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Docosahexaenoic Acid Alleviates Atopic Dermatitis by Generating Treg and IL-10/TGF- β -Modified Macrophage via TGF- β Dependent Mechanism

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Full title:

Docosahexaenoic acid alleviates atopic dermatitis by generating Treg and IL-10/TGF- β -modified macrophage via TGF- β dependent mechanism

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Short title:

DHA induces Foxp3⁺ T cells and M2 macrophages

Abbreviations

AD, atopic dermatitis; DHA, docosahexaenoic acid; Treg, regulatory T cell; Th cell, helper T cell; TGF- β , transforming growth factor- β ; Foxp3, forkhead box P3; M1 macrophage, commonly-activated macrophage; M2 macrophages, IL-10/TGF- β -modified macrophage; TSLP, thymic stromal lymphopoietin

ABSTRACT

Regulatory T cells (Tregs) play key roles in the immune response by suppressing the differentiation and proliferation of various immune cells. The beneficial effects of docosahexaenoic acid (DHA) have been described for many diseases; however, the mechanism by which it modulates immune system is poorly understood. Therefore, the aim of this study was to examine 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 -modified macrophages (M2 macrophages) treated with DHA into a mouse model of atopic dermatitis. Here, we show that administration of DHA up-regulates the generation of TGF- β -dependent CD4⁺Foxp3⁺ Tregs. DHA 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 DHA-M2 macrophages, which deactivated effector macrophages and inhibited CD4⁺ T cell proliferation. DHA showed therapeutic effects in mice with experimental atopic dermatitis. These results show that DHA enhances the function of M2 macrophages, and that the generation of Tregs effectively protects mice against an inflammatory immune disorder. Thus, DHA may be a useful therapeutic strategy for treating chronic inflammatory diseases.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with cutaneous hyper-reactivity, including edema and itching, which affects approximately 10-20% of children, and 1-3% of adults 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, such as mast cells, basophils, eosinophils, and T cells (Li *et al.*, 2010; Wahlgren, 1999; Jakasa *et al.*, 2011).

Regulatory T cells (Tregs) play a key role in immune responses, 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 such as CD4⁺ and CD8⁺ T 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 key regulator of Treg development 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; Tjui *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 reduce the sensitization to allergens, alleviate the severity of 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 aim of this study was to examine

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.

RESULTS

DHA suppresses the development of experimental AD

To induce experimental AD, mice were sensitized by applying 1 % 2, 4-Dinitrochlorobenzene (DNCB) to the abdomen. They were then re-sensitized by applying 0.3 % DNCB to the ears on every other day for up to 41 days. Starting on Day 12, the mice received a daily dose of DHA (100 mg/kg) via the drinking water (Figure 1a). IgE is an important therapeutic target for AD as it is the major activator of mast cells, which release 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-only group (mice exposed to DNCB but not fed DHA; Figure 1b). The skin lesions associated with AD are characterized by an inflammatory cell infiltrate (de Vries *et al.*, 1997). Therefore, we next tested whether DHA reduced the level of inflammatory cell infiltration in the ears of mice with experimental AD. We also examined cutaneous edema as a measure of AD progression. We found 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 produced by epidermal keratinocytes, dermal fibroblasts, and mast cells, 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) staining and 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-treated group than in the induction-only group (Figure 1e and f).

CD4⁺Foxp3⁺ T cells are enriched at sites of inflammation and are associated with AD suppression

The lymph nodes (LNs) play an important role in cell-mediated immunity by regulating the activity of mature T and B cells (Debes *et al.*, 2004). Therefore, we examined the morphologic changes in the LN of AD mice. The LNs from mice in the induction-only group were very swollen, whereas 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 in the number of 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).

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 processes by preventing or suppressing the differentiation, proliferation, and function 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 (Supplementary Figure S1a online). Although DHA also increased the CD4⁺Foxp3⁺ population in the presence of exogenous TGF- β , which induces the generation of Tregs *in vitro* (Figure 3c, Supplementary Figure S1b and c online), we found no difference in the size of the CD4⁺CD25⁺ population between the different groups (Figure 3d). 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 3f), 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 3e). 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 3f). 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.

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-1 β ($P < 0.01$ 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 Supplementary Figure S2a online). 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 4d and Supplementary Figure S2b online). 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 4e and Supplementary Figure S2c online). 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 .

Transfusion of DHA-M2 macrophages protects against experimental AD

To induce experimental AD, mice were sensitized by applying 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 in the number of 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.

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 such as CD4⁺ and CD8⁺ T 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 (IFN- γ , IL-4, IL-5, IL-13, IL-31, IL-17 and IL-6) 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.

MATERIALS AND METHODS

Experimental animals

BALB/c mice (7 weeks old) were purchased from Orient Bio and maintained under pathogen-free conditions in the animal facility of Jeju National University. All animal experiments were approved by the Jeju National University Animal Care and Use Committee.

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.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Titles and legends to figures

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-embedded 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 $\pm$ S.D. ** $P < 0.01$; *** $P < 0.001$.

