBS. After centrifugation, mouse splenocytes and peritoneal cell were harvested and the resulting cell suspension was filtered through 40 μm nylon mesh and purified by magnet anti-$CD11b^+$ antibody flowcomp dynabeads (BD Biosciences, Franklin Lakes, NJ, USA and Invitrogen). For further purification of $CD11b^+$ macrophages, $CD11b^+$ cells were incubated at 37°C for 40 minutes, and then the culture supernatant that contained floating cells (*e.g.*, T cells, B cells, dendritic cells and NK cells) was discarded. The adherent peritoneal and spleen-derived macrophages were rinsed three times in medium. Macrophages used to all the experiment were macrophages isolated from spleen and peritoneal macrophages rolled into one.

**2-9. Cell culture and stimulation**

The isolated $CD4^+$ T cells and macrophages were cultured in RPMI 1640 (Gibco, Uxbridge, UK) containing L-Glutamine and 25 mM HEPES and supplemented with 10% (vol/vol) FBS (Gibco), 100 U/ml penicillin-streptomycin (Gibco), and 0.05 mM 2-β-mercaptoethanol (Sigma). For the cytokine analysis and $CD4^+$ T cells proliferation assays, isolated $CD4^+$ T cells were responded to anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) in the presence or absence of TGF-β (10 ng/mL) for 72 h. For macrophage polarization, macrophages were cultured in normal medium for 48 h to yield M0 macrophages, cultured with LPS (100 ng/mL; Sigma) to yield M1 macrophages, or cultured with IL-10/TGF-β (both at 10 ng/mL; eBioscience) to yield M2 macrophages.

**2-10. Enzyme-linked immunosorbent assay (ELISA)**

The levels of IgE (Biolegend) and histamine (Labor Diagnostika Nord, Nordhom, Germany) in mouse serum, and the levels of IL-6 and TNF-α (R&D Systems, St. Louis, MO, USA) in the supernatant of cells, were measured to commercial ELISA kits.

**2-11. Flow cytometric analysis**

To analyze Foxp3 expression, cells were first permeabilized using a Foxp3 fixation/permeabilization kit (BD Biosciences) and then stained with anti-Foxp3-FITC

(eBioscience). Briefly, the cells were responded to CD16/CD32 (BD Biosciences) for 15 min to block mouse Fc receptors, and then incubated in fixation/permeabilization buffer for 20 min followed by anti-Foxp3-FITC for 30 min. To measure the $CD4^+CD25^+$ T cells, $CD4^+$ T cells were first stained with anti-CD4-FITC or CD25-PE (both eBioscience). The cell suspension was incubated with CD16/CD32 for 15 min, followed by anti-CD4-FITC and anti-CD25-PE for 30 min. Finally, T cells were stained with anti-CD4-PE and Foxp3-FITC (both eBioscience) to measure the $CD4^+Foxp3^+$ population.

**2-12. Co-culture experiments**

For the T cell suppression assay, $CD4^+CD25^+$T cells (Tregs, suppressor cells) were isolated from normal or DHA-fed mice and co-cultured at different ratios with carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience)-labeled $CD4^+CD25^-$T cells (responder cells; isolated from the LN of WT BALB/c mice) in the presence of anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL). To test suppression sensitivity of $CD4^+CD25^-$T cells by $CD4^+CD25^+$ Tregs, $CD4^+CD25^+$T cells isolated from LNs of normal mice were cultured at different ratios with CFSE-labeled $CD4^+CD25^-$Tcells (isolated from each group) in the presence of anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) and in the presence or absence of TGF-β (10 ng/mL). Each sample culture was maintained up to 72 h. After 72 h, cells were harvested and examined with

by FACS.

For the proliferation assays, $CD4^+$T cells were isolated from the LN of WT mice and labeled with CFSE. Cells (1 x $10^6$ per well) were then co-cultured different ratios with pre-adherent M0, M1, or M2 macrophages isolated from the WT mice or DHA-fed mice and stimulated by anti-CD3/CD28 (each at 1 and 0.5 μg/mL) for 72 h. Cell proliferation was examined by FACS.

For the Treg induction experiments, $CD4^+CD25^-$T cells were co-cultured with M1 or M2 macrophages isolated from WT mice or DHA-fed mice for 7 days. For some experiments, $CD4^+CD25^-$T cells and IL-10/TGF-β M2 were co-cultured in 24-well plates, or cultured separately in Transwell chambers in the presence or absence of anti-IL-10 (10 μg/mL; BD PharMingen) and anti-TGF-β (10 μg/mL; R&D Systems)-neutralizing antibodies for 7 days. $CD4^+$T cells were then stained with an anti-Foxp3 antibody and analyzed by FACS.

**2-13. Co-culture M2 macrophages with M1 macrophages**

The two cell monolayers were co-cultured as described previously. Briefly, isolated macrophages were grown on 12-mm plastic coverslips (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and stimulated with various cytokines to generate M2 macrophages. Macrophages also were seeded onto 24-well plates and then induced to differentiate into M1 macrophages. The coverslips harboring monolayers of M2 macrophages were then placed over

the confluent monolayers of M1 macrophages and co-cultured for 24 hours. The various cytokines of M1 macrophages were then analyzed by ELISA.

**2-14. Macrophage labeling and adoptive transfer into BALB/c mice**

Macrophages of various phenotypes were labeled with Vybrant® CM-Dil cell-labeling solution (Invitrogen). CM-DiI is used extensively as a long lasting tracer for neuronal and other cells. Mice were sensitized by applying 1 % DNCB to the abdomen, followed by re-sensitization by applying 0.5 % DNCB to the ears every other day for up to 26 days. Starting on Day 12, the ears of some mice were treated with hydrocort cream (Green Cross, Korea) containing 2 mg/g hydrocortisone valerate. This was performed every other day. These mice acted as a positive control. Starting on Days 12 and 19, CM-DiI-labeled DHA-M2 macrophages ($1 \times 10^7$ cells/mL) were injected into AD mice via the tail-vein. All mice were sacrificed on Day 27. The mice were divided into four groups ($n$=5 per group): saline (normal), AD (induction-only), AD + Hydrocort cream, and AD + DHA-M2 macrophages (Figure 5A). Ear, spleen, and lymph node tissues were isolated and examined microscopically for morphological changes, and by real-time PCR, western blot analysis, IHC, and IF.

**2-15. Statistical analysis**

The student's *t* test was used to determine the statistical significance of samples. Data is expressed as the mean ± standard deviation (SD). *P*-values less than 0.05 were considered significant.

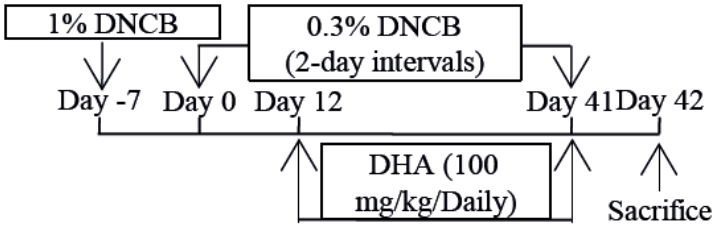
# 3. Results

### 3-1. DHA suppresses the development of experimental AD.

To induce experimental AD, mice were stimulated with 1% DNCB to the abdomen. They were then re-stimulated with 0.3% DNCB on every other day. Starting on Day 12, the mice received a daily dose of DHA (100 mg/kg) via the drinking water (Figure 1a). IgE is a key therapeutic target for inflammatory skin diseases as it is the major stimulator of mast cells, which release cytokines and histamine (Levin *et al*., 2006). Therefore, we measured the levels of serum IgE and histamine in mice with dermatitis. The DHA-treated group showed significantly reduced levels of IgE and histamine (both $P < 0.01$) compared with the induction group (mice applied to DNCB but not fed DHA; Figure 1b). The skin lesions with AD patients are characterized by infiltration of inflammatory cells (de Vries *et al*., 1997). Therefore, we next tested whether DHA alleviated the level of inflammatory cell infiltration in AD mice. We also evaluated ear edema as a measure of AD progression. We identified that the ear thickness in DHA-fed mice was reduced at Days 33 and 42 (both $P < 0.01$) compared with that in induction-only mice (Figure 1c and d). Thymic stromal lymphopoietin (TSLP), which is expressed by epidermal keratinocytes and dermal fibroblasts, induces both the maturation of antigen presenting cells

(APCs) and allergic inflammatory reactions (Bogiatzi *et al*., 2007; Soumelis *et al*., 2002). Therefore, we next examined the effect of DHA on the infiltration of inflammatory cells and the expression of TSLP by hematoxylin and eosin (H&E) or immunohistochemical (IHC) staining of ear tissue sections, and by western blotting. Epidermal thickness, the degree of inflammatory cell infiltration, and the expression of TSLP were significantly lower in the DHA-administered group than in the induction group (Figure 1e and f).

(a)

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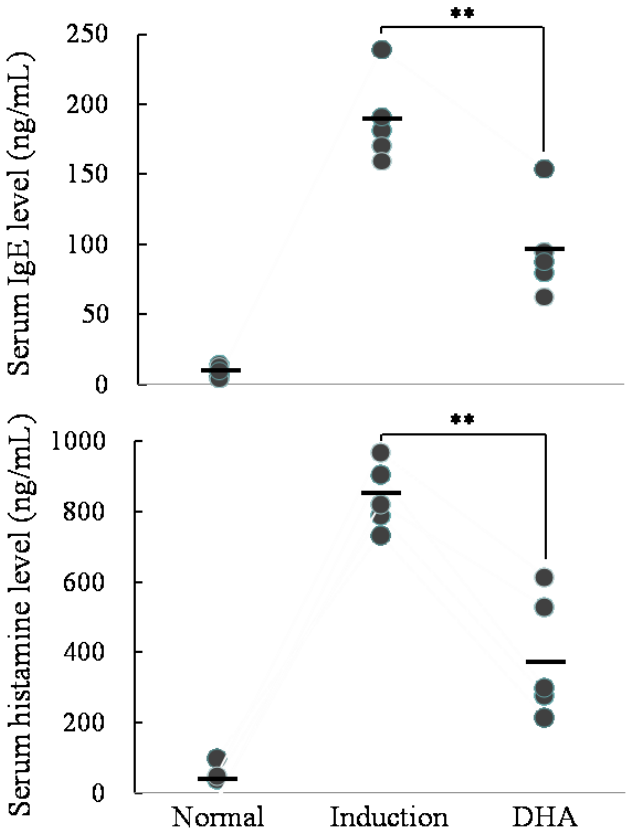

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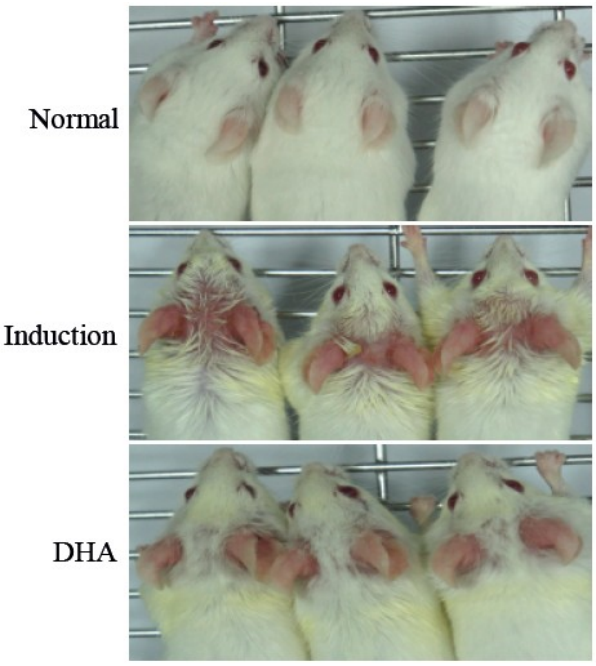


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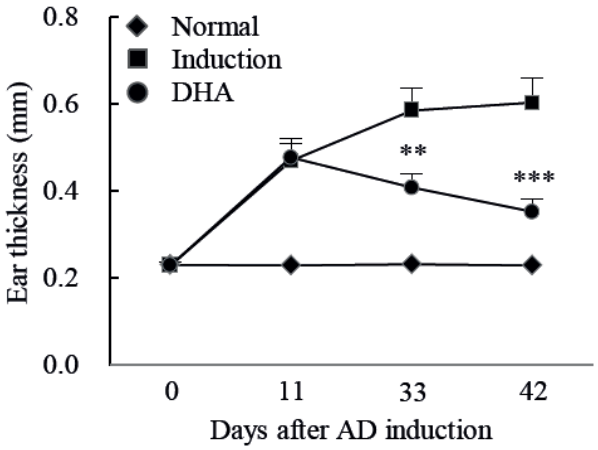

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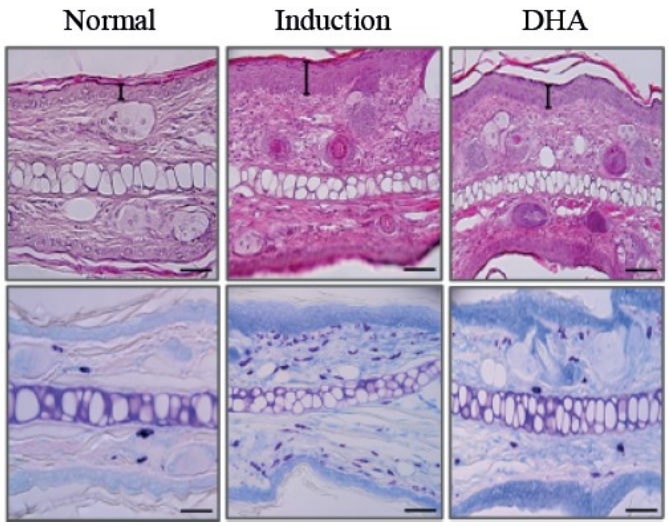



(f)

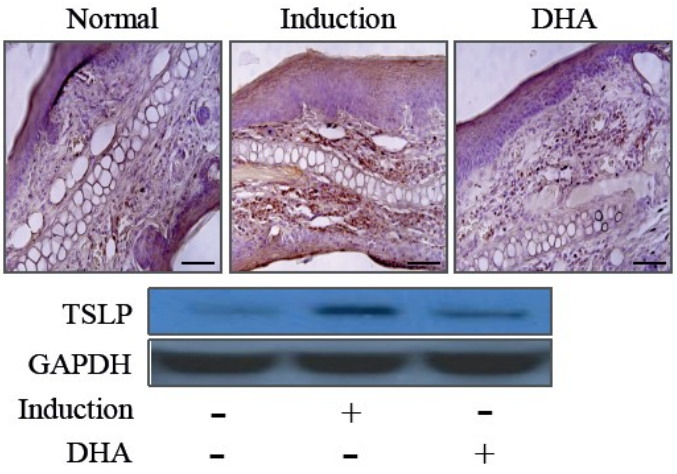
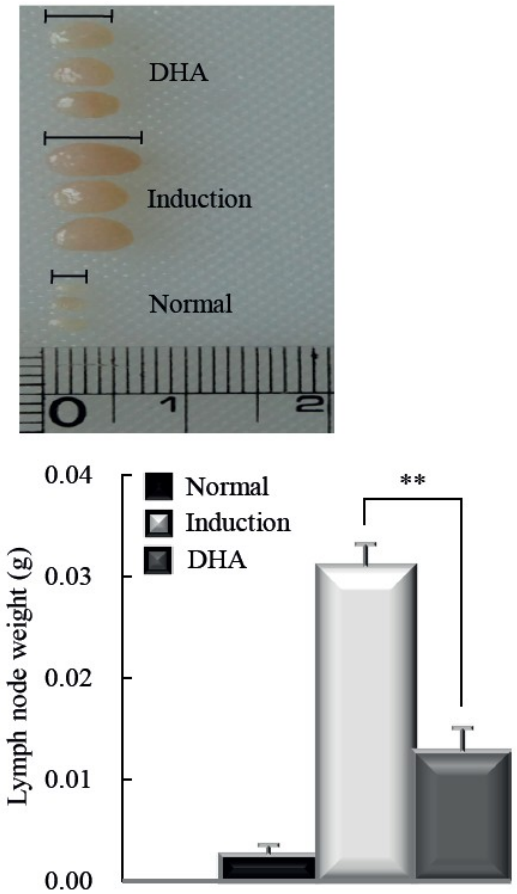


**Figure 1. DHA suppresses experimental AD. (a)** Mice were challenged with DNCB. From Day 12, mice were fed a daily dose of DHA (100 mg/kg) in the drinking water. **(b)** The IgE and histamine levels in serum were measured by ELISA. **(c)** Macroscopic views of the ears and **(d)** ear thickness measured on Days 0, 11, 33 and 42. **(e)** Paraffin-embeded sections of ear tissue stained with H&E and toluidine blue. **(f; upper panel)** IHC staining was performed with a anti-TSLP antibody and HRP/DAB detection IHC kit. **(f; lower panel)** TSLP levels were measured in ear tissues by western blotting. (n = 10 mice per group). Scale bar = 0.1 mm. Values represent the mean ± S.D. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with mice in the induction group.

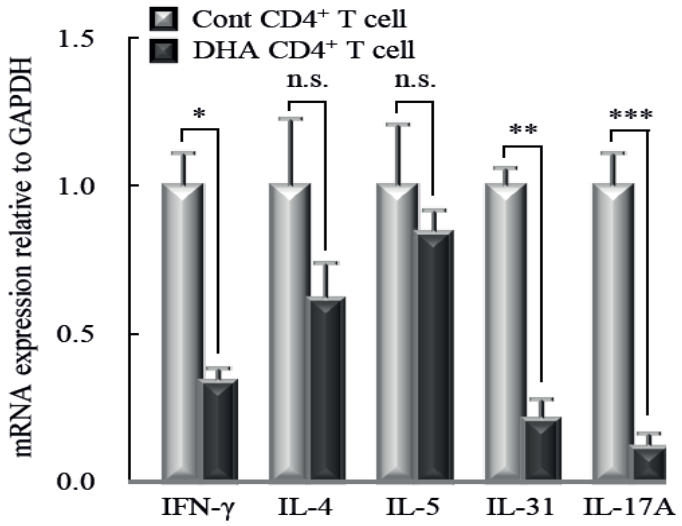
**3-2. $CD4^+Foxp3^+$ T cells are enriched at sites of inflammation and are associated with AD suppression.**

Next, we evaluated the morphologic changes in the lymph nodes (LNs) of mice. The LNs isolated from AD mice in the induction group were very swollen, but those from DHA mice were smaller and weighed less (Figure 2a). We also examined whether DHA suppresses the function of mature $CD4^+$T cells in the experimental AD model. We measured the relative expression of IFN-γ (Th1), IL-4, IL-5 and IL-31 (Th2), and IL-17 (Th17) transcripts by real-time PCR. Compared with the induction group, DHA-fed mice showed reduced IFN-γ ($P < 0.05$), IL-31 ($P < 0.01$), IL-17A ($P < 0.001$), IL-4, and IL-5 mRNA levels (Figure 2b). Because $CD4^+Foxp3^+$Tregs suppress the activity and generation of pathogenic effector cells at the site of inflammation via direct cell-to-cell contact or by secreting TGF-β and IL-10 (Vignali and Collison, 2008; Huehn and Hamann, 2005), we examined the population of $Foxp3^+$Tregs at the inflammatory sites (the LN in AD mice) by FACS analysis and immunofluorescence (IF) staining. FACS analysis showed a significant increase $Foxp3^+$T cells in DHA mice compared with Cont mice (30.8 % *vs*. 19.7 %, respectively; Figure 2c). This was confirmed by IF analysis of LN section (Figure 2d).