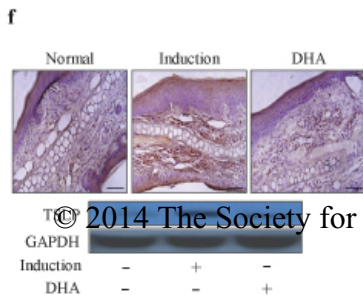
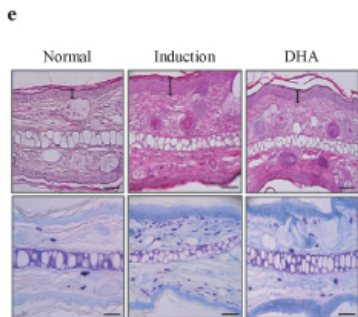
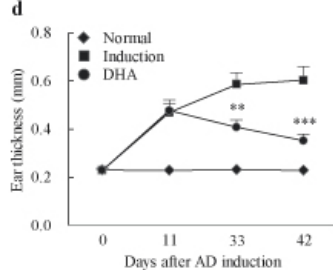
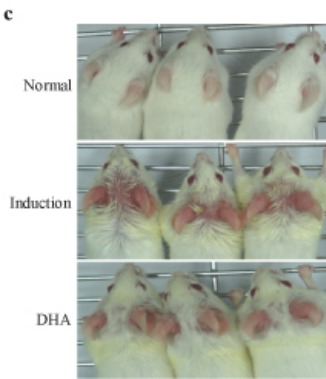
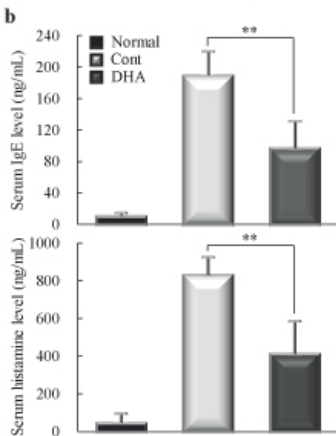
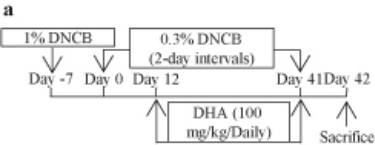
Figure 2. CD4⁺Foxp3⁺ T cells are enriched at sites of inflammation. (a) The LNs were photographed and measured to record morphologic changes. (b) Cytokines and (c) Foxp3 levels expressed by CD4⁺ T cells isolated from lymphocytes were analyzed by real-time PCR and FACS. (d) Sections of LN were stained 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 $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ compared with mice in the induction group.

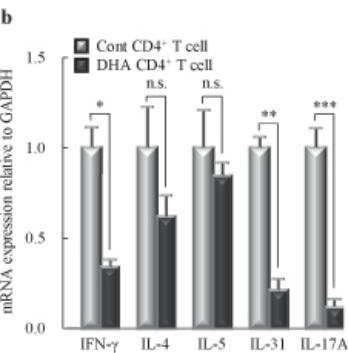
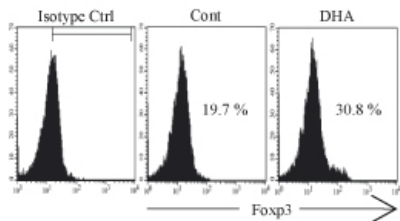
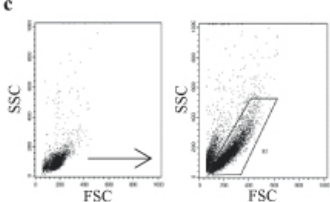
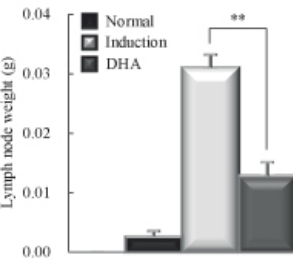
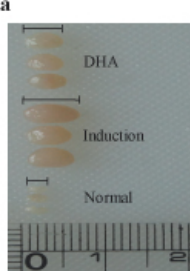
Figure 3. DHA induces the differentiation of CD4⁺Foxp3⁺ Tregs from CD4⁺ T cells in a TGF- β -dependent manner. (a) Naïve mice received DHA for 30 days. (b) The expression of mRNA for various factors in CD4⁺ T cells was measured by real-time PCR. (c and d) Cells were stimulated with anti-CD3/-CD28 in the presence of TGF- β for 72h. (e) Tregs from each treatment group was co-cultured with responder cells (CD4⁺CD25⁻) labeled with CFSE at different Treg/responder ratios. (f) 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 $\pm$ S.D. * $P < 0.05$; and *** $P < 0.001$.

Figure 4. DHA induces Foxp3⁺ Tregs and stronger ability of IL-10/TGF- β M2 macrophages. (a) Macrophages (MC) 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, d and e) Macrophages were co-cultured with CD4⁺ T cells in plates or cultured separately in Transwell chambers for 7 days. (f) CD4⁺ T cells-labeled with CFSE were co-cultured with macrophages, and

stimulated by anti-CD3/-CD28 for 72h. Values represent the mean $\pm$ S.D. Data are representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

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 $\pm$ S.D. * $P < 0.05$; and ** $P < 0.01$.





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