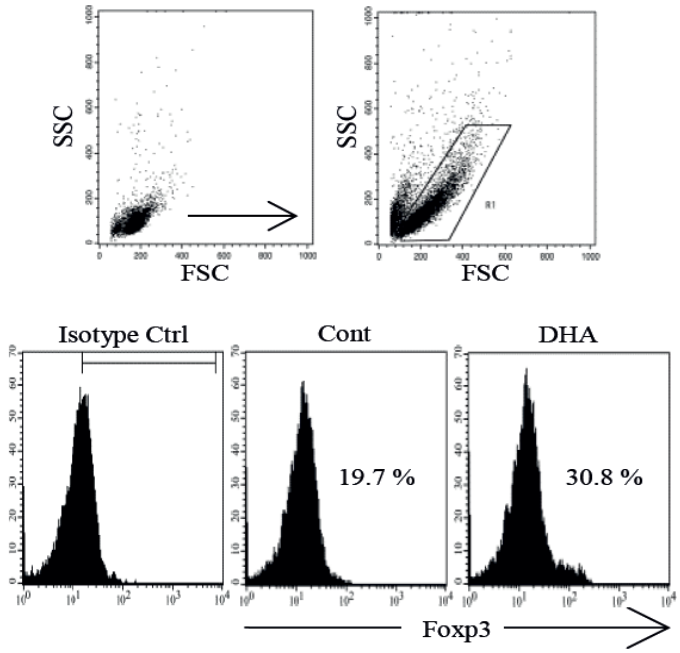
(a)




(b)



(c)

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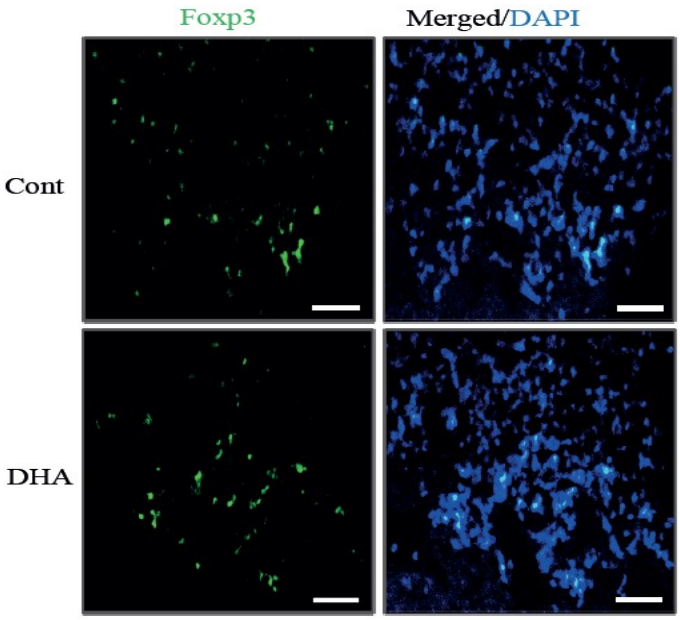


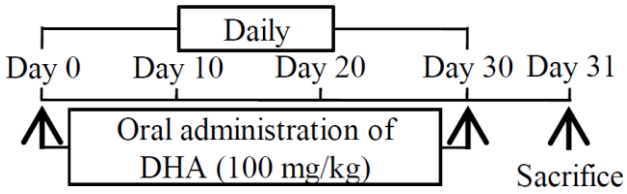
**Figure 2. $CD4^+Foxp3^+$ T cells are enriched at sites of inflammation. (a)** The LNs were photographed and measured to record morphologic changes. **(b)** Cytokines and **(c)** Foxp3 levels expressed by $CD4^+$ T cells isolated from lymphocytes were analyzed by real-time PCR and FACS. **(d)** Sections of LN were stained with a rabbit anti-Foxp3 antibody and then incubated with DyLight488-conjugated donkey anti-rabbit IgG. Tissue sections were analyzed using confocal microscopy; Foxp3 is green and the nuclei are blue. (n = 10 mice per group). Scale bar = 0.1 mm. Values represent the mean ± S.D. *$P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ compared with mice in the induction group.

### 3-3. DHA administration induces the differentiation of $CD4^+Foxp3^+$ Tregs from $CD4^+$ T cells in a TGF-β-dependent manner

Tregs play a key role in immune mechanism by suppressing the differentiation and proliferation of $CD4^+$T cells (Sakaguchi *et al*., 2009). Therefore, we examined whether DHA affects the expression of factors related to Treg cell differentiation during T cell activation. DHA was fed to normal BALB/c mice for 30 days and the mice were sacrificed on Day 31 (Figure 3a). Compared with the control group (non-DHA-fed mice), DHA suppressed the expression of IFN-γ, IL-4, IL-5 (all $P < 0.05$), IL-31 ($P < 0.001$), IL-13 and IL-17A mRNA and increased the expression of TGF-β and cytotoxic T-lymphocyte associated (CTLA)-4 (both $P < 0.05$) mRNA (Figure 3b). In addition, the $CD4^+$T cells of the DHA group showed increased expression of Foxp3 and TGF-β in the presence of TGF-β (Figure 3c). Although DHA also increased the $CD4^+Foxp3^+$ population in the presence of exogenous TGF-β, which induces the generation of Tregs *in vitro* (Figure 3d and e), we found no difference in the size of the $CD4^+CD25^+$ population between the different groups (Figure 3f). Compared with the control group, $CD4^+CD25^-$T cells (responder) from the DHA group were slightly more sensitive to suppression by $CD4^+CD25^+$Tregs (suppressors; Figure 3h), although the inhibitory effects of Treg isolated from DHA-fed mice on normal $CD4^+$T cells proliferation were no different from

that of Tregs isolated from control mice (Figure 3g). However, $CD4^+CD25^-$T cells from DHA-fed mice were significantly more susceptible to suppression by $CD4^+CD25^+$Tregs in the presence of exogenous TGF-β (Figure 3h). These observations indicate that DHA induced the generation of $CD4^+Foxp3^+$Tregs from the $CD4^+CD25^-$ cell pool and inhibited the proliferation of $CD4^+$T cells via a TGF-β-dependent mechanism.

(a)

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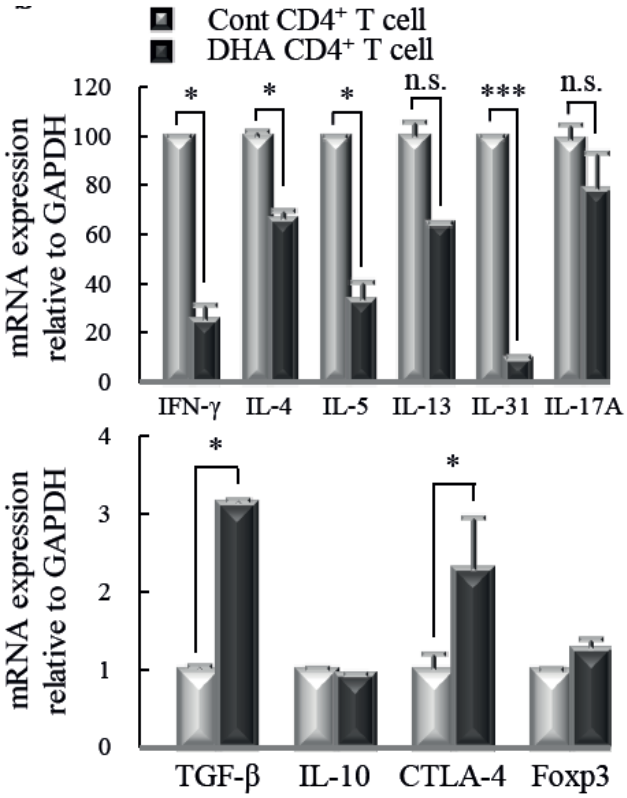

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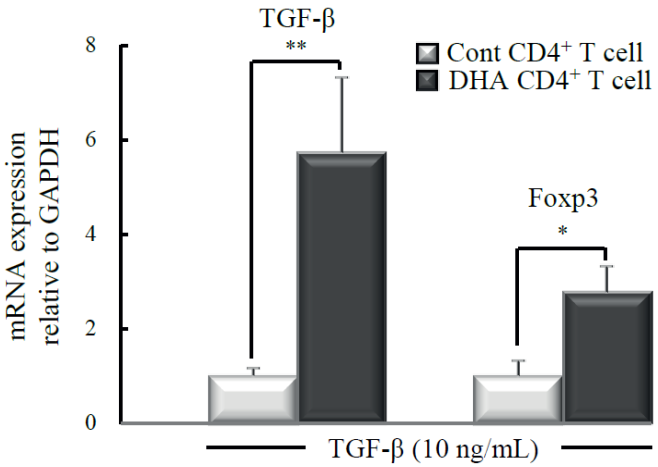


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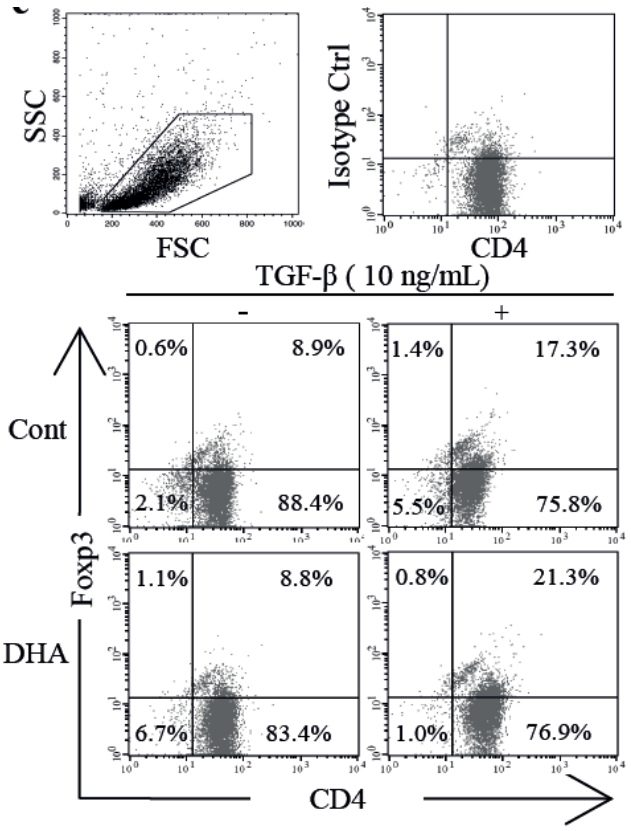

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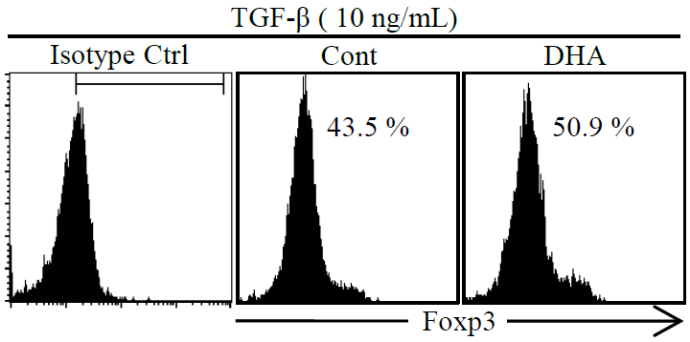


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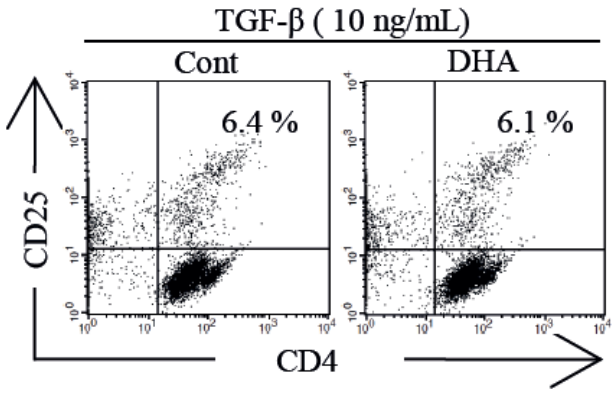


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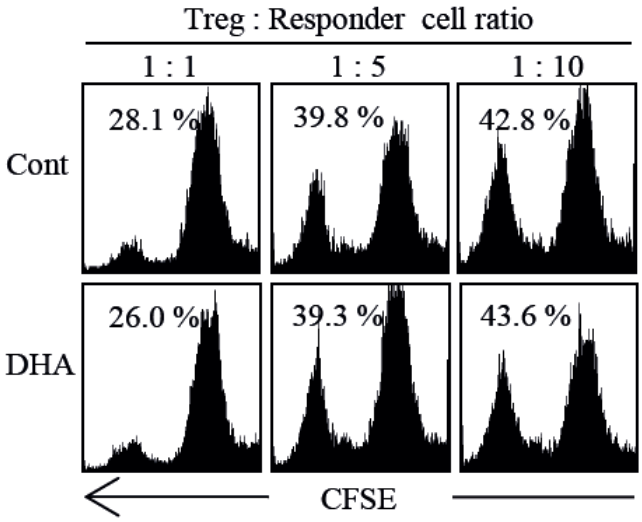

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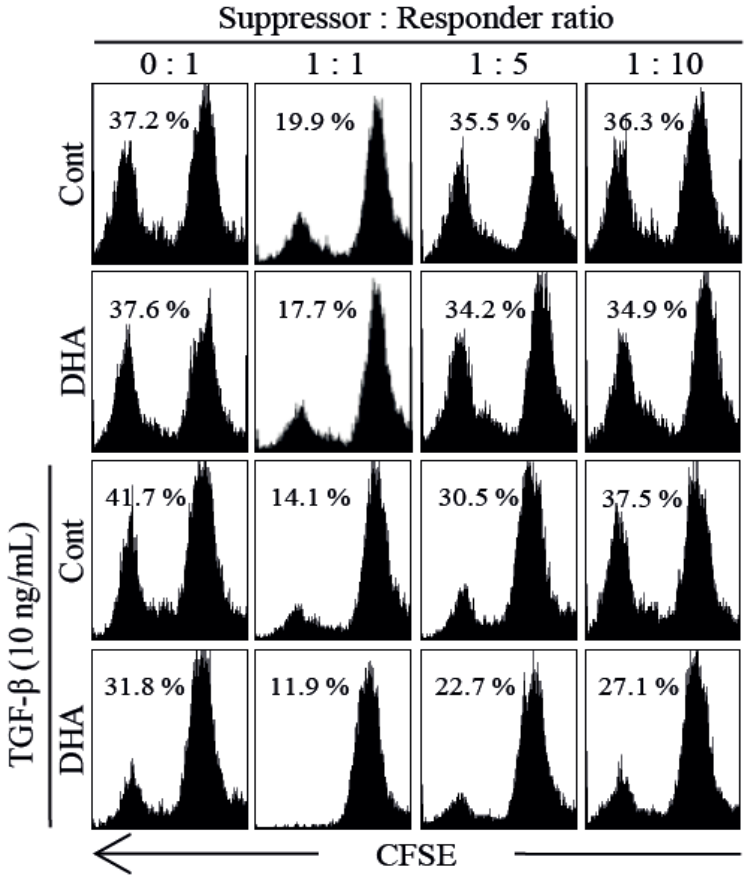

**Figure 3. DHA induces the differentiation of $CD4^+Foxp3^+$ Tregs from $CD4^+$ T cells in a TGF-β-dependent manner. (a)** Naïve mice received DHA for 30 days. **(b)** The expression of mRNA for various factors in $CD4^+$T cells was measured by real-time PCR. **(c ~ f)** The $CD4^+$T cells isolated from each lymph node (LN) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) in the presence of TGF-β (10 ng/mL) for 72 h. The expression of mRNA for TGF-β or Foxp3 in $CD4^+$T cells was measured by real-time PCR or FACS. **(g)** Tregs from each treatment group was co-cultured with responder cells ($CD4^+CD25^-$) labeled with CFSE at different Treg/responder ratios. **(h)** The suppression sensitivity of CFSE-labeled $CD4^+CD25^-$T cells (responder) by $CD4^+CD25^+$Tregs (suppressors) was measured by culturing them with WT nTregs at different ratios in the presence or absence of TGF-β for 72h. Values represent the mean ± S.D. *$P < 0.05$; and *** $P < 0.001$ compared with mice in the control group.

### 3-4. DHA strongly up-regulates the function of M2 macrophages, which induce the generation of $CD4^+Foxp3^+$ T cells

We next examined the role of M2 macrophages in the DHA-induced generation of $CD4^+Foxp3^+$ Tregs. $CD11b^+$ macrophages were isolated from normal BALB/c mice and stimulated with LPS, IL-10, TGF-β, or IL-10/TGF-β, and the expression of inflammatory cytokines was measured. Compared with control mice, DHA inhibited the expression of IL-6 ($P < 0.001$) by LPS-stimulated macrophages; however, treatment with other anti-inflammatory cytokines had no effect in either groups (Figure 4a). In addition, DHA-M2 macrophages expressed higher levels of IL-10 and TGF-β than M0 (non-stimulation), M1 (LPS stimulation), IL-10-, TGF-β-stimulated macrophages, or control-M2 macrophages from both groups (Figure 4a). We also compared the inhibitory effects of M2 macrophages between control and DHA-treated groups on the expression of pro-inflammatory cytokines by M1 macrophages. Compared with the co-culture of M1 macrophages with control-M2 macrophages, co-culture of M1 macrophages with DHA-M2 macrophages for 24 h led to a significant reduction in the expression of IL-6 ($P < 0.01$ DHA *vs*. $P < 0.05$ control), TNF-α (both $P < 0.05$) and IL-1β ($P < 0.01$ DHA *vs*. $P < 0.05$ control; Figure 4b). We then tested whether DHA treatment of M2 macrophages had the potential to induce $Foxp3^+$Tregs. M0, M1, and M2 macrophages were

isolated from each group and co-cultured with $CD4^+$ T cells for 7 days. The $CD4^+Foxp3^+$ Tregs populations were then analyzed by flow cytometric analysis. When compared with control-M2 macrophages, DHA-M2 macrophages induced the preferential differentiation of $CD4^+$ T cells into $CD4^+Foxp3^+$ T cells (14.4 % DHA *vs*. 8.0 % Cont; 44.3 % DHA *vs*. 39.4 % Cont, Figure 4c and d). By contrast, neither M0 nor M1 macrophages induced the transformation of $CD4^+$ T cells into Tregs (Figure 4c). In addition, Transwell and neutralizing antibodies studies showed that DHA-M2 macrophages-mediated Tregs induction required cell-to-cell contact and TGF-β (Figure 4e and f). The proliferation of $CD4^+$ T cells co-cultured with DHA-M2 macrophages was significantly reduced, as shown by the higher ratio (macrophages : $CD4^+$ T cells) ratio in the DHA-M2 macrophages group compared with that in the M0, M1, and Cont-M2 macrophages groups (this was due to the increased Tregs population in the DHA-M2 macrophages group). By contrast, M1 macrophages induced significantly greater T cell proliferation than M0 or M2 macrophages in all groups (Figure 4g and h). Collectively, these results suggest that DHA strongly up-regulates the function of M2 macrophages, which have the capacity to convert $Foxp3^-$ T cells into $Foxp3^+$ Tregs through cell-to-cell contact, and the secretion of TGF-β.

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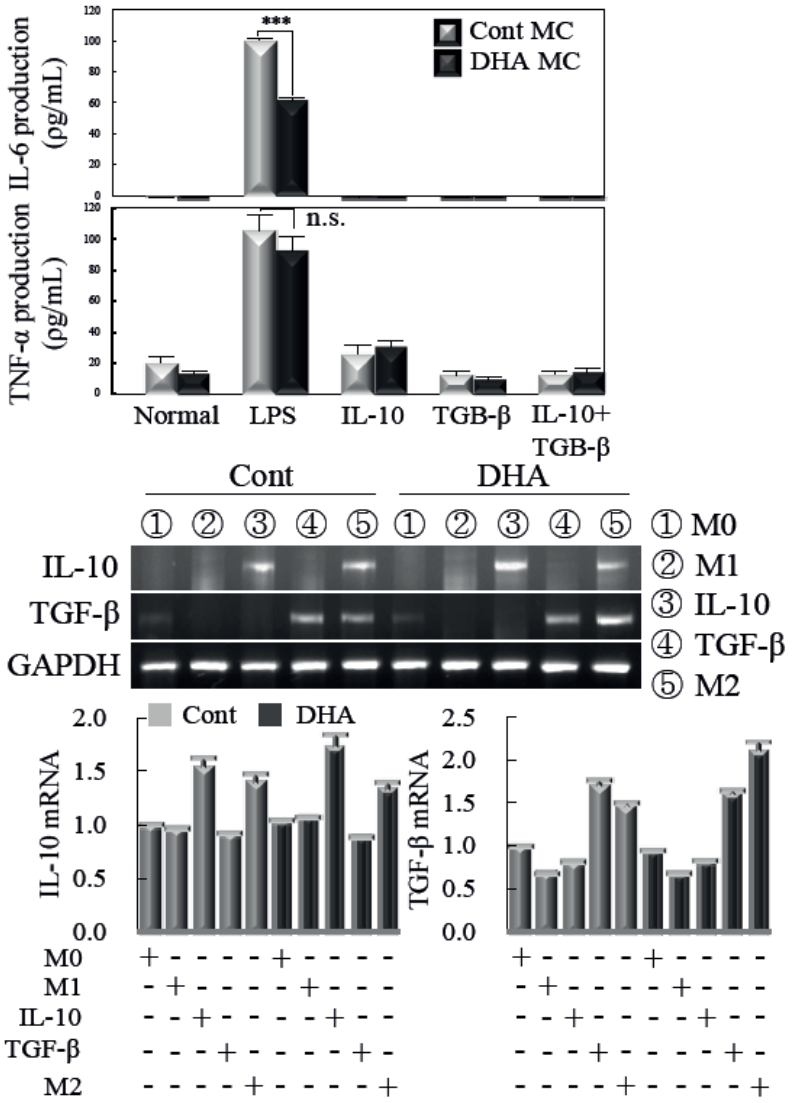

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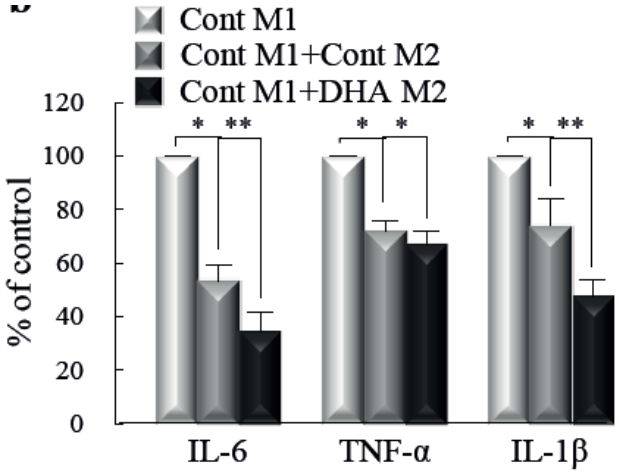


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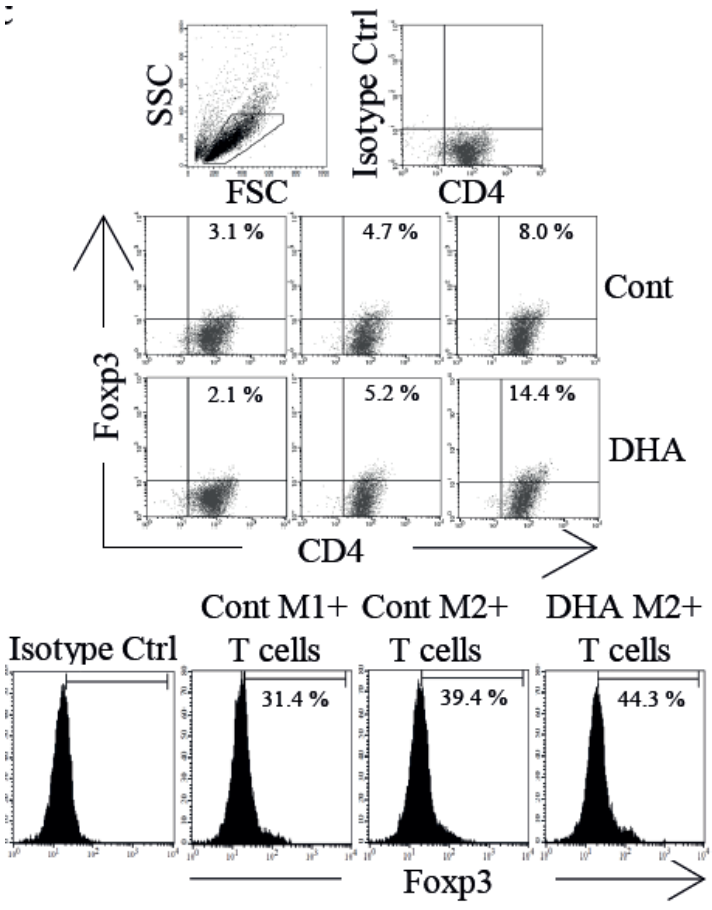

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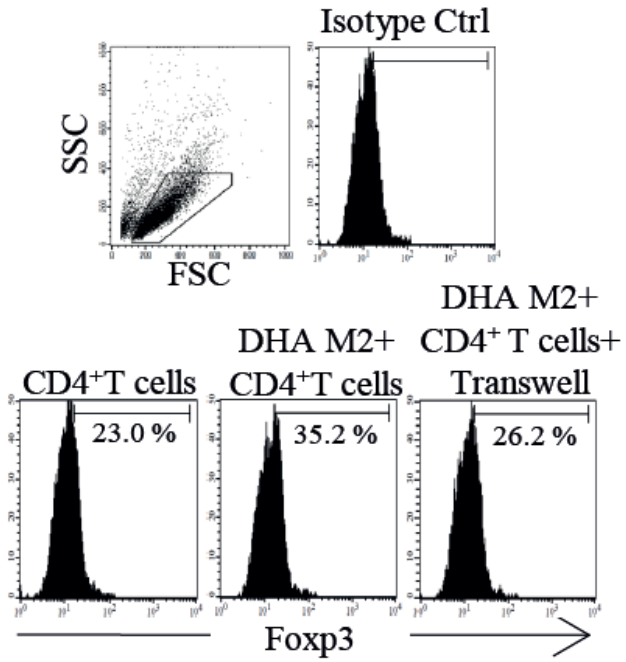


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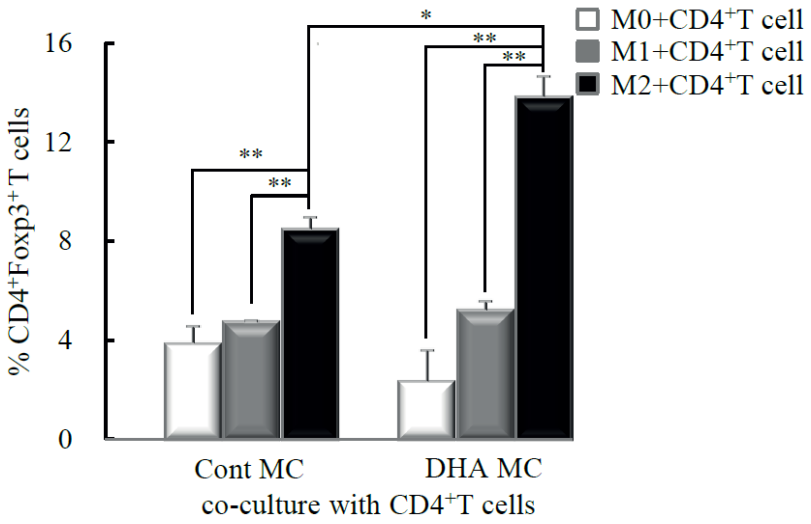

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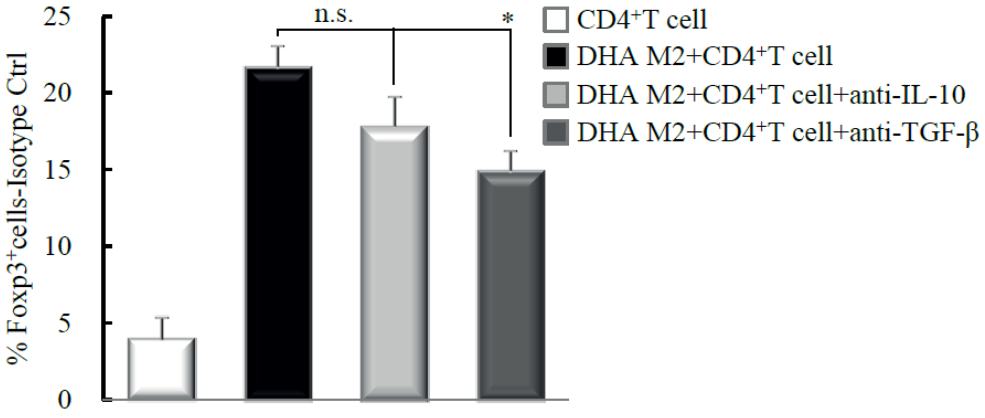


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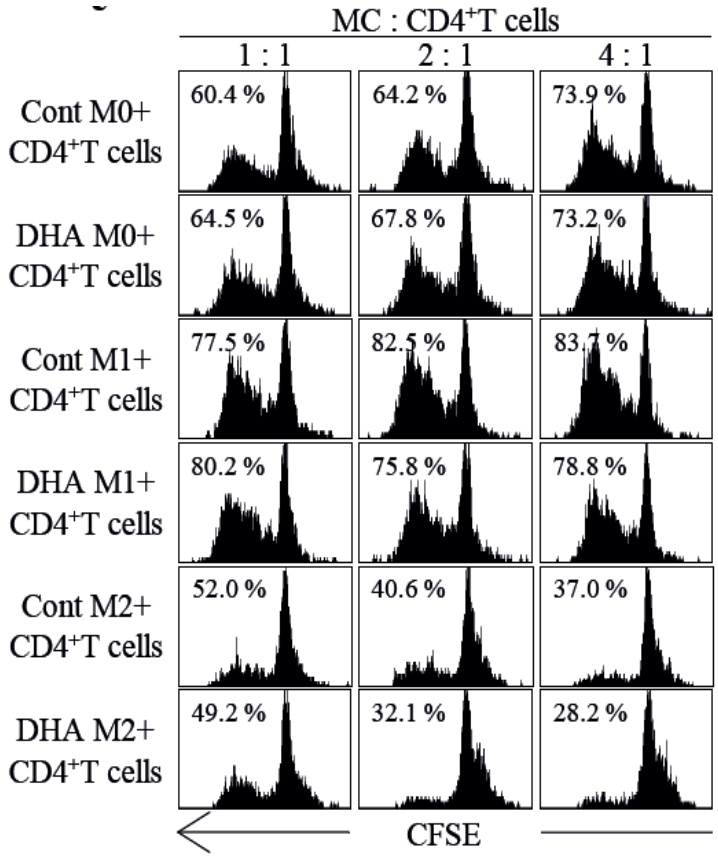

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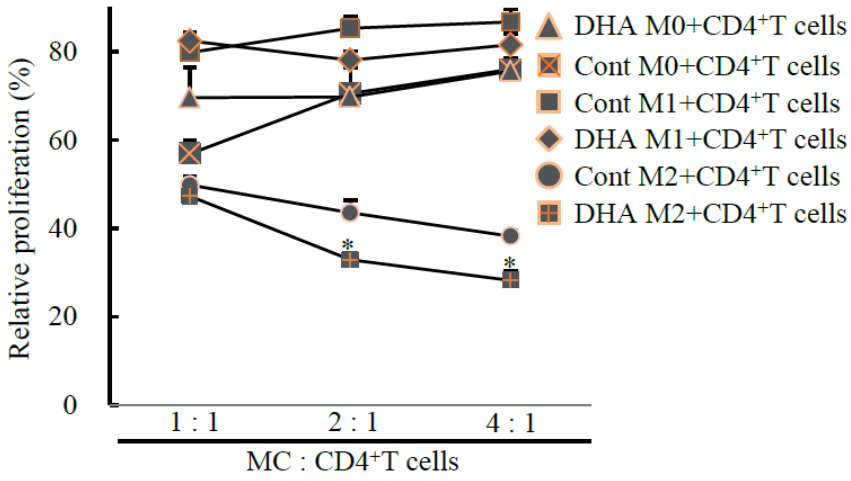

**Figure 4. DHA induces Foxp3$^+$ Tregs and stronger ability of IL-10/TGF-β M2 macrophages. (a)** Macrophages (MCs) were cultured for 48h in normal medium, containing LPS, IL-10, TGF-β, or IL-10+TGF-β, and then analyzed by ELISA, RT-PCR and image J. **(b)** The coverslips seeded with M2 macrophages were placed onto the confluent of M1 macrophages for 24h. The cytokines of M1 macrophages were analyzed by ELISA. **(c and d)** Macrophages were co-cultured with CD4$^+$T cells in plates or cultured separately in Transwell chambers for 7 days. **(e and f)** Macrophages isolated from Cont- or DHA-fed mice were cultured in normal medium for 48 h to yield M0 macrophages, cultured with LPS (100 ng/mL) to yield M1 macrophages, or cultured with IL-10/TGF-β (both at 10 ng/mL) to yield M2 macrophages. M0, M1 or M2 macrophages were co-cultured with CD4$^+$T cells in the presence or absence of anti-IL-10 (10 μg/mL) and anti-TGF-β (10 μg/mL)-neutralizing antibodies. After co-culture, the alteration of Foxp3$^+$ population was measured by FACS. **(g and h)** CD4$^+$T cells-labeled with CFSE were co-cultured with macrophages, and stimulated by anti-CD3/-CD28 for 72h. Values represent the mean ± S.D. Data are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$; and *** $P < 0.001$.

### 3-5. Transfusion of DHA-M2 macrophages protects against experimental AD

To induce AD in mice, mice were stimulated with 1 % DNCB to the abdomen, and then re-sensitized by applying 0.5 % DNCB to their ears on every other day for up to 26 days. Starting on Days 12 and 19, mice received a single injection of CM-DiI-labeled DHA-M2 macrophages into the tail-vein. All mice were sacrificed on Day 27 (Figure 5a). Compared with the induction-only group, DHA-M2 macrophages injected mice showed a significant reduction in ear thickness at Days 18 and 26 ($P < 0.01$; Figure 5b and d), and reduced IgE levels ($P < 0.01$; Figure 5e). We also found that the LNs from DHA-M2 macrophages injected mice were smaller than those from mice in the induction-only group (Figure 5c). In addition, transfusion of DHA-M2 macrophages suppressed the expression of IFN-γ, IL-4 (both $P < 0.05$), IL-13, and IL-17A mRNA, and increased that of TGF-β, IL-10, and Foxp3 ($P < 0.05$) compared with that in the induction-only group (Figure 5f). Next, we examined the number of transfused DHA-M2 macrophages present at sites of inflammation in AD mice. Many fluorescently-labeled cells were detected in the ear, LN, and spleen (SP), although most were present in the LN (Figure 5g). We also measured the population of $Foxp3^+$ Tregs in the LN and SP by IHC and western blot analyses. DHA-M2 macrophages injected mice showed a significant increase $Foxp3^+$T cells compared with the Cont and Hydrocort groups (Figure. 5h). These results suggest that DHA-M2

macrophages suppress inflammation by inducing the generation of $Foxp3^+$ Tregs.

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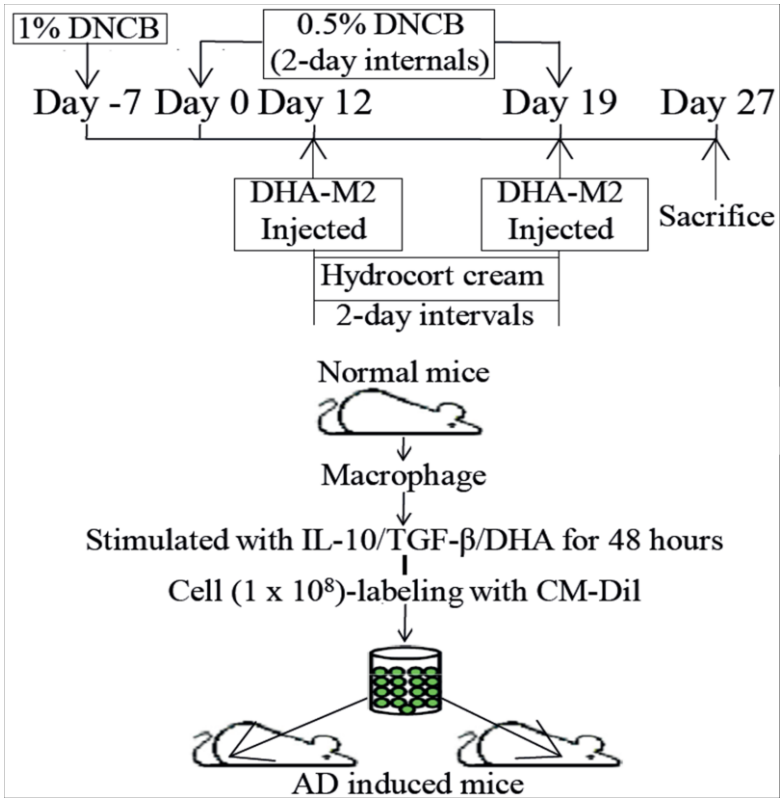


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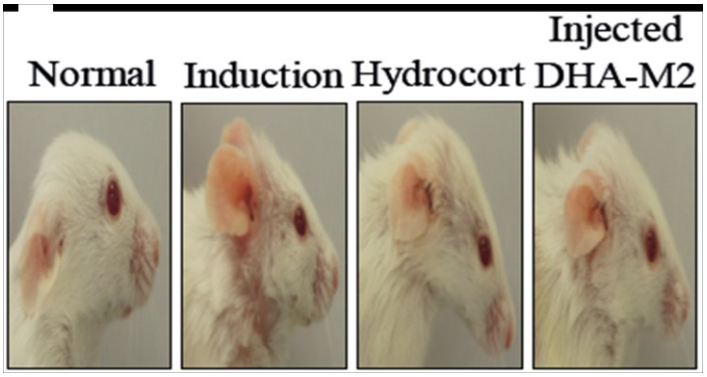

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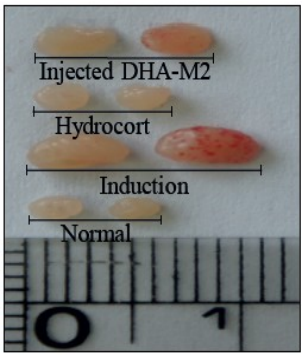


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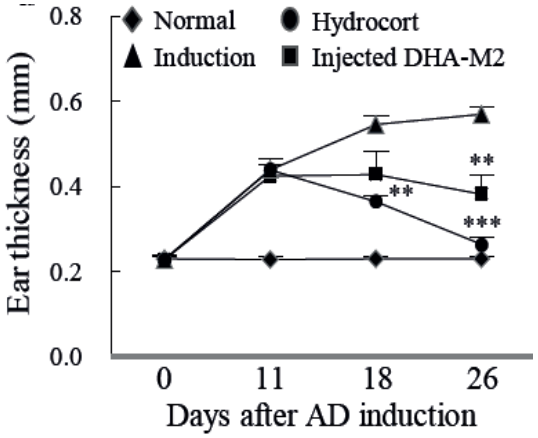


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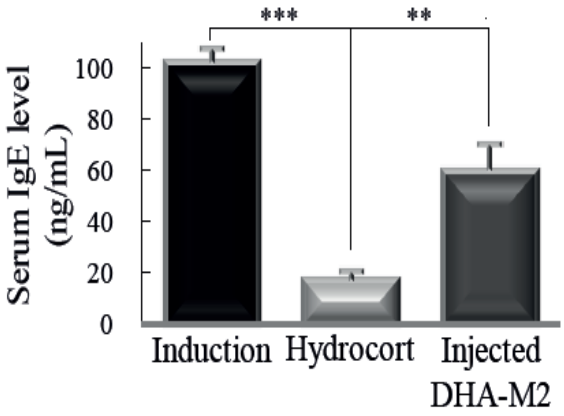

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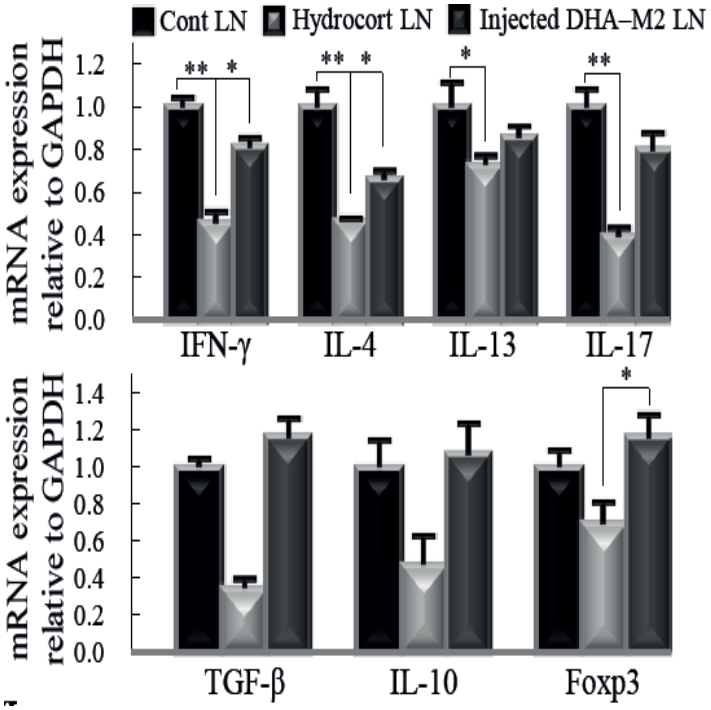


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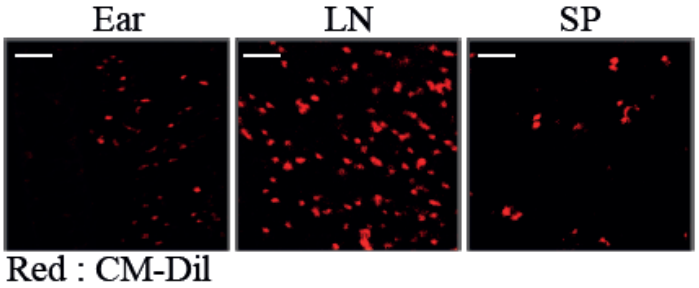

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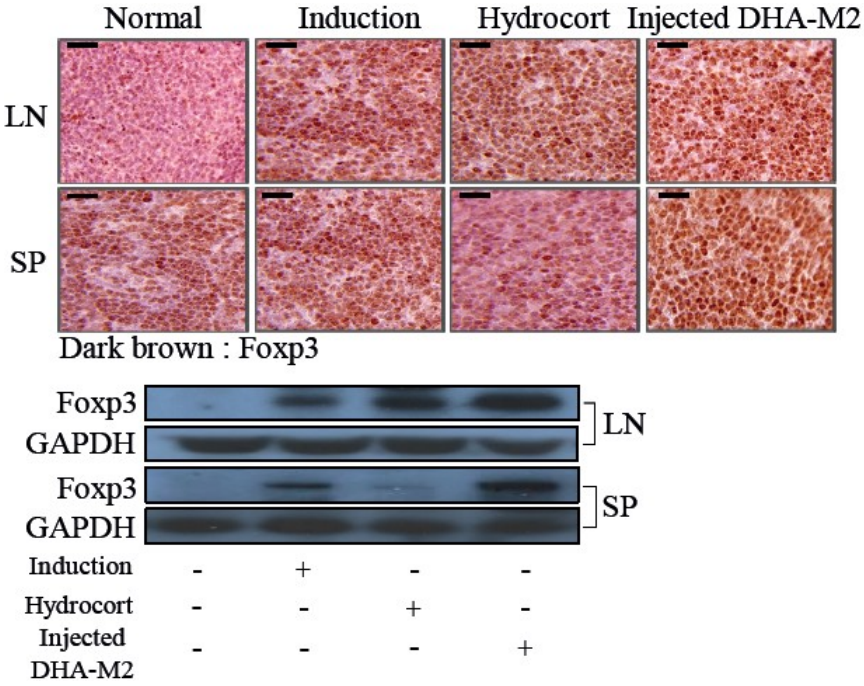


**Figure 5. Transfusion of DHA-M2 macrophages protect against experimental AD. (a)** Mice were challenged with DNCB. On Days 12 and 19, CM-DiI-labeled DHA-M2 macrophages were injected into AD mice. **(b and c)** Macroscopic views of the ears, LNs and **(d)** ear thickness. **(e)** IgE levels in the serum were measured by ELISA. **(f)** Real-time PCR was used to measure the expression of mRNA for cytokines and transcription factors in $CD4^+$ T cells. **(g)** CM-DiI-labeled macrophages in ear, SP, and LN were examined by confocal microscopy. **(h)** The expression of Foxp3 was measured in LN and SP tissues by IHC staining and western blotting. Scale bar = 0.1 mm. Values represent the mean ± S.D. $*P < 0.05$; and $**P < 0.01$ compared with mice in the induction group.

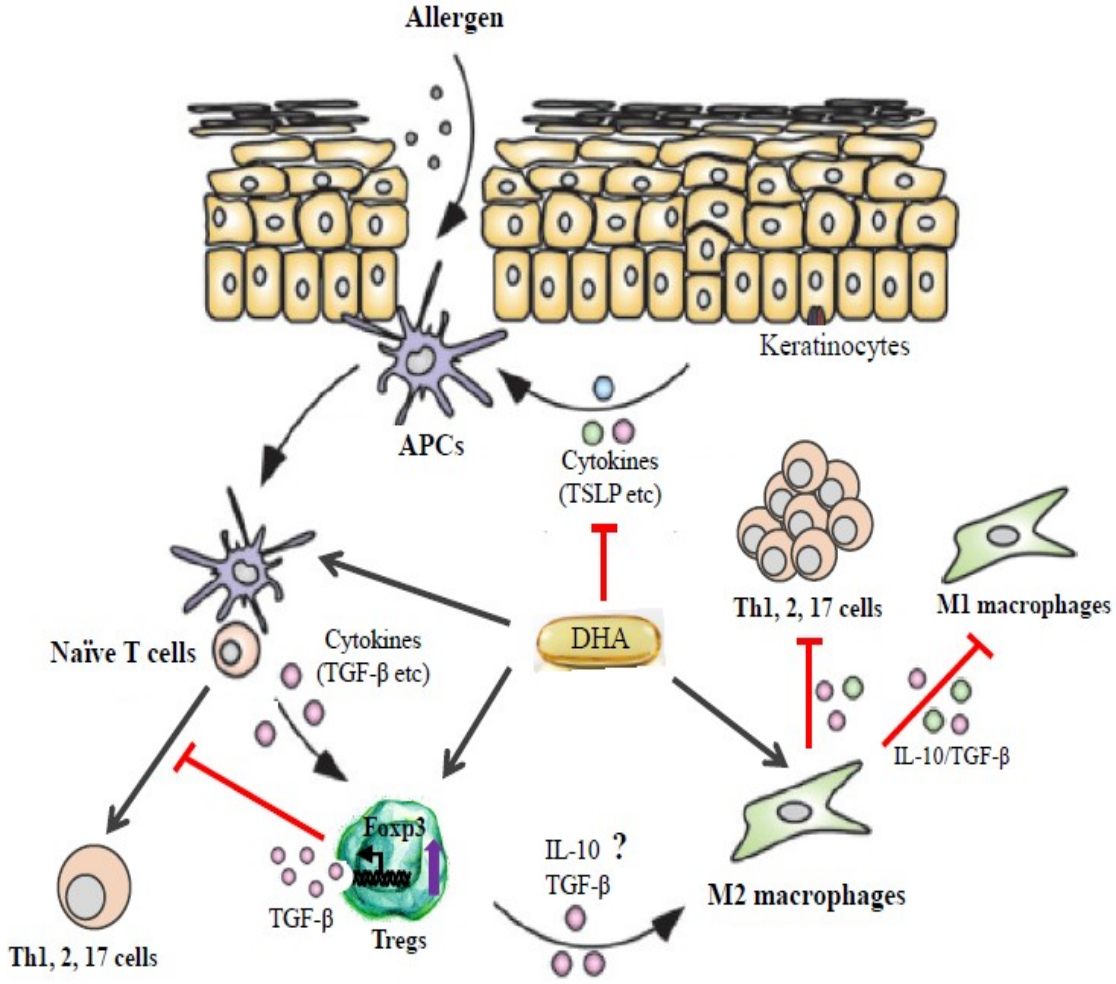
# 4. Discussion

Recent, study reports that the patients suffering from atopic eczema aged 18-40 years were received the high dose of DHA (5.4 g/daily) for 8 weeks, and DHA resulted in a clinical improvement of atopic eczema (Koch *et al*., 2008). Here, we examined how the potent anti-inflammatory properties of DHA affect the progression of experimental AD. DHA also increased the number of adaptive Tregs in healthy mice and in AD. The protective effect of DHA was associated with up-regulated levels of $CD4^{+}Foxp3^{+}$Tregs at the sites of inflammation. Tregs, control immune homeostasis in AD, suppress immune responses by interacting with effector T cells or APC (Vignali *et al*., 2008). In experimental AD models, the lack of Tregs leads to elevated serum IgE levels and production of Th2 cytokines (Lin et al., 2005). When comparing Treg cells populations in the peripheral blood of AD patients with those in the blood of healthy donors, several groups reported increased numbers of Tregs in AD patients (Ou *et al*., 2004). However, Tregs in AD patients do not protect against or attenuate the differentiation, proliferation, and function of immune cells (Hijnen *et al*., 2009; Ito *et al*., 2009). The enriched $CD4^{+}Foxp3^{+}$Tregs population induced by DHA may suppress pro-inflammatory effector T cells (Th1/2/17 cells) at the sites of inflammation, resulting in a protective effect in AD models. DHA strongly up-regulates the function of M2 macrophages, which induce the generation of

$CD4^+Foxp3^+$ Tregs. The enrichment of $CD4^+Foxp3^+$ Tregs at sites of inflammation is associated with the up-regulation of TGF-β, which inhibits the progression of several immune disorders. The immuno-modulatory actions of DHA are achieved via the increased expression of immuno-suppressive cytokines such as TGF-β, and by suppressing the secretion of pro-inflammatory cytokines by $CD4^+$T cells and macrophages. DHA did not increase the number of $CD4^+CD25^+$ Tregs; however, it did increase CTLA-4 expression by $CD4^+CD25^-$T cells by more than 2-fold. DHA may not generate adaptive Tregs directly; rather, it may promote the expression of TGF-β, which then induces T cells to differentiate into Tregs to inhibit disease progression. The DHA-M2 macrophages regulate pro- and anti-inflammatory cytokines, and there are a number of potential mechanisms. Currently we are trying to identify and amplify the role of the pattern recognition receptors (Toll-like receptors and B7-H4). Among the various receptors, B7-H4 is the inhibitory molecule of the B7 family and M2 macrophages were found to express high levels of B7-H4, whereas M1 or IL-4/13-modified macrophages did not (Sica *et al*., 2003; Prasad *et al*., 2003). Recent, studies report that two novel observations about B7-H4 and M2 macrophages; first, M2 macrophages can be distinguished from M1 or IL-4/13-modified macrophages by their high level expression of B7-H4. Next, B7-H4 on M2 macrophages not only suppresses $CD4^+$T cell proliferation but also can induce Tregs (Cao *et al*., 2010). Macrophages can act as APCs, which present pathogens-derived antigens (from viruses or

bacteria) directly to T cells, and interact with both T and B cells to maintain anti-inflammatory immune responses via various mechanisms (Huang *et al*., 2000). $CD11b^+$ M2 macrophages protect the kidneys from structural and functional injury by secreting high levels of TGF-β (Cao *et al*., 2010). TGF-β is a powerful anti-inflammatory cytokine that induces the differentiation of $Foxp3^+$ Tregs from $CD4^+$T cells (Fu *et al*., 2004). M2, but not LPS-modified (M1), macrophages induce the differentiation of Treg cells (Savage *et al*., 2008). Therefore, we postulated that DHA generates stronger function of M2 macrophages, which express higher levels of TGF-β. Indeed, we showed that treatment of M2 macrophages with DHA drives $CD4^+CD25^-$T cells to differentiate into $CD4^+Foxp3^+$T cells; however, Transwell assays showed that inhibition of cell-to-cell contact strongly suppressed the generation of Tregs. Previous studies in experimental disease models showed that the adoptive transfer of M1 macrophages exacerbated inflammation and induced marked renal injury (Ikezumi *et al*., 2003). Here, *in vitro* co-culture of DHA-M2 macrophages with M1 macrophages showed that DHA-M2 macrophages deactivated pro-inflammatory macrophages (M1 phenotype) more potently than normal M2 macrophages. Also, the results so far show that the transfusion untreated-M2 macrophages may protect mice against experiment AD, but not nearly as much as DHA-M2 macrophages. To our knowledge, it is previously unreported that deactivation of inflammatory macrophages by DHA–M2 macrophages may explain why DHA-M2 macrophages protect against AD.

We also examined various omega-3 polyunsaturated fatty acids (n-3 PUFAs) to detect which had the most potent immuno-regulatory effect (data not shown). Recently, we developed an *in vitro* screening system to identify immuno-regulatory n-3 PUFAs that induce Foxp3 but suppress pro-inflammatory cytokines. Thus, we compared the difference between DHA and eicosapentaenoic acid, both n-3 PUFAs that modulate the immune system. After co-culturing lymphocytes with each of the n-3 PUFAs for 3 days, we found that DHA suppressed the expression of IFN-γ and IL-4. In addition, we determined the optimal dosage and treatment duration of DHA based on the criteria that enhanced Foxp3 expression. In summary, M2 macrophages recognize and process DHA. During this process, normal $CD11b^{+}$M2 macrophages induce the differentiation of $CD4^{+}Foxp3^{-}$T cells into $CD4^{+}Foxp3^{+}$ Tregs in a TGF-β-dependent manner. Finally, the Tregs down-regulate the pro-inflammatory functions of helper T cells, which leads to the suppression of disease progression.



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# ABSTRACT IN KOREAN

아토피성 피부염은 유전적, 환경적 요인에 의한 비정상적으로 나타나는 면역 반응의 결과로, 부종, 가려움, 염증반응 등의 증상을 동반하는 피부질환이다. 현재 아토피성 피부염은 스테로이드계, 항히스타민계와 같은 치료제가 사용되고 있으며 이는 일시적인 완화 효과가 있으나 지속적인 사용 시 심각한 부작용을 유발하고 있다. 아토피성 피부염은 항원지시세포에 의해 포획된 알레르겐이 Th (T helper) 세포를 Th1 (T helper 1) 세포 또는 Th2 (T helper 2) 세포로 분화를 유도하여 다양한 염증성 사이토카인을 발현함으로써 지속적인 아토피 피부염이 유지된다. 또한, Th 세포는 과도한 면역반응의 결과로서 발생하는 자가면역질환, 조직이식 거부반응을 억제하는 Treg (Regulatory T 세포)으로도 분화가 된다. 특이적 전사인자로 Foxp3 (forkhead box P3)를 갖고있는 Treg 은 세포-세포간 접촉이나 IL-10 (interleukin-10), TGF-β (Transforming growth factor-beta)를 분비함으로써 알레르겐에 의한 면역 항상성과 관용을 유지하고 다양한 면역세포들의 분화, 증식, 기능을 억제함으로써 면역 반응을 조절한다. 대식세포는 항원지시세포의 한 종류로 항원이 침입하면 이를 포획하여 림프구에 항원을 전달하므로서 면역반응을 일으킨다. 대식세포는 사이토카인의 자극에 의해 활성화되며, 자극의 종류에 따라 다양한 기능을 가지는

대식세포로 분화된다. M1 대식세포는 LPS (Lipopolysaccharides) 또는 IFN-γ (Interferon-gamma) 자극에 의해서 활성화된 대식세포로서 염증반응을 증가시키고, M2 대식세포는 IL-10, TGF-β 자극에 의해서 활성화된 대식세포로서 염증반응을 감소시키는 역할을 한다. 오에가-3 지방산은 불포화지방산으로서 EPA (Eicosapantaenoic acid)와 DHA (Docosahexaenoic acid)가 대표적이며 α-linolenic acid 로부터 합성된다. 최근 오메가-3 지방산 관련 연구결과에 의하면 DHA 는 수지상 세포의 성숙 및 항원 특이적 Th 세포의 분화를 억제하여 대장염, 피부염, 자가면역성 뇌척수염 등의 개선 효과를 보고하였다. 이렇게 오메가-3 지방산이 염증 질환을 개선하는 효능이 있으며, 위에서 제시한 활성기전을 나타내지만 면역계에 대한 작용, 특히 Th 세포의 분화 및 조절에 대한 연구 결과는 매우 적다. 그러므로 본 논문에서는 DHA 가 Th 세포와 Treg 의 분화 및 조절, M2 대식세포의 항염증 능력에 대해 미치는 영향을 조사하고, 이러한 기전과 마우스 아토피성 피부염 개선 효과를 연결시켜 연구함을 목적으로 하였다. 그 결과, DHA 는 DNCB (2,4-Dinitrochlorobenzene)로 유도된 마우스 아토피성 피부염 증상 (피부두께, 태선화, 조직학적 소견)을 완화하였고 CD4+T 세포의 증식, 분화, 사이토카인 생성 억제 및 TGF-β 에 의존적인 작용기전을 통해 naïve T 세포로부터 Treg 분화를 유도하였다. DHA 는 IL-10/TGF-β 로 유도된 M2 대식세포의 항염증성 기능을 강화시켰고 이러한 DHA-M2 대식세포는 TGF-β 발현 및 세포-세포 간 접촉 기전을 통해 naïve T

세포로부터 Treg 의 분화를 유도하여 CD4+T 세포 증식을 억제하였다. 또한 DHA-M2 대식세포를 아토피성 피부염이 유발된 마우스에 투여한 결과 증상이 완화되었고 Treg 의 생성도 증가 하였다. 이러한 결과는 향후 천연물을 이용한 아토피성 피부염 개선소재의 기전연구에 중요한 기초 자료가 되라라 사료된다.